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Milnesium (Tardigrada: Apochela) in Japan: The First Confirmed Record of *Milnesium tardigradum* s.s. and Description of *Milnesium pacificum* sp. nov.

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Presently, more than 40 species of the genus *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela: Milnesiidae) have been described. In Japan, however, almost all records of milnesiid tardigrades should be re-examined with the current criteria on the taxonomy of this genus, except for one species, the recently described *Milnesium inceptum* Morek, Suzuki, Schill, Georgiev, Yankova, Marley, and Michalczyk, 2019. In this study, we found two species, *Milnesium pacificum* sp. nov. and *Milnesium tardigradum* Doyère, 1840, from three southern islands and two cold regions in Japan, respectively. *Milnesium pacificum* sp. nov., having dorsal sculpturing, exhibits an early positive change in claw configuration. On the other hand, *M. tardigradum* s.s. from Japan has an early negative claw configuration change, as has been reported in a recent study on the neotype population of this species. We performed DNA barcoding for both species, which indicated that *M. pacificum* sp. nov. has a close affinity with an undescribed *Milnesium* species collected from Brazil, and that *M. tardigradum* from Japan represents the recently described subclade that contains specimens from Poland, Hungary, and Russia. The chromosome numbers were 2n = 14 in *M. pacificum* sp. nov. and 2n = 10 in *M. tardigradum*. We detected at least three species of the genus *Milnesium* present in Japan. Our results advance the investigation of the relationship between phylogenetic position and characteristic morphology as well as expand the known geographic range of *M. tardigradum*.

Key words: circum-Pacific, molecular phylogeny, new species, ontogenetic morphology, phylogeography

INTRODUCTION

Until the early 20th century, three species, *Milnesium tardigradum* Doyère, 1840, *Milnesium alpigenum* Ehrenberg, 1853, and *Milnesium quadrifidum* Nederström, 1919, had been described in the genus *Milnesium* (Apochela, Apotardigrada). However, Marcus (1928) advocated synonymizing *M. alpigenum* and *M. quadrifidum* with *M. tardigradum*, based on his opinion that the difference in the number of points on the secondary claw branches, now designated as the claw configuration (CC), indicated intraspecific variants. After that, *Milnesium* was considered as a monospecific genus for more than 60 years. Although *Milnesium tardigradum* var *granulatum* Ramazzotti, 1962 was distinguished by fine 'granules' on the cuticle, which is actually reticulated with many depressions (Michalczyk et al., 2012a), another three decades passed until new species were described in this genus. Since the redescription of *M. tardigradum* establishing the neotype with DNA analysis (Michalczyk et al., 2012a, b), investigators have been able to compare milnesiid specimens with the type species based on the morphometrics as well as DNA barcoding. Recently, Morek et al. (2019a) provided important new traits for *M. tardigradum*, including the description of ontogenetic CC change, which made more detailed taxonomic studies possible. Moreover, *M. alpigenum* was integratively redescribed, which now allows for a confident identification of the species (Morek et al., 2019b). Presently, more than 40 species of the genus *Milnesium* have been described (Degma et al., 2019; Kaczmarek et al., 2019; Surmacz et al., 2019; Moreno-Talamantes et al., 2019).

Milnesium inceptum Morek et al., 2019b, described together with the redescription of *M. alpigenum*, has long been cultured in two distant laboratories, one in Japan (Suzuki, 2003) and the other in Germany (Schill et al., 2004). Each culture had a different origin: one from Yokohama, Japan and the other from Tübingen, Germany. The Japanese strain of *M. inceptum* (H-1 strain) originated in 2000 from a female that lived in the moss *Bryum argenteum* Hedw. on a building wall in Hiyoshi, Yokohama, and was tentatively identified as *M. tardigradum* at the time of publication (Suzuki, 2003). Although many localities of '*Milnesium*

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tardigradum' have been reported from Japan (Mathews, 1936–1937; Hatai, 1956, 1959; Utsugi, 1985a, b, 1986, 1987, 1988, 1990, 1991, 1992, 1994a, b, 1996; Utsugi et al., 1997; Ito, 1997, 1999; Ito and Abe, 1997, 2001; Abe and Tanaka, 2000; Biserov et al., 2001; Ishimaru, 2011), these reports lacked detailed morphometrics and DNA barcodes. Therefore, all of these records should be treated as unconfirmed and re-examined with the current criteria. Kume et al. (2012) reported that *Milnesium* species in Japan were classified into at least three different clades by molecular analyses of the cytochrome *c* oxidase subunit I (COI) and internal transcribed spacer 2 (ITS-2) regions.

Milnesium granulatum, elevated to the species level by Michalczyk et al. (2012a), has not only different CC ([3-3]-[3-3] in *M. granulatum*) from *M. tardigradum*, but also the dorsal cuticular sculpturing in the form of reticulation with shallow depressions. Until now five species with this character were collectively called the *granulatum* group (Michalczyk, 2012a; Morek et al., 2019a), which is not monophyletic (Morek and Michalczyk, 2020). In this study, we describe a new member of the *granulatum* group, as well as report *M. tardigradum* s.s. in Japan for the first time, based on their morphometrics, DNA barcodes, and karyotypes. These new records provide new insights into the biodiversity and phylogeography of *Milnesium.*

MATERIALS AND METHODS

Collecting and culturing

Moss and lichen samples were obtained from several localities (Fig. 1; Table 1). They were soaked in water for over 20 min and examined under a stereomicroscope (Leica Mz95) to extract tardigrades. Collected *Milnesium* specimens were placed in 3.2 cm plastic dishes coated with 1.2% agar and filled with Volvic water, and cultured at 20°C with prey, the rotifer *Lecane inermis* (Bryce, 1892) (see Suzuki, 2003). Water was changed every day, and the dishes were replaced every week. To establish isogenic strains, one clutch of laid eggs was separated into individual parthenogenetic cultures. To record ontogenetic morphological changes, tardigrades were classified into three stages by ontogenetic tracking as in Morek et al. (2016): hatchling (the first instar), juvenile (the second instar) and adult (the third and older instars). The terminology for the CC change was determined according to Morek et al. (2019a).

Morphometrics

All examined tardigrades in this study were from the strains we established. Each specimen was mounted in a drop of Hoyer's medium on a microscope slide and secured with a cover slip. Slides were dried at 60°C for 5 days and sealed with transparent nail polish. They were observed with a phase contrast microscope (Axio Imager M1, ZEISS) and each structure was measured by ImageJ (https://imagej.nih.gov/ij/). All measurements were made in micrometers (μm). 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 Pilato (1981). The diameter (width) of the buccal tube was measured at the anterior, standard, and posterior positions (Michalczyk et al., 2012a). Other measurements were made according to Tumanov (2006). The *pt* index is the percentage of the length of each structure relative to the buccal tube length (Pilato, 1981). The diameter and density of dorsal cuticular depressions were measured from SEM photos with ImageJ. Observations under ultraviolet (UV) were performed with a fluorescent microscope (Axio Imger M1) with a 360 nm excitation wavelength.

Scanning electron microscopy (SEM)

More than 10 specimens of each species were washed with 10% phosphate buffered saline (PBS) with 1% Tween20 and fixed with 4% formaldehyde in water. Fixed tardigrades were dehydrated

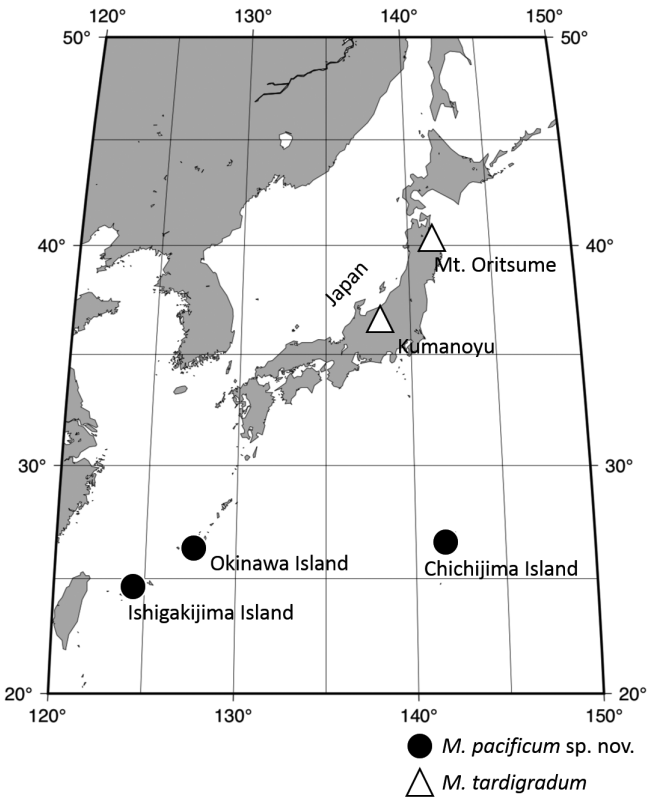

Fig. 1. Map of Japan showing localities of *Milnesium pacificum* sp. nov. (solid circles) and *Milneisum tardigradum* Doyère, 1840 s.s. (hollow triangles) in this study.

Table 1. Collection data for moss samples containing the two *Milnesium* species.

Species	Strain	Sampling site	Geographical coordinates	Collector	Date
Milnesium pacificum sp. nov.	OGA7	Ogasawara Island, Tokyo	27°04′01″N, 142°12′17″E	Kenta Sugiura	Mar. 13. 2017
Milnesium pacificum sp. nov.	MW	Manko Wetland, Okinawa	26°11′33″N, 127°41′13″E	Atsushi C. Suzuki & Kenta Sugiura	Dec. 12. 2017
Milnesium pacificum sp. nov.	i30	Ishigakijima Island, Okinawa	24°20′25″N, 124°09′09″E	Akira Sugiura	Jan. 30. 2018
Milnesium tardigradum	OTM	Mt. Oritsume, Iwate	40°16′07″N, 141°22′18″E	Hiroki Minato	May. 23. 2017
Milnesium tardigradum	KNY	Kumanoyu, Nagano	36°41′02″N, 138°29′46″E	Hiroki Minato	Aug. 3. 2017

in 50%, 60%, 70%, 80%, 90%, 95%, 99.5% ethanol for 15 min each, and rinsed 3 times in 100% t-butyl alcohol for 15 min each. Specimens were maintained in 100% t-butyl alcohol at 10°C overnight to be frozen and lyophilized using a JFD-320 (JEOL). The specimens were mounted on aluminum stubs under a stereomicroscope, sputter-coated with gold, and observed with a scanning electron microscope (JSM 6510, JEOL) at 10 or 15 kV.

DNA extraction and sequencing

To extract DNA from single specimens, the method described in Arakawa et al. (2016) was followed. Each tardigrade was maintained separately in 1% streptomycin in Volvic water without feeding for 24 h. A single tardigrade was then transferred into a 200 µl tube with a small amount of water (< 2 µl), to which 100 µl of 1% 2-mercaptoethanol/Cell Lysis Solution (QIAGEN) was added. The animal was lysed using 3 freeze-thaw cycles between −80°C and 42°C. Genomic DNA was purified with a Mini Plus Column (VIOGENE). Five specimens per strain were sequenced.

18S rDNA, 28S rDNA, COI, and ITS-2 regions were amplified by PCR. The PCR products were cloned into the pGEM-T (easy) Vector (Promega) and sequenced (Eurofins Genomics). Primers used were as follows: SSU_F_04: 5′-GCTTGTCTCAAAGATTAAGCC-3′ (Kiehl et al., 2007), rev960: 5′-GCTTTCGTAAACGGTTCGGAC-3′, fw390: 5′-AATCAGGGTTCGATTCCGGAGA-3′, rev18S: 5′-TGATCCTTCCGCAGGTTCACCT-3′ (Dabert et al., 2014), for 18S rDNA; 28SF0001: 5′-ACCCVCYNAATTTAAGCATAT-3′, 28SR0990: 5′-CCTTGGTCCGTGTTTCAAGAC-3′ for 28S rDNA (Mironov et al., 2012); HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′, LCO1490: 5′-GGTCAACAAATCATAAAGATATTG-3′ for COI (Folmer et al., 1994), ITS3: 5′-GCATCGATGAAGAACGCAGC-3′, ITS4:

Table 2. Sequence profiles in genetic analyses of *Milnesium* species.

Species	Sample	COI	ITS-2	References
M. tardigradum	CH.002	MG923562	MG923552	Morek et al. (2019a)
	DE. 002	JN664950	JF951049	Michalczyk et al. (2012a)
		MG923559		Morek et al. (2019a)
	DK.001	MG923560	MG923552	Morek et al. (2019a)
	FR.072	MG923560	MG923554–5	Morek et al. (2019a)
	HU.001	MG923564	MG923553	Morek et al. (2019a)
	PL.023	MG923561	MG923552	Morek et al. (2019a)
	PL.028	MG923563	MG923551–2	Morek et al. (2019a)
	PL.034	MG923565	MG923553	Morek et al. (2019a)
	PL.142	MG923558	MG923552	Morek et al. (2019a)
	RU.028	MK492292	MK484011	Morek and Michalczyk (2020)
	OTM	LC511100	LC511101	This study
	KNY	LC511103	LC511104	This study
M. inceptum	Germany	EU244603	GQ403681	Schill (unpublished)
	Japan	EU244604	GQ403682	Schill (unpublished)
	DE.001	KU513422	MH000386	Morek et al. (2019b)
	BG.058	MH000381	MH000387	Morek et al. (2019b)
M. alpigenum		MH000380	MH000382	Morek et al. (2019b)
M. lagniappe		MH751518	MH746111	Jackson and Meyer (2019)
M. cf. *granulatum*		MH751517	MH745034	Jackson and Meyer (2019)
M. berladnicorum		KT951659	KT951662	Morek et al. (2016)
M. dornensis		MG923566	MG923557	Morek et al. (2019a)
M. variefidum		KT951663	KT951666–7	Morek et al. (2016)
Milnesium sp.	*M. hisatsinomorum*	KX306950		Fox et al. (unpublished)
	CJS-2007a	EF632553		Sands et al. (unpublished)
	Ta46-01	KJ857001		Velasco-Castrillón et al. (2015)
	Ta47-01	KJ857002		Velasco-Castrillón et al. (2015)
	Miln06_124	KP013598		Velasco-Castrillón et al. (2015)
	Mil06_123	KP013601		Velasco-Castrillón et al. (2015)
	Mlln06_224	KP013613		Velasco-Castrillón et al. (2015)
	MC-2013	JX683822–5		Vicente et al. (2013)
	PH.014	MK484029	MK492303	Morek and Michalczyk (2020)
	BR.007	MK484019	MK492306	Morek and Michalczyk (2020)
M. pacificum sp. nov.	OGA7	LC511094	LC511095	This study
	MW	LC511090	LC511091	This study
	i30	LC511096	LC511097	This study
Diploechiniscus oihonnae		MG063724	MG923556	Gąsiorek et al. (2017) & Morek et al. (2019a)

5′-TCCTCCGCTTATTGATATGC-3′ for ITS-2 (White et al., 1990). The PCR setting was preheat at 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. The PCR solution consisted of 10 μl Quick-Taq (TOYOBO), 2 μl each primer, 5 μl distilled H_2O, and 1 μl genomic DNA. For amplifying the ITS-2 region, 3 μl genomic DNA was added. Sequencing was performed by Eurofins genomics (Japan), then checked and trimmed with GENETYX-MAC v17.0.0.

Phylogenetic analyses

For phylogenetic analyses, we used the published sequences of COI and ITS-2 from GenBank (Table 2). A published sequence tagged as *Milnesium tardigradum* isolate Tar235 (FJ435810) was removed from our analyses (see Morek et al., 2019a). The sequences were aligned using MAFFT v7.222 (Katoh et al., 2002; Katoh and Toh, 2008) with the default setting. The alignments were checked by using MEGA7 (Kumar et al., 2016), and trimmed manually to 509 bp in COI and 524 bp in ITS-2. Then they were concatenated manually to 1033 bp. The p-distances were calculated with MEGA7. The aligned sequences were applied to choose the best-fit evolution model by using PartitionFinder v2.1.1 (Lanfear et al., 2016) under the Bayesian Information Criterion. GTR+I+G was chosen for COI and concatenated alignments, while GTR+G was

Fig. 2. *Milnesium pacificum* sp. nov. while molting and preoviposition, turning its face frontally inside exuviae.

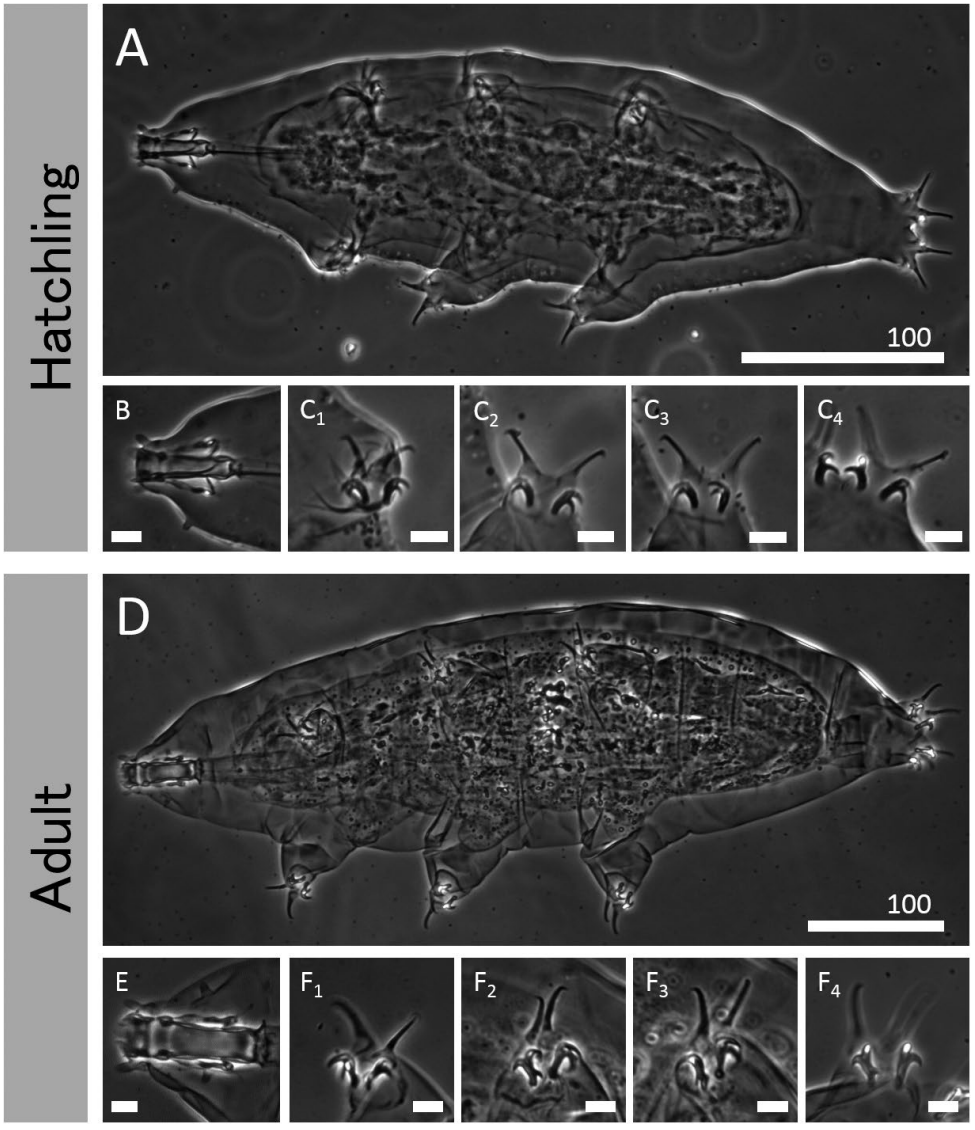

Fig. 3. *Milnesium pacificum* sp. nov., phase-contrast micrographs of buccal tube and claws, ventral view. **(A–C)** Hatchling (paratype). **(D–F)** Adult (holotype). Scale bars in μm.

used as a suitable model for ITS-2 alignment. Maximum Likelihood (ML) topologies were constructed by using RAxML v8.0.0 (Stamatakis, 2014), and strength of support for nodes was calculated by 1000 rapid bootstrap replicates. For calculating Bayesian Inference probabilities, we used MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) and the analyses were run for 5,000,000 generations, sampling the Markov chain every 1000 generations. Moreover, an average standard deviation of split frequencies of < 0.01 was confirmed. The trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). 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 (Zhang et al., 2013). To this end, we constructed a TCS haplotype network by using PopART v1.7 (Clement et al., 2002; Leigh and Bryant, 2015) based on the alignments of COI and ITS2, respectively. The designations of haplotypes and subclades followed those described in Morek et al. (2019a).

Chromosomal analyses

To examine mitotic chromosomes, we used the 4′, 6-diamidino-2-phenylindole (DAPI) staining method described by Sugiura et al. (2019). Laid eggs were fixed in methanol:acetic acid (3:1) for 3 h, placed on slides in a drop of 60% acetic acid, and air dried. The eggs were stained with 1/1000 DAPI (Wako) in PBS for 5 min, washed twice with PBS, and mounted with Fluoro-KEEPER Antifade Reagent Non-Hardening Type (Nacalai Tesque). More than 10 eggs were examined for each staining. We also analyzed the chromosomes of the *M. inceptum* H-1 strain in the same way.

RESULTS

Taxonomic account

Milnesium pacificum sp. nov.
(Figs. 2–7, 8A; Table 3, 4)

Material examined. A cultured strain of *M. pacificum* sp. nov., collected in March 2017 at the type locality and designated as OGA7 (OGAsawara-7), has been established. Type series from the strain is deposited in National Museum of Nature and Science, Tsukuba, Japan (accompanied with NSMT-Tg number) and a part of paratypes are also deposited in Natural History Museum of Denmark, Copenhagen (with NHMD number). The holotype (slide number: NSMT-Tg-241). Eleven paratypes: five adult females (NSMT-Tg-242–244, NHMD-666570 and 666571), two juveniles (NSMT-Tg-245, NHMD-666572) and four hatchlings (NSMT-Tg-246–248, NHMD-666573) of OGA7. Two other cultured strains, MW (Manko Wetland) and i30 (Ishigaki January 30th), from Okinawa prefecture were also examined, with four adult females (NSMT-Tg-249–252), two juveniles (NSMT-Tg-253 and 254) and six hatchlings (NSMT-Tg-255–260) of MW deposited in the same place. OGA7 and MW strains have been maintained in Keio University, while the i30 strain became extinct after data acquisition.

Description. Live adult females (Fig. 2) show a creamy white body under dark-field illumination, or transparent under transmitted light, with three brownish longitudinal stripes. Eyes present but not visible in mounted specimens. Adult body length 501–853 μm (593.2 μm in the holotype). All measurements shown in Table 3A. Six peribuccal and two lateral papillae (Figs. 3B, 3E, 4B). The ventral peribuccal papilla is shortest (Fig. 4B). Six (4 + 2) peribuccal lamellae (Fig. 4B). Buccal tube wide and long (Fig. 3E). Buccal apparatus *Milnesium* type, pharynx without placoids or septula. The CC in adult and juvenile specimens was [2-3]-[3-2] (Fig. 3F), while the [2-2]-[2-2] CC was observed in hatchlings (Fig. 3C); therefore, the new species showed early positive CC

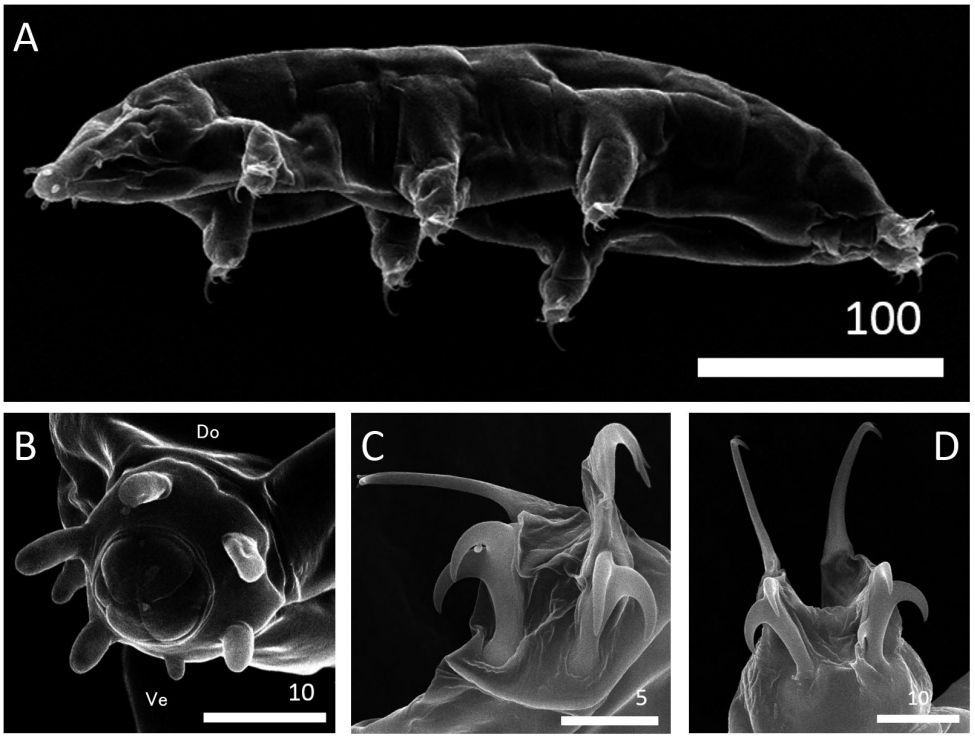

Fig. 4. *Milnesium pacificum* sp. nov., SEM images (paratypes). **(A)** Lateral view. **(B)** Mouth configurations. **(C)** Claws of hatchling. **(D)** Claws of adult. Abbreviations: Do, dorsal; Ve, ventral. Scale bars in μm.

change. Morphometrics of the OGA7 strain are shown in Table 3A, B and the entire dataset is available online in Supplementary Table S1.

Cuticular sculpturing in the form of a reticulum composed of depressions present on whole dorsum except on the head. The full diameter of dorsal depressions on hatchlings 0.61–0.82 μm, smaller and irregular circular depressions in adults (0.50–0.65 μm, Fig. 5). Density of the sculpturing was 1.44 depressions/μm^2 in hatchlings, 1.88 depressions/μm^2 in adults. The pseudoplate was recognizable dorsally by observations with both phase-contrast and fluorescent microscopy (Figs. 3, 6).

Type locality. Tokyo Prefectural Road 240, Chichijima Island, Ogasawara, Tokyo, Japan (27°04′01″N, 142°12′17″E).

Other localities. Two other localities of the new species: Manko wetland, Okinawa Island, Okinawa prefecture, Japan (26°11′33″N, 127°41′13″E), and Ishigakijima Island, Okinawa prefecture, Japan (24°20′25″N, 124°09′09″E).

Habitats. Specimens from Ogasawara and Ishigakijima Island were collected from moss samples on a concrete wall, and the Okinawa population was obtained from lichen samples on concrete on the ground.

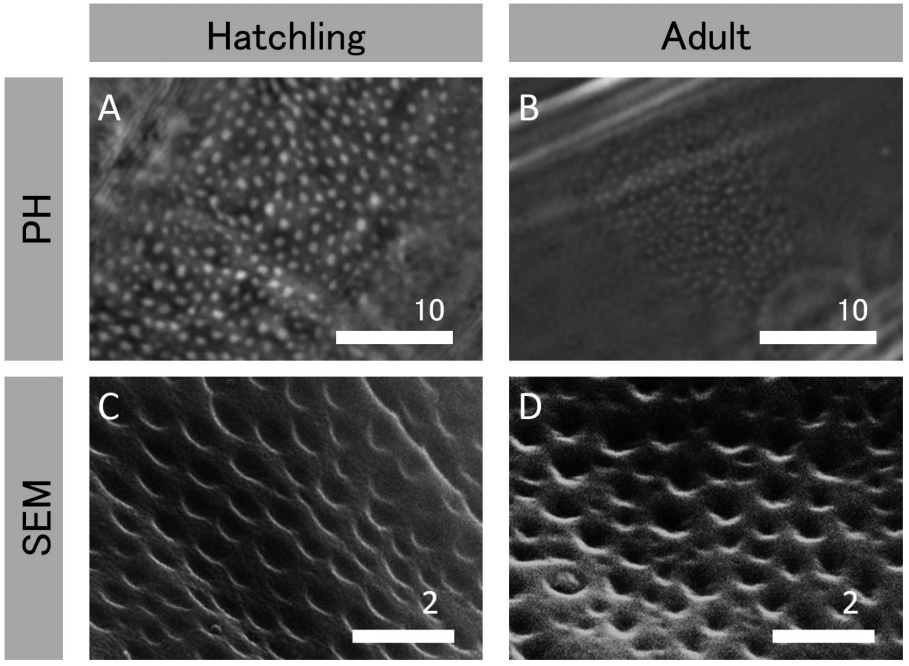

Fig. 5. *Milnesium pacificum* sp. nov., phase-contrast micrographs **(A, B)** and SEM images **(C, D)**, showing details of cuticular sculpturing on dorsum. **(A, C)** Hatchling. **(B, D)** Adult. Scale bars in μm.

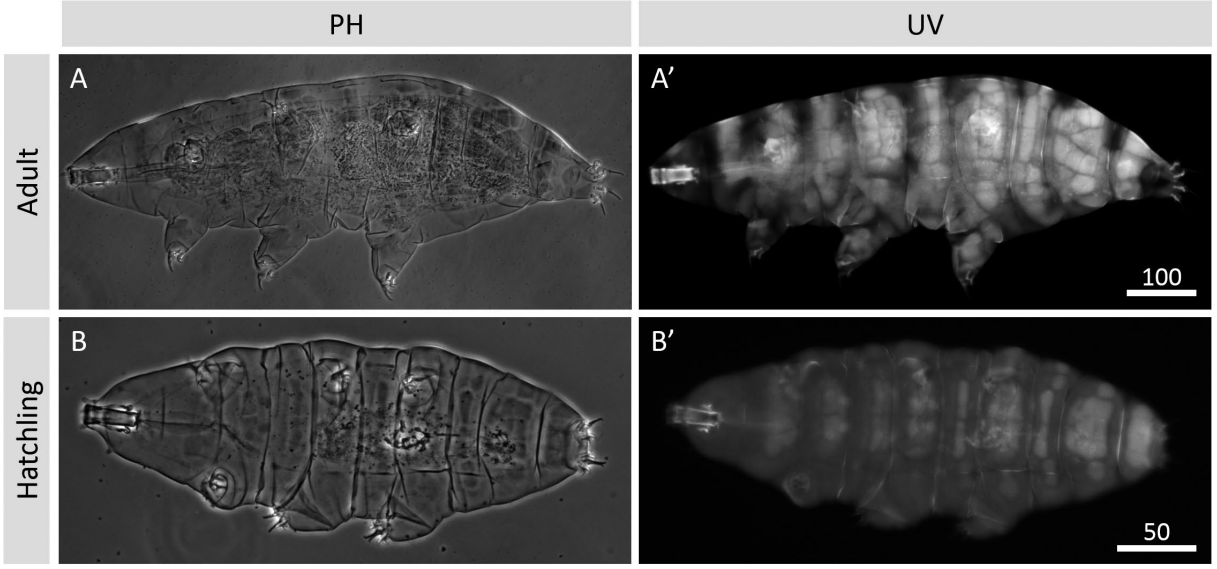

Fig. 6. *Milnesium pacificum* sp. nov., phase-contrast **(A, B)** and UV fluorescence **(A′, B′)** micrographs showing pseudoplates. **(A, A′)** Adult. **(B, B′)** Hatchling. Scale bars in μm.

Etymology. The specific name "*pacificum*" is an adjective, referencing the Pacific Ocean, the region where the species was collected.

Aberrant CC. Two adult specimens with an irregular CC, i.e., a [3-3] claw among otherwise normal [2-3]-[3-2] CC, were found in the cultures. One adult from the i30 strain showed [3-3] on left leg II (Fig. 7). The other case from the MW strain was found on right leg IV.

Life history. The early stages in the life history of *M. pacificum* sp. nov. were observed. Clutch size (eggs/clutch), duration of embryogenesis, and days required for first oviposition after hatching are shown in Table 4. The ranges were 6–13 eggs/clutch, 6–8 days for embryogenesis, and 26–27 days for the first oviposition in the OGA7 strain. The ranges in the MW strain were 8–12 eggs/clutch, 5–8 days, and 50–64 days, respectively.

Chromosomes. DAPI staining for laid eggs at the 1-cell stage showed 14 chromosomes (Fig. 8A).

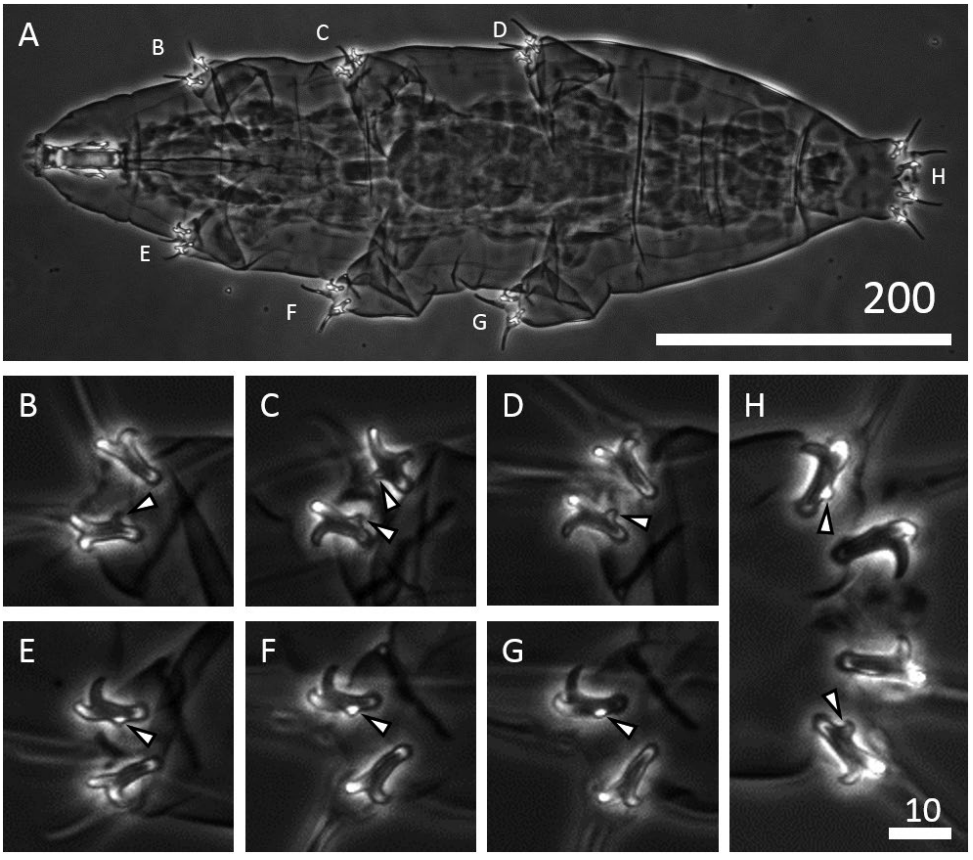

Fig. 7. *Milnesium pacificum* sp. nov., phase-contrast micrographs of an adult female from the i30 strain showing irregular CC. **(A)** Entire body. **(B, D–H)** Magnification of claws in **(A)**, all of which show [2-3]-[3-2] configuration. **(C)** Magnification of left leg II with an irregular CC [3-3]; white arrowheads indicating spurs on the secondary branches. Scale bars in μm **(A)**; 10 μm for **(B–H)**.

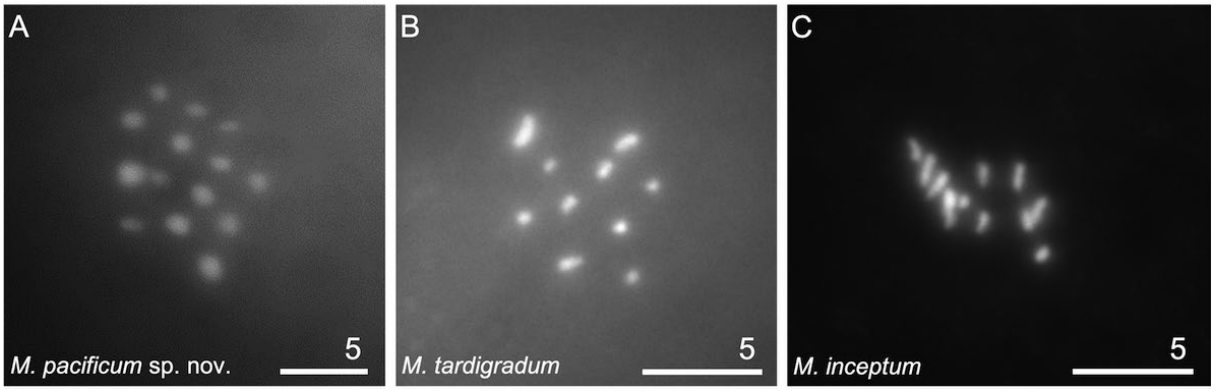

Fig. 8. Chromosomes stained with DAPI. **(A)** *Milnesium pacificum* sp. nov. OGA strain, single cell stage. **(B)** *Milnesium tardigradum* OTM strain. **(C)** *Milnesium inceptum* H-1 strain. Scale bars in μm.

Table 3A. Measurements of adults and holotype of *Milnesium pacificum* sp. nov. OGA7 strain.

	Adult				Holotype	
	n	μm		*pt*		μm	*pt*
		Range	Mean ± SD	Range	Mean ± SD		
Body length	6	501–853	684 ± 145	*1450–1929*	*1623 ± 211*	593	*1450*
Peribuccal papillae length	5	5.5–9.5	8.0 ± 1.7	*16.3–22.4*	*18.7 ± 2.4*	7.1	*17.3*
Lateral papillae length	5	4.6–8.3	6.0 ± 1.5	*10.8–18.0*	*14.1 ± 3.1*	5.0	*12.3*
Buccal tube							
Length	6	33.7–50.0	41.9 ± 5.7	*100.0–100.0*	*100.0 ± 0.0*	40.9	*100.0*
Stylet support	6	20.8–31.2	26.1 ± 3.8	*57.1–67.8*	*62.2 ± 4.1*	23.9	*58.3*
Anterior width	6	13.1–19.5	16.4 ± 2.6	*36.1–42.3*	*39.2 ± 2.1*	14.8	*36.1*
Posterior width	6	12.2–18.9	15.4 ± 2.8	*29.8–41.1*	*36.6 ± 3.8*	12.2	*29.8*
Standard width	6	12.9–18.8	15.9 ± 2.6	*33.0–40.8*	*37.9 ± 2.9*	13.5	*33.0*
Standard/length	6	33.0–40.8	37.9 ± 2.9			33.0	
Posterior/anterior	6	82.5–101.5	93.3 ± 6.5			82.5	
Claw1							
External primary branch	6	12.8–23.5	19.0 ± 4.1	*38.1–51.1*	*45.0 ± 5.8*	20.1	*49.2*
External base + secondary branch	6	11.4–19.6	15.5 ± 3.1	*29.3–45.8*	*37.0 ± 6.2*	18.7	*45.8*
External spur							
Internal primary branch	6	12.7–22.1	17.0 ± 3.4	*34.4–48.1*	*40.4 ± 5.4*	15.7	*38.5*
Internal base + secondary branch	6	11.7–19.4	15.9 ± 3.2	*28.6–46.0*	*38.1 ± 6.7*	11.7	*28.6*
Internal spur	3	3.4–4.8	4.1 ± 0.7	*8.8–9.5*	*9.1 ± 0.4*	4.0	*9.8*
Basal thickening width	6	2.2–5.3	3.3 ± 1.0	*6.3–11.5*	*7.9 ± 1.9*	3.3	*8.0*
Claw2							
External primary branch	6	17.8–23.8	19.4 ± 2.3	*36.8–52.7*	*46.9 ± 6.5*	18.0	*44.1*
External base + secondary branch	6	12.5–18.3	16.5 ± 2.1	*35.5–43.6*	*39.5 ± 2.9*	17.9	*43.6*
External spur							
Internal primary branch	6	17.5–24.5	20.2 ± 3.0	*42.8–54.7*	*48.2 ± 4.8*	17.5	*42.8*
Internal base + secondary branch	6	13.8–18.5	15.3 ± 1.8	*32.4–42.1*	*36.8 ± 3.5*	13.8	*33.7*
Internal spur	6	3.4–4.9	4.1 ± 0.5	*7.8–10.9*	*9.7 ± 1.1*	4.3	*10.5*
Basal thickening width	6	2.5–4.4	3.7 ± 0.7	*5.3–11.3*	*8.9 ± 2.2*	3.3	*8.1*
Claw3							
External primary branch	6	16.1–27.7	20.9 ± 4.1	*40.1–60.2*	*50.0 ± 7.4*	18.2	*44.5*
External base + secondary branch	6	11.2–19.4	16.2 ± 2.8	*33.2–42.3*	*38.6 ± 3.8*	17.0	*41.5*
External spur							
Internal primary branch	6	16.4–23.3	19.7 ± 3.3	*42.1–51.3*	*46.9 ± 4.0*	17.2	*42.1*
Internal base + secondary branch	6	12.7–21.7	18.1 ± 3.4	*37.6–51.3*	*43.0 ± 5.5*	19.3	*47.1*
Internal spur	5	3.3–5.7	4.2 ± 0.9	*8.0–12.2*	*10.0 ± 1.8*	4.0	*9.8*
Basal thickening width	6	3.2–4.4	3.9 ± 0.4	*7.8–11.3*	*9.4 ± 1.5*	3.2	*7.9*
Claw4							
Anterior primary branch	6	15.0–25.5	21.2 ± 3.9	*41.6–60.3*	*50.6 ± 6.9*	21.9	*53.5*
Anterior base + secondary branch	6	15.9–21.3	17.5 ± 2.3	*32.8–47.1*	*42.1 ± 5.5*	16.0	*39.2*
Anterior spur	6	2.6–5.8	4.4 ± 1.7	*5.6–13.8*	*10.5 ± 2.9*	4.0	*9.7*
Posterior primary branch	6	15.3–27.8	22.3 ± 4.6	*41.1–61.1*	*53.1 ± 8.7*	24.5	*59.8*
Posterior base + secondary branch	6	18.6–23.5	20.5 ± 2.0	*39.1–55.2*	*49.3 ± 6.0*	18.6	*45.4*
Posterior spur							
Basal thickening width	6	2.5–5.0	3.8 ± 1.0	*7.4–10.9*	*8.9 ± 1.4*	3.5	*8.5*

Sequence data. All sequences are available online in Supplementary Text S1 and GenBank (LC511088 to LC511097); a p-distances matrix is available online in Supplementary Table S2. We found two haplotypes in both 18S rDNA and 28S rDNA sequences, and three haplotypes of the new species in both COI and ITS-2 sequences (Fig. 9A, B). A maximum of 13 mutations of the COI sequence were observed between the OGA7 and MW strains. In *M.*

Table 3B. Measurements of hatchlings and juveniles of *Milnesium pacificum* sp. nov. OGA7 strain.

	Hatchling					Juvenile				
	n	μm		*pt*		*n*	μm		*pt*	
		Range	Mean ± SD	Range	Mean ± SD		Range	Mean ± SD	Range	Mean ± SD
Body length	4	294–364	316 ± 33	*1216–1524*	*1302 ± 149*	2	464–465	464 ± 1	*1403–1466*	*1435 ± 44*
Peribuccal papillae length	4	3.8–5.1	4.4 ± 0.6	*15.8–20.9*	*18.2 ± 2.5*	2	6.4–6.9	6.6 ± 0.3	*20.3–20.7*	*20.5 ± 0.3*
Lateral papillae length	4	2.9–4.1	3.5 ± 0.6	*12.1–17.1*	*14.4 ± 2.3*	2	3.7–4.7	4.2 ± 0.7	*11.7–14.2*	*13.0 ± 1.8*
Buccal tube										
Length	4	23.7–25.4	24.3 ± 0.8	*100.0–100.0*	*100.0 ± 0.0*	2	31.6–33.1	32.4 ± 1.1	*100.0–100.0*	*100.0 ± 0.0*
Stylet support	4	14.6–17.6	16.3 ± 1.2	*61.5–69.2*	*67.0 ± 3.7*	2	19.4–20.2	19.8 ± 0.6	*58.7–63.9*	*61.3 ± 3.7*
Anterior width	4	7.3–9.7	8.5 ± 1.0	*30.7–39.9*	*34.8 ± 4.0*	2	12.5–12.8	12.6 ± 0.2	*38.5–39.5*	*39.0 ± 0.7*
Posterior width	4	5.9–7.6	7.0 ± 0.8	*24.7–31.6*	*29.0 ± 3.0*	2	10.8–12.4	11.6 ± 1.2	*32.5–39.2*	*35.9 ± 4.8*
Standard width	4	7.2–8.6	7.7 ± 0.7	*30.1–35.8*	*31.9 ± 2.6*	2	11.8–12.5	12.1 ± 0.5	*35.5–39.4*	*37.4 ± 2.8*
Standard/length	4	30.1–35.8	31.9 ± 2.6			2	35.5–39.4	37.4 ± 2.8		
Posterior/anterior	4	69.5–98.2	84.0 ± 12.5			2	84.4–99.3	91.8 ± 10.5		
Claw1										
External primary branch	2	11.4–14.0	12.7 ± 1.9	*44.7–58.8*	*51.8 ± 10.0*	2	18.2–18.7	18.5 ± 0.3	*55.1–59.1*	*57.1 ± 2.8*
External base + secondary branch	3	9.2–12.7	11.1 ± 1.8	*38.0–53.2*	*45.4 ± 7.6*	2	11.2–11.4	11.3 ± 0.1	*34.5–35.5*	*35.0 ± 0.7*
External spur										
Internal primary branch	2	10.0–14.1	12.1 ± 2.9	*39.5–59.0*	*49.2 ± 13.8*	2	14.5–16.6	15.6 ± 1.5	*45.8–50.2*	*48.0 ± 3.1*
Internal base + secondary branch	3	5.7–9.5	7.1 ± 2.1	*23.7–37.4*	*28.7 ± 7.5*	2	11.2–11.6	11.4 ± 0.3	*35.1–35.5*	*35.3 ± 0.3*
Internal spur						2	2.4–3.8	3.1 ± 1.0	*7.6–11.5*	*9.5 ± 2.7*
Basal thickening width	4	1.6–2.9	1.9 ± 0.6	*6.1–12.0*	*7.9 ± 2.7*	2	2.3–3.1	2.7 ± 0.6	*6.8–9.9*	*8.4 ± 2.2*
Claw2										
External primary branch	4	7.0–12.5	9.3 ± 2.4	*29.6–52.1*	*38.3 ± 10.6*	2	14.1–14.8	14.4 ± 0.5	*44.5–44.6*	*44.6 ± 0.0*
External base + secondary branch	4	7.0–11.2	9.0 ± 1.8	*29.7–47.1*	*37.2 ± 7.8*	2	14.6–15.6	15.1 ± 0.7	*43.9–49.2*	*46.5 ± 3.7*
External spur										
Internal primary branch	3	7.8–15.7	10.4 ± 4.6	*30.5–65.8*	*43.1 ± 19.7*	2	14.7–16.1	15.4 ± 1.0	*46.6–48.6*	*47.6 ± 1.4*
Internal base + secondary branch	4	6.2–9.5	7.8 ± 1.4	*24.4–39.8*	*32.4 ± 6.4*	2	11.3–14.3	12.8 ± 2.1	*34.2–45.1*	*39.6 ± 7.7*
Internal spur						2	3.2–4.0	3.6 ± 0.6	*10.3–12.2*	*11.2 ± 1.4*
Basal thickening width	4	1.6–2.6	1.9 ± 0.4	*6.5–10.8*	*7.9 ± 2.0*	2	2.6–3.1	2.9 ± 0.3	*8.0–9.8*	*8.9 ± 1.3*
Claw3										
External primary branch	4	9.3–15.9	12.0 ± 3.0	*39.0–66.7*	*49.5 ± 13.2*	2	14.8–15.3	15.1 ± 0.4	*44.6–48.5*	*46.6 ± 2.8*
External base + secondary branch	4	7.5–10.3	8.9 ± 1.3	*31.6–43.3*	*36.6 ± 5.9*	2	11.7–12.1	11.9 ± 0.3	*36.6–37.0*	*36.8 ± 0.3*
External spur										
Internal primary branch	4	7.4–12.0	10.0 ± 2.2	*31.0–49.7*	*41.3 ± 9.1*	2	14.4–16.9	15.6 ± 1.8	*45.5–50.9*	*48.2 ± 3.9*
Internal base + secondary branch	4	6.8–9.3	8.2 ± 1.2	*26.9–38.6*	*33.9 ± 5.4*	2	12.5–13.5	13.0 ± 0.7	*37.6–42.6*	*40.1 ± 3.5*
Internal spur						2	3.0–4.6	3.8 ± 1.1	*9.6–13.8*	*11.7 ± 3.0*
Basal thickening width	4	1.7–2.2	2.0 ± 0.2	*7.2–8.9*	*8.1 ± 0.8*	2	1.6–2.2	1.9 ± 0.5	*5.0–6.7*	*5.8 ± 1.3*
Claw4										
Anterior primary branch	4	9.7–12.0	10.8 ± 1.0	*40.0–50.3*	*44.7 ± 4.6*	2	15.7–18.6	17.1 ± 2.1	*49.5–56.2*	*52.8 ± 4.7*
Anterior base + secondary branch	4	6.6–9.5	8.1 ± 1.2	*27.9–39.6*	*33.3 ± 4.9*	2	12.9–17.6	15.3 ± 3.3	*39.1–55.7*	*47.4 ± 11.8*
Anterior spur						2	3.1–5.0	4.1 ± 1.4	*9.4–15.9*	*12.7 ± 4.6*
Posterior primary branch	3	10.3–12.3	11.4 ± 1.0	*40.6–51.5*	*46.6 ± 5.5*	2	14.9–21.4	18.1 ± 4.6	*47.0–64.5*	*55.7 ± 12.4*
Posterior base + secondary branch	4	6.7–9.7	8.3 ± 1.2	*28.3–40.7*	*34.1 ± 5.2*	2	12.5–17.2	14.8 ± 3.3	*39.5–51.9*	*45.7 ± 8.8*
Posterior spur										
Basal thickening width	4	1.5–2.3	1.9 ± 0.4	*6.2–9.9*	*8.0 ± 1.8*	2	2.9–3.1	3.0 ± 0.1	*8.9–9.7*	*9.3 ± 0.6*

Table 3C. Measurements of *Milnesium pacificum* sp. nov. MW strain.

	Hatchling					Juvenile			Adult				
	n	μm		*pt*		*n*	μm	*pt*	*n*	μm		*pt*	
		Range	Mean ± SD	Range	Mean ± SD		Range	Range		Range	Mean ± SD	Range	Mean ± SD
Body length	4	314–377	358 ± 29	*1181–1503*	*1375 ± 137*	2	456–493	*1507*	3	640–795	721 ± 78	*1663–1776*	*1727 ± 58*
Peribuccal papillae length	5	3.1–6.1	4.8 ± 1.1	*12.8–26.7*	*19.4 ± 5.1*	1	6.4	*21.1*	2	8.8–10.9	9.9 ± 1.5	*20.2–24.4*	*22.3 ± 3.0*
Lateral papillae length	3	3.0–3.8	3.5 ± 0.4	*11.7–15.2*	*13.6 ± 1.8*	1	4.9	*16.2*	1	4.6		*10.6*	
Buccal tube													
Length	6	23.0–26.6	25.2 ± 1.5	*100.0–100.0*	*100.0 ± 0.0*	1	30.3	*100.0*	3	36.8–44.8	41.8 ± 4.4	*100.0–100.0*	*100.0 ± 0.0*
Stylet support	6	15.5–18.2	17.0 ± 1.0	*65.8–69.4*	*67.3 ± 1.4*	1	19.3	*63.8*	3	23.7–27.7	25.9 ± 2.0	*60.1–64.4*	*62.1 ± 2.2*
Anterior width	6	8.1–9.4	8.4 ± 0.5	*31.7–35.5*	*33.5 ± 1.7*	1	11.4	*37.6*	3	15.4–18.5	17.4 ± 1.0	*37.6–46.6*	*41.8 ± 4.6*
Posterior width	6	6.6–8.5	7.3 ± 0.7	*25.0–33.7*	*29.1 ± 3.5*	1	8.1	*26.9*	3	15.4–16.3	15.7 ± 0.5	*34.3–42.0*	*37.8 ± 3.9*
Standard width	6	7.4–8.3	7.9 ± 0.4	*28.5–33.5*	*31.3 ± 2.1*	1	9.8	*32.2*	3	16.3–16.9	16.5 ± 0.3	*37.4–44.3*	*39.8 ± 3.9*
Standard/length	6	28.5–33.5	31.3 ± 2.1			1	32.2		3	37.4–44.3	39.8 ± 3.9	*84.4–120.5*	
Posterior/anterior	6	76.7–104.7	87.1 ± 10.9			1	71.6		3	83.2–99.0	90.8 ± 7.9	*185.7–245.1*	
									3				
Claw1									3				
External primary branch	6	9.8–13.1	11.2 ± 1.1	*39.2–49.3*	*44.5 ± 4.2*	1	15.1	*49.8*	3	19.5–27.9	23.4 ± 4.2	*52.4–62.2*	*55.9 ± 5.5*
External base + secondary branch	6	7.4–12.3	9.5 ± 1.7	*29.6–53.3*	*37.9 ± 8.1*	2	7.9–12.0	*26.1*	3	16.6–20.1	18.5 ± 1.7	*42.0–45.8*	*44.4 ± 2.0*
External spur									3				
Internal primary branch	6	6.3–11.6	9.8 ± 1.9	*27.6–45.2*	*38.8 ± 6.8*	2	12.6–19.2	*41.5*	3	16.5–21.0	18.7 ± 2.3	*42.5–47.0*	*44.8 ± 2.2*
Internal base + secondary branch	6	8.4–10.5	9.0 ± 0.8	*32.4–39.5*	*35.8 ± 2.4*	2	11.9–12.7	*39.2*	3	17.6–20.8	19.2 ± 1.6	*44.1–47.9*	*46.2 ± 1.9*
Internal spur						1	1.6	*5.3*	2	3.9–4.3	4.1 ± 0.3	*8.8–11.7*	*10.3 ± 2.1*
Basal thickening width	6	1.9–2.3	2.1 ± 0.2	*7.2–10.1*	*8.5 ± 1.0*	2	2.4–3.4	*7.9*	3	2.7–4.6	4.0 ± 1.1	*7.5–10.3*	*9.4 ± 1.7*
Claw2													
External primary branch	6	9.8–15.4	12.5 ± 2.4	*40.6–57.9*	*49.5 ± 7.8*	2	16.2–18.0	*53.7*	3	20.4–23.3	22.2 ± 1.6	*51.2–55.6*	*53.3 ± 2.2*
External base + secondary branch	6	7.9–12.1	10.1 ± 1.5	*34.3–46.3*	*39.9 ± 5.1*	2	10.7–11.1	*35.2*	3	16.0–26.7	20.7 ± 5.5	*43.1–61.1*	*49.2 ± 10.3*
External spur													
Internal primary branch	6	9.1–11.7	10.5 ± 0.9	*37.9–48.0*	*41.7 ± 3.7*	2	11.6–17.5	*38.4*	3	19.0–26.7	22.7 ± 3.9	*50.9–59.6*	*54.0 ± 4.9*
Internal base + secondary branch	6	8.3–9.5	8.7 ± 0.4	*31.2–38.3*	*34.8 ± 3.0*	2	13.8–15.6	*45.6*	3	17.5–20.4	19.1 ± 1.4	*44.1–47.7*	*45.8 ± 1.8*
Internal spur						2	1.8–2.9	*6.0*	3	3.2–4.3	3.7 ± 0.6	*7.1–10.2*	*9.1 ± 1.7*
Basal thickening width	6	1.8–2.6	2.1 ± 0.3	*7.3–10.0*	*8.4 ± 0.9*	2	2.5–3.2	*8.1*	3	2.5–3.9	3.2 ± 0.7	*6.9–8.7*	*7.5 ± 1.0*
Claw3													
External primary branch	5	12.6–15.4	14.0 ± 1.2	*48.3–61.6*	*55.0 ± 5.3*	2	18.0–18.8	*62.2*	3	21.1–28.2	24.2 ± 3.7	*51.7–64.5*	*57.9 ± 6.4*
External base + secondary branch	6	7.1–11.9	10.0 ± 2.0	*29.6–46.3*	*39.4 ± 6.4*	2	9.2–13.3	*30.3*	3	19.3–23.0	21.0 ± 1.9	*43.1–55.9*	*50.5 ± 6.6*
External spur													
Internal primary branch	5	10.3–14.6	12.2 ± 2.0	*41.2–58.0*	*47.8 ± 7.4*	2	14.7–15.7	*48.6*	3	19.4–25.3	22.0 ± 3.0	*47.6–57.7*	*52.7 ± 5.1*
Internal base + secondary branch	6	8.8–11.2	10.3 ± 1.1	*38.2–44.3*	*40.9 ± 2.5*	2	11.4–13.4	*44.3*	3	15.9–20.5	18.2 ± 2.3	*41.0–46.8*	*43.7 ± 2.9*
Internal spur						2	2.3–2.3	*7.5*	3	3.4–5.4	4.3 ± 1.0	*7.5–12.4*	*10.5 ± 2.6*
Basal thickening width	6	1.9–2.5	2.2 ± 0.3	*7.8–9.4*	*8.8 ± 0.7*	2	2.6–2.8	*8.6*	3	2.2–4.2	3.0 ± 1.0	*5.9–9.4*	*7.1 ± 1.9*
Claw4													
Anterior primary branch	5	10.7–13.1	12.1 ± 1.0	*42.4–49.7*	*47.0 ± 3.3*	2	15.5–20.0	*65.9*	3	21.7–23.5	22.6 ± 0.9	*48.4–61.7*	*54.6 ± 6.7*
Anterior base + secondary branch	6	8.8–12.4	9.6 ± 1.4	*33.2–49.5*	*38.1 ± 6.0*	2	10.1–11.3	*33.2*	3	15.3–22.2	19.6 ± 3.7	*41.5–49.5*	*46.5 ± 4.4*
Anterior spur						2	1.4–3.9	*4.8*	3	3.7–4.8	4.2 ± 0.6	*8.9–11.1*	*10.0 ± 1.1*
Posterior primary branch	5	12.4–15.8	13.6 ± 1.4	*47.7–59.4*	*52.8 ± 5.0*	2	16.5–16.5	*54.5*	3	17.2–29.2	23.2 ± 6.0	*38.4–66.8*	*55.9 ± 15.3*
Posterior base + secondary branch	6	8.4–11.1	9.4 ± 1.0	*31.6–42.5*	*37.4 ± 3.6*	2	10.2–11.2	*33.8*	3	12.5–22.1	18.4 ± 5.1	*34.1–49.3*	*43.4 ± 8.1*
Posterior spur													
Basal thickening width	6	1.9–2.8	2.3 ± 0.4	*7.1–11.7*	*9.3 ± 1.8*	2	2.9–3.0	*10.1*	3	2.4–4.9	3.6 ± 1.2	*6.6–10.9*	*8.5 ± 2.2*

Table 4. Life history of *Milnesium pacificum* sp. nov.

	OGA7	MW
clutch size (eggs/clutch)	6, 7, 7, 7, 9, 10, 11, 13	8, 9, 9, 9, 9, 19, 11, 11, 12, 12, 12
duration of embryogenesis (days)	6, 6, 7, 7, 7, 8, 8	5, 5, 6, 6, 7, 8
day of first oviposition (days)	26, 26, 27, 27	50, 55, 62, 64

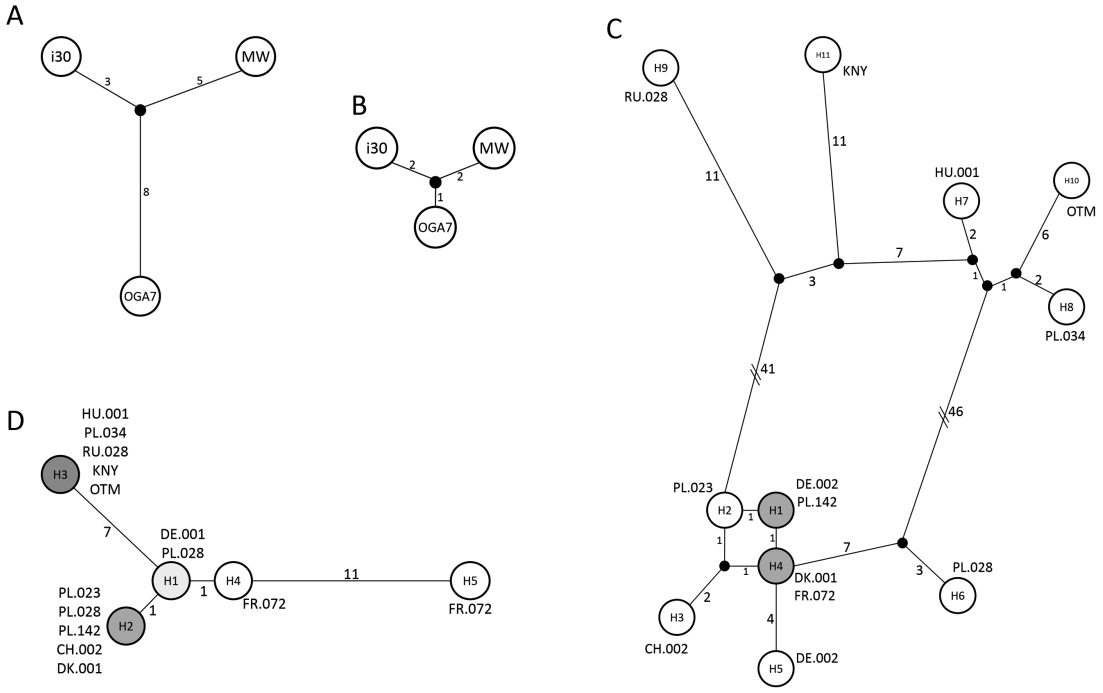

Fig. 9. Haplotype networks. **(A)** *Milnesium pacificum* sp. nov., COI. **(B)** *Milnesium pacificum* sp. nov., ITS-2. **(C)** *Milnesium tardigradum* Doyère, 1840 s.s., COI. **(D)** *Milnesium tardigradum* Doyère, 1840 s.s., ITS-2. The size of circles is proportional to the number of haplotypes. The numbers on the branches indicate the number of mutations between each haplotype.

pacificum sp. nov., the mean intra-species p-distances were 0.3% (18s rDNA), 0.4% (28s rDNA), 6.6% (COI) and 1.6% (ITS-2).

Genetic differential diagnosis. Based on the 18S rDNA sequences, the most similar species to *M. pacificum* sp. nov. (LC511088 and LC511092) were *Milnesium* sp. BR.007 and *M. tardigradum* OTM (MK484086 and LC511098, 0.0–0.3%, respectively); the least similar was *Milnesium* sp. (HM187581, 1.9–2.2%).

Based on the 28S rDNA sequences, the most similar species to *M. pacificum* sp. nov. (LC511089 and LC511093) was *Milnesium* sp. (AY210826, 0.6–0.8%); the least similar was *Milnesium* sp. PH.014 (MK484004, 10.3–10.5%).

Based on the COI sequences, the most similar species to *M. pacificum* sp. nov. (LC511090, LC511094, and LC511096) was *Milnesium* sp. BR.007 (MK492306, 10.0–10.4%); the least similar was *Milnesium* sp. (KJ857002, 24.6–25.1%).

With ITS-2 sequences, the most similar species to the new species (LC511091, LC511095, and LC511097) was *Milnesium* sp. BR.007 (MK484019, 1.9%), and the least similar was *Milinesium variefidum* Morek et al., 2016 (KT951666–7, 24.9–26.1%).

Phylogeny. The ML and BI trees were expected to be similar; however, the supporting bootstrap values of the ML tree were often low for COI, ITS-2, and concatenated trees. Thus, we provide the concatenated tree constructed by BI (Fig. 10), which showed that the new species belongs to a clade that includes *Milnesium* sp. BR.007 (MK484019+MK492306) and *Milnesium lagniappe* Meyer et al., 2013 (MH751518+MH746111).

Species delimitation. Genetic species delimitations indicated that the new species and *Milnesium* sp. BR.007 were different species in the COI and concatenated trees (PTP supports: 87.2–100%); however, the four populations (OGA7, MW, i30, and BR.007) were recognized as a cluster in the ITS-2 tree (PTP support: 86.8%).

Milnesium tardigradum Doyère, 1840
(Figs. 8B, 11, 12; Table 5)

We also identified *M. tardigradum* s.s. in Japan for the first time. Two strains of *M. tardigradum* s.s. named KNY (KumaNoYu) from Kumanoyu and OTM (OriTsuMe) from Mt. Oritsume were cultured and examined. Both strains have been maintained in Keio University.

Material examined. The following 11 specimens of KNY were examined and deposited in National Museum of Nature and Science, Tsukuba, Japan: five adult females (NSMT-Tg-261–265), a juvenile (NMST-Tg-266) and five hatchlings

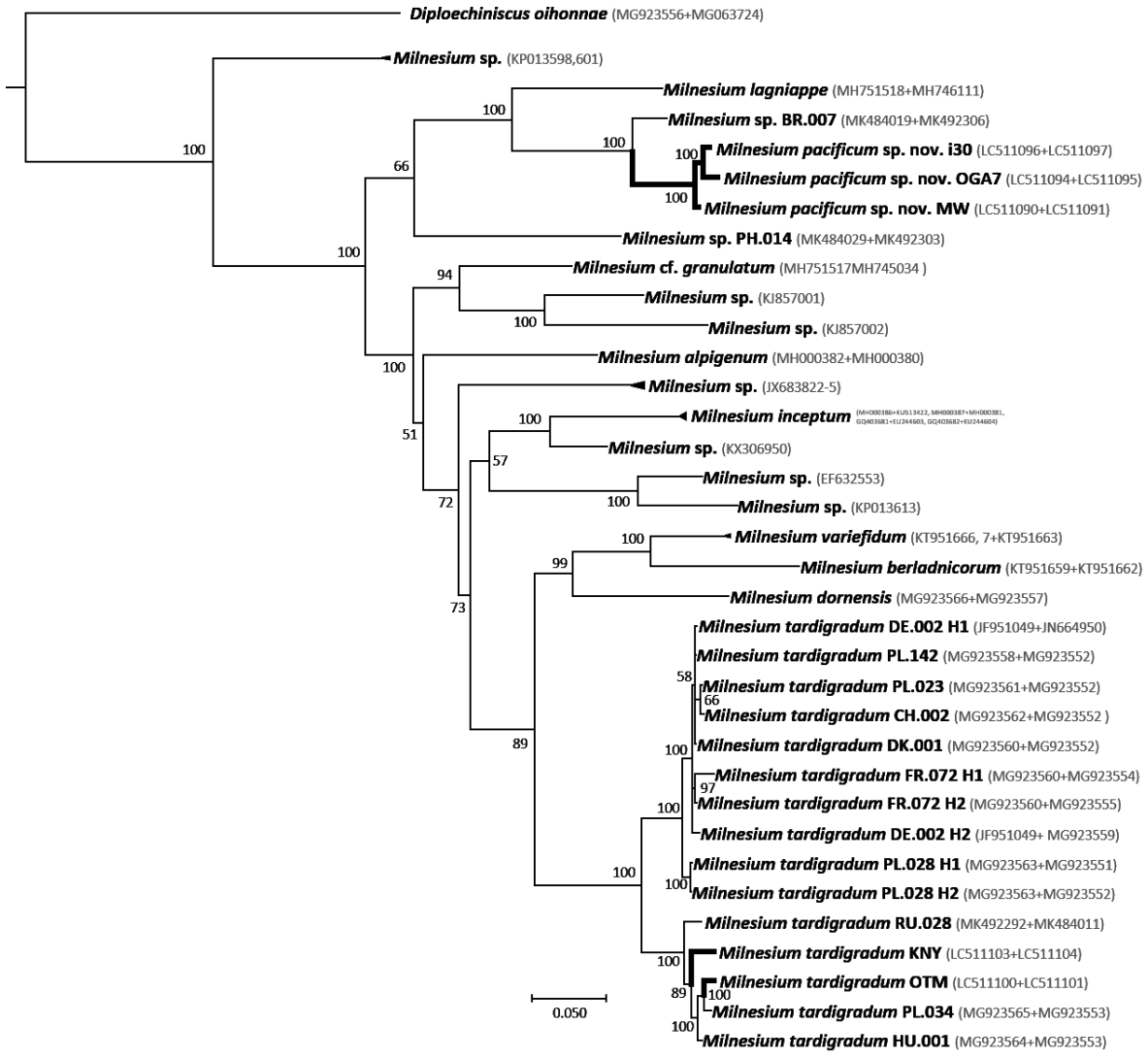

Fig. 10. A Bayesian phylogenetic tree based on COI+ITS-2 sequences. The number on each node indicates Bayesian posterior probability.

(NSMT-Tg-267–271). In addition, nine specimens of OTM were also examined: two adult females (NSMT-Tg-272 and 273), one juvenile (NMST-Tg-274), and six hatchlings (NSMT-Tg-275–280).

Description. Adult *M. tardigradum* appears transparently brownish yellow under dark-field illumination, or transparent under transmitted light, with three brownish longitudinal stripes. Eyes present but not visible in mounted specimens. Six peribuccal and two lateral papillae and six (4+2) peribuccal lamellae (Fig. 12B, F). The adult and juvenile CC were [2-3]-[3-2]; however, all of the hatchlings of both strains showed [3-3]-[3-3], indicating early negative CC change (Figs. 11, 12). Morphometric data are available in Table 5 and Supplementary Table S1.

Localities. The *Milnesium tardigradum* KNY strain was from Kumanoyu, Yamanouchi City, Nagano Prefecture, Japan (36°41′02″N, 138°29′46″E), and the OTM strain was from Mt. Oritsume, Ninohe City, Iwate Prefecture, Japan (40°16′07″N, 141°22′18″E).

Habitat. All specimens were obtained from moss samples on concrete on the ground.

Sequence data. DNA sequences are available online in Supplementary Text S1 and in GenBank (LC511098 to LC511104).

We obtained one 18S rDNA (LC511098) from the OTM strain, showing 20 mutations between OTM and other *M. tardigradum* (MG9125541+MK484076) in the haplotype network. The two obtained sequences of 28S rDNA (LC511102 and LC511099 from KNY and OTM, respectively) represented the same haplotype as that of *M. tardigradum* from Russia (RU.028, MK483984). The two new haplotypes of COI sequences (LC511103 and LC511100 from KNY and OTM, respectively) were included in the subclade γ, showing 41–46 mutations among the tested populations (Fig. 9C). The ITS-2 sequences of KNY and OTM (LC511104 and LC511101) belonged to haplotype 3 (Fig. 9D). In *M. tardigradum*, the mean p-distances between the haplotypes were 0.3% (18s rDNA), 0.6% (28s rDNA), 1.9% (COI) and 0.7% (ITS-2), respectively.

Genetic distance. The ranges of p-distances between the previously sequenced European and Asian populations of the *M. tardigradum* populations KNY and OTM strains

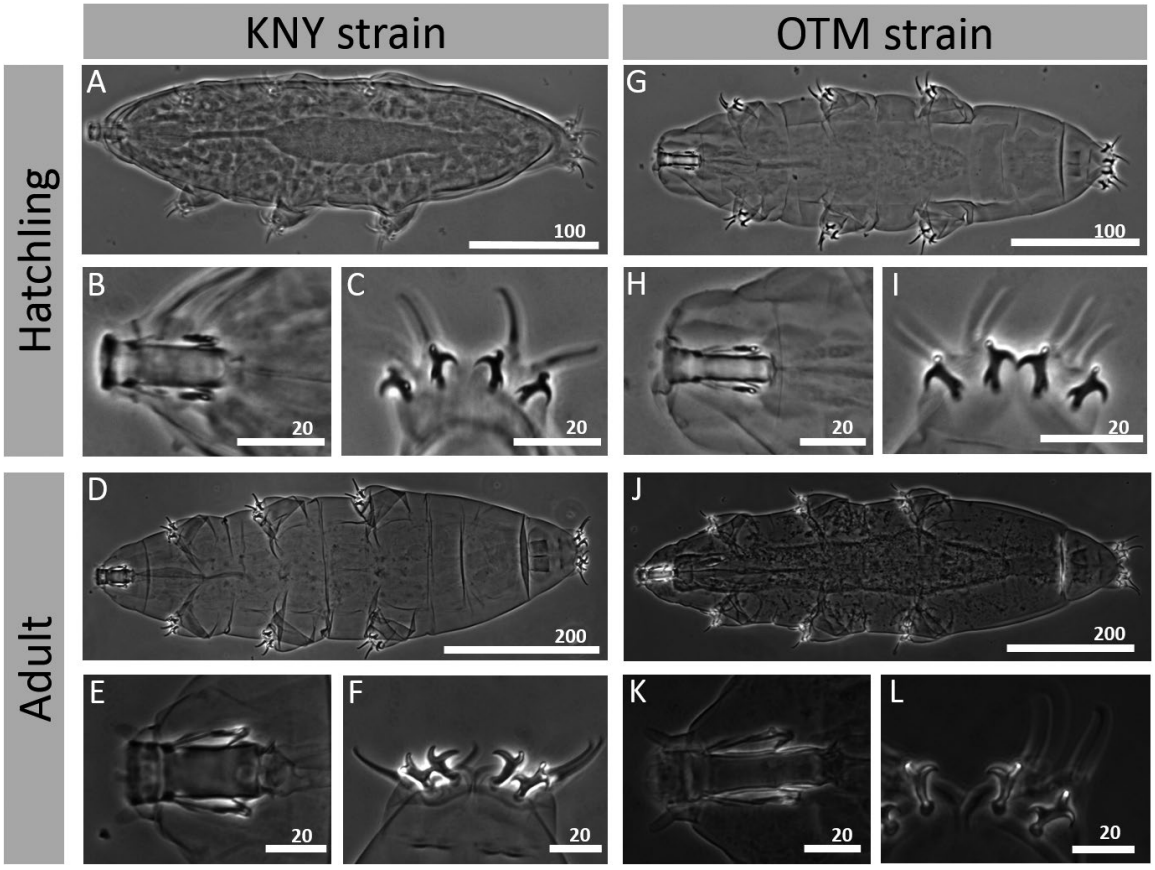

Fig. 11. *Milnesium tardigradum* Doyère, 1840 s.s., phase-contrast micrographs. **(A–F)** KNY strain. **(G–L)** OTM strain. **(A, G)** Hatchling, ventral view. **(B, H)** Hatchling, buccal tube. **(C, I)** Hatchling, claw IV. **(D, J)** Adult, ventral view. **(E, K**) Adult, buccal tube. **(F, L)** Adult, claw IV. Scale bars in μm.

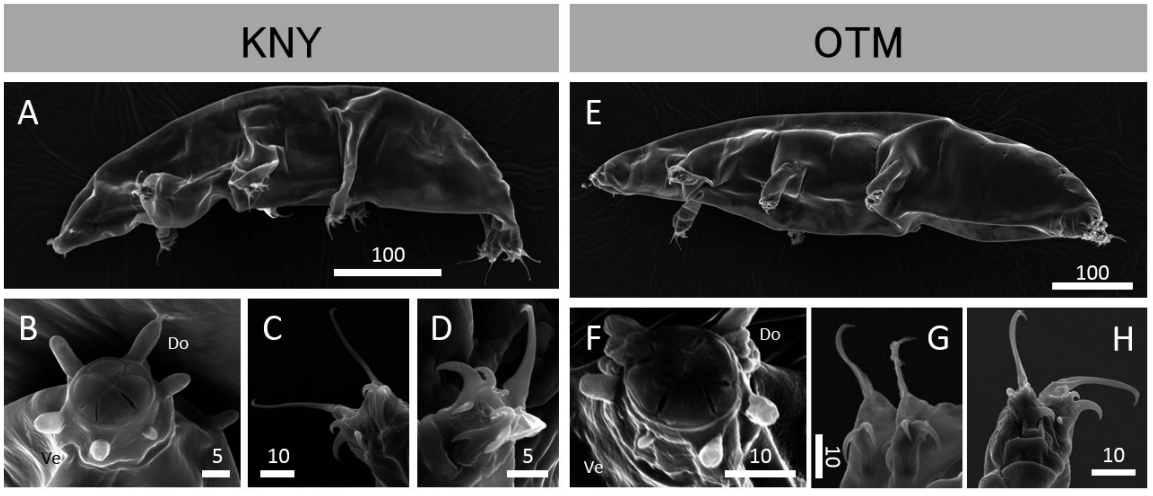

Fig. 12. *Milnesium tardigradum* Doyère, 1840 s.s., SEM images. **(A–D)** KNY strain. **(E–H)** OTM strain. **(A, E)** Entire body, lateral view. **(B, F)** Mouth configuration. **(C, G)** Claws in hatchlings. **(D, H)** Claws in adults; white arrowheads indicating accessory points on the primary branches. Abbreviations: Do, dorsal; Ve, ventral. Scale bars in μm.

were 0.3–0.7% in 18S rDNA (mean 0.5%), 0.0–0.8% in 28S rDNA (mean 0.32%), 0.9–12.3% in COI (mean 12.3%), and 0.0–5.1% in ITS-2 (mean 1.8%), respectively.

Species delimitation. Genetic species delimitations in the COI tree showed that the subclade α+β, *M. tardigradum* RU.028, KNY, and OTM+subclade γ were delimited (PTP supports: 47.9%, 97.0%, 92.5% and 87.9% respectively); in contrast, all strains of *M. tardigradum* were clustered in a group by the ITS-2 tree (PTP support: 76.2%). The delimitations were supported by 71.6% and 91.6% in the subclade γ+OTM+KNY and subclade α+β in the concatenated tree (Fig. 10).

Table 5A. Measurements of *Milnesium tardigradum* KNY strain.

	Hatchling					Juvenile			Adult				
	n	µm		*pt*		*n*	µm	*pt*	*n*	µm		*pt*	
		Range	Mean ± SD	Range	Mean ± SD		Range	Range		Range	Mean ± SD	Range	Mean ± SD
Body length	5	278–376	327 ± 42	*1050–1536*	*1296 ± 177*	1	516	*1393*	5	537–636	573 ± 39	*1524–1856*	*1648 ± 144*
Peribuccal papillae length	5	4.5–5.4	4.8 ± 0.4	*17.7–19.7*	*18.8 ± 0.9*	0			4	7.7–8.9	8.5 ± 0.6	*22.7–26.1*	*24.7 ± 1.6*
Lateral papillae length	5	2.7–5.3	3.7 ± 1.0	*10.0–19.9*	*14.7 ± 4.0*	1	7.0	*18.8*	5	4.8–7.0	6.3 ± 0.8	*14.2–21.3*	*18.0 ± 2.6*
Buccal tube													
Length	5	22.8–27.4	25.3 ± 1.8	*100.0–100.0*	*100.0 ± 0.0*	1	37.1	*100.0*	5	32.9–37.3	34.8 ± 1.7	*100.0–100.0*	*100.0 ± 0.0*
Stylet support	5	15.4–18.5	17.0 ± 1.4	*61.1–74.3*	*67.5 ± 5.3*	1	23.8	*64.1*	5	23.0–24.2	23.5 ± 0.5	*61.6–71.3*	*67.7 ± 4.4*
Anterior width	5	10.0–12.4	10.8 ± 1.0	*39.0–48.1*	*42.7 ± 3.9*	1	15.5	*41.8*	5	15.5–19.1	17.9 ± 1.5	*43.5–55.8*	*51.5 ± 4.9*
Posterior width	5	7.4–10.3	8.8 ± 1.1	*28.0–41.1*	*34.9 ± 5.0*	1	12.3	*33.1*	5	9.5–18.0	13.7 ± 3.6	*26.5–52.5*	*39.6 ± 11.0*
Standard width	5	6.7–9.2	7.9 ± 1.1	*25.5–37.5*	*31.3 ± 5.3*	1	14.4	*38.9*	5	12.1–16.6	14.2 ± 1.9	*34.4–48.3*	*40.7 ± 6.0*
Standard/length	5	25.5–37.5	31.3 ± 5.3			1	38.9		5	34.4–48.3	40.7 ± 6.0		
Posterior/anterior	5	71.9–85.6	81.4 ± 5.5			1	79.1		5	60.9–94.1	76.1 ± 15.2		
Claw1													
External primary branch	5	11.4–12.9	12.0 ± 0.6	*44.5–56.6*	*47.8 ± 5.0*	1	18.7	*50.4*	5	16.3–19.0	17.9 ± 1.1	*47.9–55.8*	*51.4 ± 3.1*
External base + secondary branch	5	7.3–9.4	8.4 ± 0.8	*29.0–36.9*	*33.3 ± 2.9*	1	13.0	*35.1*	5	12.8–13.5	13.0 ± 0.3	*34.3–38.8*	*37.4 ± 1.9*
External spur	4	3.0–3.6	3.3 ± 0.3	*11.4–14.0*	*12.9 ± 1.1*								
Internal primary branch	5	10.5–11.7	11.1 ± 0.5	*39.5–51.3*	*44.3 ± 4.5*	1	16.7	*45.2*	5	13.8–18.1	16.5 ± 1.7	*40.6–52.8*	*47.5 ± 5.6*
Internal base + secondary branch	5	7.6–9.0	8.4 ± 0.6	*30.0–35.8*	*33.3 ± 2.2*	1	12.6	*33.9*	5	12.4–14.4	13.5 ± 0.8	*33.3–43.7*	*38.9 ± 4.0*
Internal spur	5	3.1–4.1	3.7 ± 0.4	*13.6–15.6*	*14.8 ± 0.8*	1	5.1	*13.8*	5	4.0–5.7	4.9 ± 0.6	*11.3–16.7*	*14.2 ± 2.0*
Basal thickening width	5	1.7–2.7	2.2 ± 0.4	*6.9–9.9*	*8.6 ± 1.1*	1	2.8	*7.6*	5	2.9–3.7	3.2 ± 0.4	*8.1–10.9*	*9.3 ± 1.1*
Claw2													
External primary branch	5	11.3–13.8	12.4 ± 1.0	*44.0–54.5*	*49.3 ± 4.2*	1	18.0	*48.5*	5	16.5–18.4	17.6 ± 0.7	*47.3–56.1*	*50.5 ± 3.5*
External base + secondary branch	5	7.2–9.7	8.5 ± 1.0	*28.4–35.5*	*33.6 ± 3.0*	1	11.9	*32.2*	5	11.4–14.3	13.2 ± 1.2	*32.0–41.9*	*37.9 ± 4.7*
External spur	4	3.4–3.7	3.6 ± 0.2	*12.9–15.3*	*13.8 ± 1.0*								
Internal primary branch	5	10.6–12.4	11.5 ± 0.7	*41.1–52.9*	*45.5 ± 4.7*	1	16.5	*44.5*	5	15.2–17.9	16.6 ± 1.2	*40.7–52.2*	*47.7 ± 4.3*
Internal base + secondary branch	5	7.6–9.4	8.5 ± 0.7	*30.0–37.5*	*33.6 ± 3.0*	1	12.4	*33.5*	5	12.6–13.3	12.9 ± 0.3	*33.9–39.8*	*37.1 ± 2.4*
Internal spur	5	3.3–4.6	4.1 ± 0.6	*13.4–18.6*	*16.3 ± 2.2*	1	6.2	*16.6*	5	5.0–6.6	6.1 ± 0.7	*14.1–19.8*	*17.6 ± 2.5*
Basal thickening width	5	1.6–2.5	2.0 ± 0.3	*6.4–8.9*	*8.0 ± 1.0*	1	3.2	*8.6*	5	3.1–3.8	3.3 ± 0.3	*8.2–11.0*	*9.5 ± 1.3*
Claw3													
External primary branch	5	10.9–14.9	12.4 ± 1.5	*43.0–56.2*	*48.9 ± 5.1*	1	18.2	*49.1*	5	17.4–20.8	18.9 ± 1.3	*48.5–60.6*	*54.4 ± 4.4*
External base + secondary branch	5	7.7–10.6	8.7 ± 1.2	*30.3–39.2*	*34.3 ± 4.4*	1	11.7	*31.6*	5	11.3–14.1	13.0 ± 1.2	*34.4–41.2*	*37.3 ± 3.5*
External spur	3	3.4–4.5	3.8 ± 0.6	*13.5–16.5*	*14.7 ± 1.6*								
Internal primary branch	5	10.0–13.4	11.7 ± 1.3	*39.5–50.7*	*46.2 ± 4.3*	1	17.0	*45.8*	5	15.4–18.1	16.8 ± 1.2	*41.2–53.3*	*48.4 ± 5.0*
Internal base + secondary branch	4	7.4–9.5	8.5 ± 0.9	*29.2–38.9*	*34.2 ± 3.9*	1	12.3	*33.1*	5	11.9–13.4	12.9 ± 0.6	*34.6–39.0*	*37.0 ± 1.9*
Internal spur	5	3.1–4.6	3.8 ± 0.7	*11.6–17.0*	*15.0 ± 2.2*	1	6.0	*16.1*	5	4.8–7.0	6.1 ± 0.8	*14.6–20.5*	*17.4 ± 2.3*
Basal thickening width	4	1.8–2.3	2.1 ± 0.2	*7.4–10.1*	*8.5 ± 1.2*	1	3.8	*10.1*	5	2.9–3.9	3.3 ± 0.4	*8.5–10.9*	*9.3 ± 1.0*
Claw4													
Anterior primary branch	5	11.7–14.4	12.9 ± 1.0	*49.1–53.3*	*51.2 ± 1.8*	1	19.7	*53.2*	5	18.7–23.8	21.2 ± 2.1	*52.5–69.6*	*61.2 ± 7.8*
Anterior base + secondary branch	5	8.7–10.3	9.5 ± 0.7	*34.5–42.0*	*37.5 ± 2.9*	1	13.9	*37.4*	5	13.5–18.4	15.6 ± 2.1	*36.1–53.7*	*45.1 ± 7.4*
Anterior spur	5	2.6–5.1	4.1 ± 1.0	*11.4–19.8*	*16.2 ± 3.5*	1	6.3	*16.9*	5	5.4–7.6	6.5 ± 0.8	*16.3–22.0*	*18.8 ± 2.2*
Posterior primary branch	5	10.3–14.4	12.7 ± 1.6	*15.0–22.6*	*19.0 ± 2.7*	1	18.1	*28.2*	5	18.6–24.8	21.1 ± 2.7	*28.0–34.9*	*31.2 ± 2.8*
Posterior base + secondary branch	5	7.7–10.8	9.8 ± 1.3	*30.6–43.8*	*39.0 ± 5.0*	1	14.8	*40.0*	5	14.8–18.0	16.0 ± 1.3	*39.7–52.4*	*46.2 ± 4.9*
Posterior spur	5	3.1–4.7	4.3 ± 0.7	*13.4–18.8*	*16.8 ± 2.1*								
Basal thickening width	5	1.8–2.5	2.2 ± 0.3	*6.5–9.9*	*8.6 ± 1.4*	1	4.2	*11.3*	5	2.7–3.6	3.1 ± 0.3	*7.3–10.8*	*8.9 ± 1.2*

Table 5B. Measurements of *Milnesium tardigradum* OTM strain.

	Hatchling					Juvenile			Adult				
	n	μm		*pt*		*n*	μm	*pt*	*n*	μm		*pt*	
		Range	Mean ± SD	Range	Mean ± SD		Range	Range		Range	Mean ± SD	Range	Mean ± SD
Body length	6	306–356	331 ± 18	*1153–1428*	*1290 ± 87*	1	491	*1265*	2	603–765	684 ± 114	*1432–1765*	*1599 ± 236*
Peribuccal papillae length	4	4.3–5.8	5.0 ± 0.8	*16.5–24.1*	*19.3 ± 3.6*	1	8.2	*21.1*	1	9.0		*20.8*	
Lateral papillae length	5	4.2–5.0	4.6 ± 0.3	*15.8–20.1*	*17.8 ± 1.5*	1	6.6	*17.0*	1	7.5		*17.4*	
Buccal tube													
Length	6	24.0–27.5	25.7 ± 1.2	*100.0–100.0*	*100.0 ± 0.0*	1	38.8	*100.0*	2	42.1–43.3	42.7 ± 0.8	*100.0–100.0*	*100.0 ± 0.0*
Stylet support	6	16.1–19.1	17.7 ± 1.1	*65.7–71.8*	*68.7 ± 2.3*	1	24.3	*62.5*	2	26.7–26.8	26.8 ± 0.0	*61.7–63.5*	*62.6 ± 1.3*
Anterior width	6	9.9–11.0	10.5 ± 0.4	*37.6–44.0*	*41.1 ± 2.8*	1	17.2	*44.2*	2	18.9–19.6	19.3 ± 0.5	*44.9–45.2*	*45.1 ± 0.2*
Posterior width	6	7.0–9.1	8.1 ± 0.7	*26.5–34.3*	*31.6 ± 2.7*	1	10.6	*27.3*	2	14.6–15.8	15.2 ± 0.9	*33.7–37.6*	*35.7 ± 2.7*
Standard width	6	7.7–8.3	7.9 ± 0.2	*27.9–32.4*	*30.7 ± 1.7*	1	11.1	*28.6*	2	13.7–16.3	15.0 ± 1.8	*31.6–38.6*	*35.1 ± 5.0*
Standard/length	6	27.9–32.4	30.7 ± 1.7			1	28.6		2	31.6–38.6	35.1 ± 5.0		
Posterior/anterior	6	70.5–87.5	77.0 ± 5.7			1	61.6		2	74.6–83.6	79.1 ± 6.4		
Claw1													
External primary branch	6	9.5–12.4	10.9 ± 1.0	*39.7–45.0*	*42.4 ± 2.5*	1	16.0	*41.1*	2	18.7–18.9	18.8 ± 0.1	*43.6–44.4*	*44.0 ± 0.5*
External base + secondary branch	6	8.1–9.7	8.8 ± 0.5	*30.9–40.4*	*34.5 ± 3.5*	1	12.7	*32.6*	2	13.8–15.0	14.4 ± 0.8	*32.8–34.6*	*33.7 ± 1.3*
External spur	6	3.0–4.8	3.8 ± 0.7	*11.2–18.7*	*14.7 ± 2.8*								
Internal primary branch	6	10.0–12.6	11.3 ± 1.0	*41.7–47.0*	*43.8 ± 2.2*	1	15.2	*39.1*	2	14.5–19.7	17.1 ± 3.7	*34.3–45.4*	*39.8 ± 7.8*
Internal base + secondary branch	6	8.7–10.1	9.2 ± 0.6	*31.8–42.3*	*35.7 ± 3.8*	1	13.0	*33.5*	2	13.0–14.3	13.7 ± 0.9	*30.9–33.1*	*32.0 ± 1.5*
Internal spur	6	3.5–4.5	3.9 ± 0.4	*13.9–16.3*	*15.1 ± 1.1*	1	4.1	*10.6*	2	5.7–5.9	5.8 ± 0.1	*13.1–13.9*	*13.5 ± 0.5*
Basal thickening width	6	1.8–3.1	2.4 ± 0.5	*7.2–12.9*	*9.3 ± 2.1*	1	3.6	*9.2*	2	3.2–4.6	3.9 ± 1.0	*7.3–10.8*	*9.0 ± 2.5*
Claw2													
External primary branch	6	10.3–13.9	12.4 ± 1.6	*40.0–53.7*	*48.1 ± 5.2*	1	18.6	*47.8*	2	19.6–20.0	19.8 ± 0.3	*46.2–46.5*	*46.3 ± 0.3*
External base + secondary branch	6	8.5–10.1	9.2 ± 0.5	*30.9–42.1*	*35.8 ± 3.7*	1	15.5	*39.8*	2	13.6–15.4	14.5 ± 1.3	*32.3–35.6*	*34.0 ± 2.4*
External spur	4	3.1–5.3	4.0 ± 0.9	*12.2–20.1*	*15.8 ± 3.3*								
Internal primary branch	6	10.0–13.5	11.4 ± 1.2	*39.1–54.0*	*44.3 ± 5.3*	1	14.8	*38.1*	1	21.7		*50.0*	
Internal base + secondary branch	6	7.7–9.9	9.1 ± 0.9	*28.2–41.0*	*35.5 ± 4.9*	1	14.1	*36.4*	2	13.5–16.0	14.8 ± 1.7	*32.1–36.9*	*34.5 ± 3.4*
Internal spur	5	3.2–4.6	4.1 ± 0.5	*13.0–18.6*	*16.1 ± 2.1*	1	4.8	*12.3*	2	5.6–5.7	5.7 ± 0.0	*13.1–13.4*	*13.2 ± 0.2*
Basal thickening width	6	1.6–2.4	2.1 ± 0.3	*6.4–9.7*	*8.2 ± 1.4*	1	3.7	*9.6*	2	3.0–3.7	3.4 ± 0.5	*7.0–8.8*	*7.9 ± 1.3*
Claw3													
External primary branch	6	11.3–13.7	12.8 ± 1.1	*43.9–54.9*	*49.7 ± 4.0*	1	16.5	*42.5*	2	18.1–22.8	20.5 ± 3.3	*43.0–52.7*	*47.8 ± 6.9*
External base + secondary branch	6	8.0–9.7	9.2 ± 0.6	*29.0–38.9*	*35.9 ± 3.7*	1	14.0	*36.1*	2	12.9–16.3	14.6 ± 2.4	*30.6–37.5*	*34.0 ± 4.9*
External spur	5	3.0–5.8	4.0 ± 1.1	*11.7–22.5*	*15.6 ± 4.4*								
Internal primary branch	6	10.4–13.3	11.8 ± 1.2	*41.9–51.5*	*46.0 ± 3.8*	1	16.4	*42.1*	2	18.3–19.3	18.8 ± 0.7	*43.4–44.6*	*44.0 ± 0.8*
Internal base + secondary branch	6	5.5–9.5	8.5 ± 1.5	*23.1–37.2*	*33.0 ± 5.1*	1	13.1	*33.6*	2	13.2–15.9	14.5 ± 1.9	*31.2–36.7*	*34.0 ± 3.9*
Internal spur	6	3.1–4.7	3.9 ± 0.6	*12.4–18.1*	*15.1 ± 2.1*	1	4.3	*11.1*	2	5.1–5.7	5.4 ± 0.4	*11.8–13.6*	*12.7 ± 1.2*
Basal thickening width	6	1.8–2.8	2.2 ± 0.4	*7.0–11.0*	*8.6 ± 1.4*	1	3.7	*9.5*	2	2.8–5.0	3.9 ± 1.6	*6.4–11.8*	*9.1 ± 3.8*
Claw4													
Anterior primary branch	6	12.1–14.2	13.2 ± 0.8	*47.0–53.8*	*51.4 ± 2.4*	1	21.5	*55.4*	1	26.5		*61.3*	
Anterior base + secondary branch	6	9.8–11.3	10.3 ± 0.5	*35.7–44.0*	*40.1 ± 3.3*	1	18.6	*47.9*	2	12.7–19.1	15.9 ± 4.6	*30.1–44.1*	*37.1 ± 9.9*
Anterior spur	6	3.2–5.6	4.4 ± 0.8	*12.4–20.2*	*17.0 ± 3.0*	1	6.8	*17.6*	2	6.3–8.0	7.1 ± 1.2	*14.4–18.9*	*16.7 ± 3.2*
Posterior primary branch	6	12.8–15.3	14.2 ± 0.9	*18.4–22.7*	*20.7 ± 1.4*	1	20.8	*33.3*	1	28.6		*46.3*	
Posterior base + secondary branch	6	10.1–11.7	10.8 ± 0.6	*36.8–46.9*	*42.0 ± 3.8*	1	19.2	*49.3*	2	13.0–19.4	16.2 ± 4.5	*30.8–44.7*	*37.8 ± 9.8*
Posterior spur	6	3.6–4.9	4.2 ± 0.5	*14.1–19.0*	*16.4 ± 2.0*								
Basal thickening width	6	2.2–3.6	2.5 ± 0.6	*8.4–14.0*	*9.8 ± 2.2*	1	4.0	*10.4*	1	3.4		*7.9*	

Chromosomes. The laid eggs of *M. tardigradum* OTM strain had 10 chromosomes in the 1-cell stage (Fig. 8B).

DISCUSSION

Differential diagnosis of *Milnesium pacificum* sp. nov.

Milnesium pacificum sp. nov. has dorsal cuticular sculpturing characteristic of the *granulatum* group in this genus, which consists of the following seven species described prior to the present study: *M. granulatum* Ramazzotti, 1962, *Milnesium reticulatum* Pilato et al., 2002, *Milnesium katarzynae* Kaczmarek et al., 2004, *Milnesium krzysztofi* Kaczmarek and Michalczyk, 2007, *Milnesium alabamae* Wallendorf and Miller, 2009 (for the five species above, see Morek et al., 2019a), and additionally *Milnesium lagniappe* and *Milnesium cassandrae* Moreno-Talamantes et al., 2019, which should also be included in the *granulatum* group. We must consider that *M. katarzynae*, for example, was described with only two specimens, both of which might have been hatchlings (294.5 and 285.0 μm, respectively, in body length, Kaczmarek et al., 2004; also see Morek et al., 2016; Surmacz et al., 2020). Therefore, we should carefully compare each species considering their ontogeny. Thus, we compare them here with the corresponding stages of the new species.

Adults and juveniles of *M. pacificum* sp. nov. are distinguished from *M. granulatum* by having [2-2/3]-[2/3-2] CC, whereas *M. granulatum* has a [3-3]-[3-3] CC.

Adults and juveniles of *M. pacificum* sp. nov. differ from *M. reticulatum* by having six (4+2) peribuccal lamellae (only four lamellae in *M. reticulatum*).

Hatchlings of *M. pacificum* sp. nov. differ from those of *M. katarzynae* by having a more anterior stylet support insertion point (14.6–18.2 μm, *pt* 61.5–69.4 in the new species vs. 20.9–23.8 μm, *pt* 73.3–78.3 in *M. katarzynae*).

Hatchlings of *M. pacificum* sp. nov. differ from those of *M. krzysztofi* by CC ([2-2]-[2-2] in the new species, [2-3]-[3-2] in *M. krzysztofi*). Adults and juveniles of *M. pacificum* sp. nov. differ slightly from *M. krzysztofi* by the length of the base + secondary branch in internal claw III (11.3–14.3 μm in *M. pacificum* sp. nov., 8.1–11.3 μm in *M. krzysztofi*).

Adults and juveniles of *M. pacificum* sp. nov. are distinguished from *M. alabamae* by having a different CC ([3-3]-[3-3] in *M. alabamae*).

Adults and juveniles of *M. pacificum* sp. nov. differ from *M. lagniappe* by having six (4+2) peribuccal lamellae (only four in *M. lagniappe*), and by a different CC ([2-3]-[3-2] in *M. lagniappe*). Moreover, these two species are significantly delimitated by genetic analyses of COI, ITS-2 and concatenated sequences (PTP supports: 100% in all analyses).

Milnesium pacificum sp. nov. differs from *M. cassandrae* by *pt* values of the anterior and standard width of the buccal tube (in the new species *30.7–42.3*, *28.5–40.8*, respectively, vs., in *M. cassandrae 47.1–66.2* and *41.6–67.2*, respectively).

Comparison with *Milnesium* sp. BR.007

Although detailed morphometrics of *Milnesium* sp. BR.007 Morek and Michalczyk, 2020 were not published, the close morphological affinity between BR.007 and *M. pacificum* sp. nov. was suggested by the combination of the important character states of BR.007, i.e., [2-3]-[3-2] CC in the adult, six (4+2) peribuccal lamellae, cuticular sculpturing, the presence of pseudoplates, and the CC ontogenetic variability. In addition to the morphological traits, both lineages reproduce by parthenogenesis in laboratory cultures. The phylogenetic analyses also indicated that the most similar species to *M. pacificum* sp. nov. was *M.* sp. BR.007 from Brazil (see Morek and Michalczyk, 2020) with a close genetic distance in the ITS-2 analysis (the mean p-distance was 1.9%). In contrast to the general morphological resemblance and similarity of ITS-2, the species delimitations were highly predicted with both the COI and concatenated trees (>87.2%). Therefore, although some of the available data suggest that *M.* sp. BR.007 could represent *M. pacificum* sp. nov., the lack of a detailed description of the *M.* sp. BR.007 population does not currently allow the identification of the population as such or delineation of it as a new species.

Patterns of ontogenetic variability in *Milnesium*

The hatchlings of *M. pacificum* sp. nov. showed the [2-2]-[2-2] CC and clear circular cuticular depressions. In contrast, the CC changed to [2-3]-[3-2] at the juvenile stage, and the cuticular sculpturing was modified to finer and irregular depressions. Thus, *M. pacificum* sp. nov. shows an early positive change in the CC, and, in addition, the new species provides the first example of a dramatic ontogenetic change also in the cuticular sculpturing in the genus *Milnesium*. Morek et al. (2016) showed that the dorsal cuticle in *Milnesium variefidum* undergoes developmental change as hatchlings and juveniles do not have pseudopores or pseudoplates, but both these traits are clearly visible in adults. However, in *M. pacificum* sp. nov. the change is more evident and if hatchlings and juveniles/adults were found in separate moss samples, they could be classified as different species under the classical taxonomy framework. This example explicitly underlines the need for an integrative approach in the genus *Milnesium* taxonomy to avoid taxonomic inflation.

Two specimens with aberrant CC were found among 23 specimens analyzed for morphometrics of *M. pacificum* sp. nov. One of the specimens showed the [3-3] configuration only on one leg II (Fig. 7), while in the other specimen, the irregular configuration appeared on one leg IV. Since aberrant CC is sometimes formed, descriptions based on only a single or a few specimens can be misleading.

According to Morek et al. (2019a), *M. tardigradum* s.s. shows an early negative CC change, such as [3-3]-[3-3] in the hatchlings changing to [2-3]-[3-2] in the juveniles. Our results also indicated that the *M. tardigradum* from Japan showed the same CC change. *Milnesium inceptum*, which also lives in Japan, shows [3-3]-[3-3] CC in hatchlings, juveniles and adults (Suzuki, 2003; Morek et al., 2019b). Neither CC change nor the cuticular sculpturing are present in *M. inceptum*. Instead, thelytokous *M. inceptum* sometimes produces a male that has modified claws on the first pair of legs (Suzuki, 2008), which is a typical secondary sexual trait of this genus (Rebecchi and Nelson, 1998). To search for the factor causing the CC change, *M. inceptum* would be a good model for comparison because *M. inceptum*, *M. tardigradum* and *M. pacificum* sp. nov. show positive, negative and sexual CC changes.

Chromosomal research on the *Milnesium* species from Japan

There are several research studies on chromosomes in eutardigrades showing that the haploid numbers are mostly n = 5 or 6 (Rebecchi, 1991; Bertolani, 2001; Rebecchi et al., 2002, 2003; Czernekova and Jönsson, 2016; Guidetti et al., 2019; Sugiura et al., 2019). However, there has been only one report on the karyotype of *Milnesium* species, which indicated that the number was 2n = 10 in a German population of "*Milnesium tardigradum*" (Glätzer et al., 2005). The OTM strain of *M. tardigradum* s.s. also showed 10 chromosomes (Fig. 8B), and *M. inceptum* had 12 chromosomes (Fig. 8C), suggesting 2n = 10 and 12 in the respective species. On the other hand, the embryos of *M. pacificum* sp. nov. showed 14 chromosomes, which indicated that the karyotype was 2n = 14 (Fig. 8A), and thus the new species had a rare number of chromosomes compared with the other analyzed Eutardigrada.

Localities of the '*granulatum*-group' species records

The distributions of the *granulatum* group have been shown from tropical to temperate regions (Fig. 13; Table 6). *Milnesium granulatum* was recorded from Fray Jorge, Chile (Ramazzotti, 1962); *Milnesium* cf. *granulatum* was recorded from Trieste, Italy (Maucci, 1973–74) and the USA (Jackson and Meyer, 2019); *M. reticulatum* was recorded from Seychelles (Pilato et al., 2002); *M. katarzynae* was recorded from China (Kaczmarek et al., 2004) and Colombia (Caicedo et al., 2014; Melo et al., 2014; Londoño et al., 2015); *M. krzysztofi* was recorded from the neotropical region, such as Costa Rica (Kaczmarek and Michalczyk, 2007), Peru (Kaczmarek et al., 2014) and Colombia (Lisi et al., 2014; Melo et al., 2014; Londoño et al., 2015); *M. alabamae* was recorded from Alabama, USA (Wallendorf and Miller, 2009), and *M. lagniappe* was found in some southern states in the USA (Meyer et al., 2013; Jackson and Meyer, 2019). In addition, *Milnesium* sp. BR.007 was found in Brazil (Morek and Michalczyk, 2020), and the most recent species (*M. cassan-*

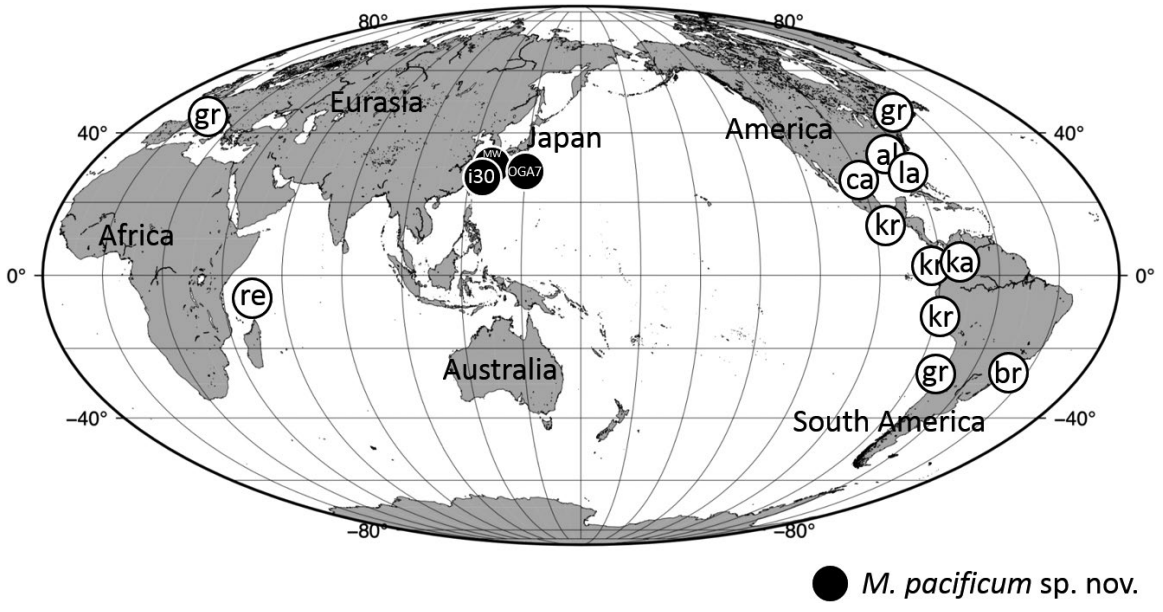

Fig. 13. World map (downloaded from GMT-4.5.18) showing distribution of the *Milnesium granulatum* group. Solid circles represent *M. pacificum* sp. nov.; alpha-numerals indicate strains. Hollow circles indicate other species. Abbreviations: al, *M. alabamae*; ca, *M. cassandrae*; gr, *M. granulatum*; ka, *M. katarzynae*; kr, *M. krzysztofi*; la, *M. lagniappe*; re, *M. reticulatum*; br, *Milnesium* sp. BR.007.

Table 6. Localities of reported species in the *Milnesium granulatum* group.

Species	Localities	References
M. granulatum	Chile, Italy	Ramazzotti, 1962; Maucci, 1973-74
M. cf. *granulatum*	USA	Jackson and Meyer, 2019
M. reticulatum	Seychelles	Pilato et al., 2002
M. katarzynae	China, Colombia	Kaczmarek et al., 2004; Caicedo et al., 2014; Melo et al., 2014; Londoño et al., 2015
M. krzysztofi	Costa Rica, Peru, Colombia	Kaczmarek and Michalczyk, 2007; Kaczmarek et al., 2014; Lisi et al., 2014; Melo et al., 2014; Londoño et al., 2015
M. alabamae	USA	Wallendorf and Miller, 2009
M. lagniappe	USA	Meyer et al., 2013; Jackson and Meyer, 2019
Milnesium sp. BR.007	Brazil	Morek and Michalczyk, 2020
M. cassandrae	Mexico	Moreno-Talamantes et al., 2019
M. tardigradum'	New Zealand	Horning et al., 1978

drae) was found in Mexico (Moreno-Talamantes et al., 2019; Fig. 13). Furthermore, "*Milnesium tardigradum*" from New Zealand (Horning et al., 1978) should also be re-examined because the cuticle was "uniformly pitted by shallow depressions".

The geographic distribution of *M. pacificum* sp. nov. and several closely related species may extend widely in the subtropical/tropical area between Japan and Brazil. Moreover, the area may also include the localities of *M. katarzynae*, *M. krzysztofi*, and *M. cassandrae*, which showed similar morphological traits to the new species. We hope that future integrative studies will unveil the relationships among the above-mentioned four species.

Distribution of *M. tardigradum*

Milnesium tardigradum is no longer considered a cosmopolitan species, as believed previously (Michalczyk et al., 2012; Morek et al., 2019a; Morek and Michalczyk, 2020; Table 7). Recent molecular analysis showed a rather limited distribution of *M. tardigradum* s.s. in Central and Western Europe, with the cautious suggestion that "the true range is most likely wider" (Morek et al., 2019a). Among the three subclades recognized so far (Morek et al., 2019a), subclade α has been found most widely from Copenhagen, Denmark in the north to Zürich, Switzerland in the south and Paris, France in the west to Nowy Sącz, Poland in the east. Subclade β was from Łękawica, Poland; subclade γ was from Kraków, Poland and Abaújszántó, Hungary (Morek et al., 2019a). Although these subclades were not indicated in Morek and Michalczyk (2020), the study showed that *M. tardigradum* s.s. is also present in eastern Russia, in the vicinity of Lake Baikal, which extended the geographic range of the species to the eastern Palaearctic. The Japanese distribution of *M. tardigradum* s.s. leads us to expect that this tardigrade species also lives in the eastern Asian continent, such as in China, Korea, and other countries, but this needs to be confirmed.

Climate conditions may also affect the animal's settlement. Kumanoyu and Mt. Oritsume, the localities of Japanese *M. tardigradum* s.s. KNY and OTM strains, are both in cold, snowy regions (the lowest temperatures were −25.7°C around Kumanoyu and −15.1°C around Mt. Oritsume in 2018; Japan Meteorological Agency). Although many specimens of *Milnesium* have been found in Japan, *M. tardigradum* s.s. has never been found in warm regions so far. Therefore, we suggest that this species exhibits habitat preference for rather cold regions. The diversity of *Milnesium*, and other tardigrades, in Japan could prove to be extensive, as the various climates in Japan, from subtropical islands to cold mountains, provide a wide range of niches for speciation.

Although the type locality of *M. tardigradum* is in Europe (Michalczyk et al., 2012), this species might not necessarily have originated in Europe. Subclade γ, which is shown as the ancestral group of the *M. tardigradum* complex in phylogenetic trees (Morek et al., 2019a), has been found in Japan, suggesting several possibilities, including that the origin of *M. tardigradum* may have been either in central Europe or in East Asia, or another place within the wide range of Eurasia.

Table 7. Localities and subclades of reported specimens of *Milnesium tardigradum*.

Subclade	Sample	Locality	Reference
α	CH.002	Switzerland	Morek et al. (2019a)
α	DE.002	Germany	Morek et al. (2019a)
α	DK.001	Denmark	Morek et al. (2019a)
α	FR.072	France	Morek et al. (2019a)
α	PL.023	Poland	Morek et al. (2019a)
α	PL.142	Poland	Morek et al. (2019a)
β	PL.028	Poland	Morek et al. (2019a)
γ	HU.001	Hungary	Morek et al. (2019a)
γ	KNY	Japan	This study
γ	OTM	Japan	This study
γ	PL.034	Poland	Morek et al. (2019a)
γ	RU.028	Russia	Morek and Michalczyk (2020)

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COMPETING INTERESTS

Not applicable.

AUTHOR'S CONTRIBUTIONS

KS, MM and ACS designed the study. KS performed the experiments with *M. pacificum* sp. nov. HM performed the experiments with *M. tardigradum*. The phylogenetic analyses and the chromosomal analyses in all tested species were performed by KS, who also prepared all figures, tables, and the text. All authors read and approved the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs190154)

Supplementary Text S1. The DNA barcodes of tested tardigrades.

Supplementary Table S1. Morphometrical data of *M. pacificum* sp. nov. and *M. tardigradum*.

Supplementary Table S2. p-distance matrix.

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