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What If Multiple Claw Configurations Are Present in A Sample? A Case Study with the Description of *Milnesium pseudotardigradum* sp. nov. (Tardigrada) with Unique Developmental Variability

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The tardigrade fauna of Iceland has been a subject of studies since mid XX century. So far, only a single species of the genus *Milnesium* has been reported from the island, *M. tardigradum*, which at the time was assumed to have a cosmopolitan distribution. However, the record comes from before the redescription of *M. tardigradum*, thus the validity of the Icelandic report is questionable. Some species of *Milnesium* are characterised by developmental variability, which is most pronounced in the morphology of secondary branches of claws, exhibited by shifts in the number of spurs. In this contribution, we present a case study in which multiple claw configurations (CC) were found in a single lichen sample from Iceland, indicating the presence of more than one species and/or ontogenetic variability. To elucidate this puzzle, we utilised a range of integrative tools, including detailed morphology, morphometry, barcoding and development tracking. We present the workflow, which enabled us to collect the data for each species/morphotype. In result, we revealed the presence of three species, two characterised by ontogenetic CC change (*M. variefidum* and a new species) and one with a stable CC (a new species). Here, we describe one of these new species, *M. pseudotardigradum*, which is extremely similar to *M. tardigradum*, but can be phenotypically differentiated by a unique, double CC change pattern.

Key words: Cryptic species, Developmental variability, Ontogeny, Pseudocrypsis, Species distribution.

BACKGROUND

Tardigrades are a phylum of microscopic invertebrates, inhabiting both aquatic and terrestrial environments. The limno-terrestrial water bears are most often found in mosses and lichens all around the world, across diverse environments (Nelson et al. 2015). So far more than 1200 species have been recognised (Guidetti and Bertolani 2005; Degma et al. 2007–2019; Degma and Guidetti 2007). Even though the tardigrade fauna of Iceland was investigated by numerous researchers (*e.g.*, de Coninck 1939; Tuxen 1941; Morgan 1980; Maucci 1996; Buda et al. 2018), all records of the genus *Milnesium* are represented by only a single species, *Milnesium tardigradum* Doyère, 1840. This is surprising given that the number of described *Milnesium* species in the genus is 37 (Degma et al. 2007 2007–2019) and recent research shows that species diversity in the genus is much greater (Morek and Michalczyk, in press). However, it is important to note that the Icelandic

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records come from before the redescription of *M. tardigradum* (Michalczyk et al. 2012a b) and therefore should be treated with caution. In fact, the verified range of this species is currently restricted to Europe (Morek et al. 2019a), with a single record from Asia, in the vicinity of Lake Baikal (Morek and Michalczyk, in press). The geographic ranges of the majority of *Milnesium* species are restricted to their type localities, with a few reported from multiple sites (*e.g.*, Kaczmarek et al. 2014 2016). Almost all, however, lack molecular support. Given that pseudocryptic diversity has been recently documented in the genus *Milnesium* (Morek et al. 2019b), records based solely on phenotypic identification are of very limited reliance and usability.

The developmental variability in tardigrades includes mainly differences between immature (first two instars) and mature life stages (third instar onwards; Morek et al. 2016a; Guidetti et al. 2016; Kosztyła et al. 2016). In *Milnesium*, the change in claw configuration (CC) was discovered for the first time in *M. variefidum* (Morek et al. 2016a) where extra points on claws develop between the last immature instar and adult. Later, the phenomenon was showed to be more common in the genus (Morek et al. 2019a; Morek and Michalczyk, in press), including examples of both increases and decreases in the number of spurs on the secondary branches that can occur during both the first and the second moulting. Apart from CC, also the appearance of a dorsal cuticle may differ between life stages (*e.g.*, pseudopores may be present only in adult animals; Morek et al. 2016a).

In this contribution, we present a case study where *Milnesium* specimens with four different CCs were found in a single lichen sample, indicating the presence of multiple species and/or multiple phenotypes representing different ontogenetic stages. We show how we dealt with this challenging material and obtained integrative data for each species. As a result, we present a description of a new, pseudocryptic species, which is most similar to *M. tardigradum*, but is characterised by an extraordinary ontogenetic CC change pattern. We also discuss the importance of our findings in relation to the assessment of geographic ranges of *Milnesium* species as well as to the taxonomy of the genus.

MATERIALS AND METHODS

Sampling and specimens handling

A lichen sample collected from stone by Małgorzata Mitan and Małgorzata Osielczak on 07.10.2015 in Reykjavík Botanic Garden, Iceland, 64°08'23"N, 21°52'09"W, 14 m asl was examined according to the standard protocol described by Stec et al. (2015). The sample yielded multiple *Milnesium* specimens exhibiting four different claw configurations (CCs), which indicated the presence of more than a single species or/and multiple developmental phenotypes. In order to identify the species and instars exhibiting particular phenotypes, we followed the workflow scheme presented in figure 1 (the detailed descriptions of particular methods are presented below).

Firstly, the dead animals were preserved on permanent microscope slides (Fig. 1. step 1) to examine general morphology of animals in phase contrast light microscopy (PCM) and to assess how many CCs and putative species are present in the sample. In the next step, the alive specimens and exuviae with eggs were individually examined on the temporary water slides (Fig. 1. step 2) to determine the CC of each specimen/exuvia. This laborious procedure allows to accurately assess how many specimens per CC (putative species/developmental stage) are available for further analysis and therefore enables a desired assignment of specimens to most important analysis. In this study, the animals representing each CC were destined for DNA sequencing (Fig. 1, step 4), whereas the remaining ones, together with the exuviae with eggs, were placed in 24-well plastic plates to establish a culture, where the animals were kept individually (Fig. 1, step 3). This kind of culture allows for developmental tracking (Fig. 1, step 6). Provided the cultures were thriving, additional animals can be used for slide preparation (Fig. 1, step 1), DNA sequencing (Fig. 1, step 4), imaging in scanning electron microscopy (SEM; Fig. 1, step 5) and developmental tracking (Fig. 1, step 6). For the exact number of specimens destined for each analyses in this study, see table 1.

Microscopy, imaging and morphometry

The specimens were mounted on permanent microscope slides in Hoyer's medium according to the method by Morek et al. (2016b) to examine general morphology in PCM and provide morphometric data. The measurements follow Tumanov (2006), the buccal tube widths were measured according to Michalczyk et al. (2012a) and the body length was measured from the anterior to posterior margin of the body, excluding the hind legs. The *pt* index is a ratio of a given structure to the length of the buccal tube (Pilato 1981), and in the text is given in *italics*. The number of measured specimens follows the recommendation of Stec et al. (2016). The morphometric data were handled using the Apochela spreadsheet ver. 1.3. available from the Tardigrada Register (Michalczyk and Kaczmarek 2013), www.tardigrada.net. All the measurements and

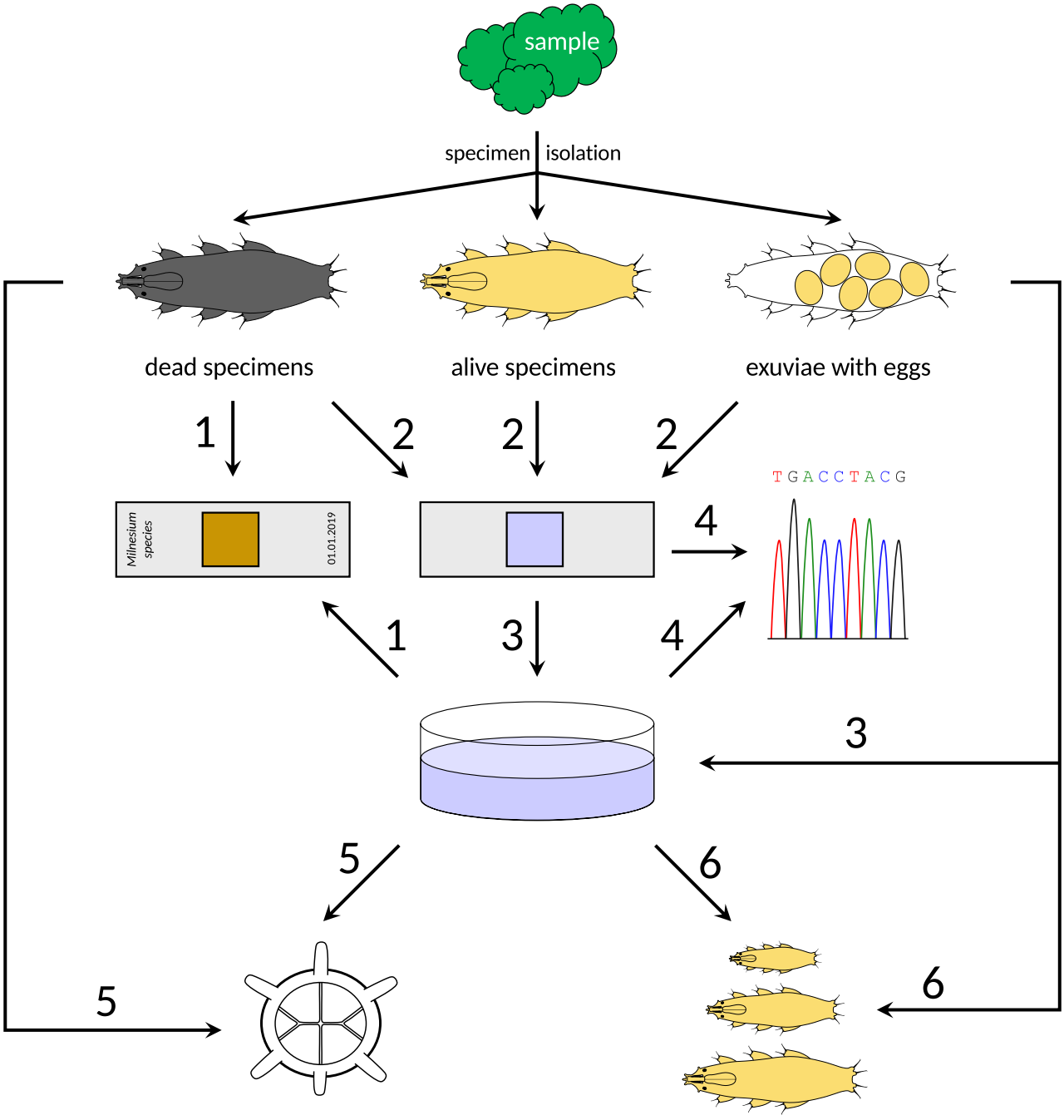

Fig. 1. The scheme illustrating the general workflow when dealing with samples containing *Milnesium* specimens characterised by multiple claw configurations (CCs). After the extraction of specimens and exuviae with eggs from the sample, the material is divided into the dead and alive specimens. The dead ones are mounted on permanent slides (1) and immediately examined under PCM to check for the potential presence of multiple species in the sample. Provided there is a large number of dead specimens, they can be also used for imaging in SEM (6) but note there can be multiple species in the sample. Next, the alive specimens and exuviae are examined on the temporary water slide to assign the CC to the specimen (2). This laborious step allows for the assessment of the exact number of specimens per CC available for further analysis. In this study we immediately set aside some specimens from each CC for DNA extraction and sequencing (4). In parallel, a separate culture for each CC is established (3) as when the culture is thriving additional specimens can be dedicated for required analysis *i.e.*, permanent slides preparation (1), DNA sequencing (4), imaging in SEM (5) or developmental tracking (6).

photographs were taken with the Olympus BX53 PCM, associated with the Olympus DP74 digital camera (PCM). For deep-focus structures a series of up to 22 pictures were taken and merged into one image using Corel Photo-Paint X8. Some specimens were processed for SEM imaging according to the protocol by Stec et al. (2015) and examined under high vacuum with HITACHI S-4700 SEM at the Institute of Geological Sciences, Jagiellonian University.

Culturing and developmental tracking

Initially, the specimens isolated from the lichen sample were individually kept on 24-well plastic plates and reared under conditions described in Kosztyła et al. (2016), at 10°C. The ontogenetic variability was assessed by developmental tracking (Morek et al. 2016a) for each CC separately. In short, individuals representing consecutive instars were mounted on permanent microscope slides and later examined under PCM. At least three specimens per each of the three putative species and each of three stages were collected (minimum 27 specimens for the entire tracking). Once the developmental tracking was complete, the remaining specimens were transferred from the plates to plastic Petri dishes to facilitate further culture growth (Stec et al. 2015).

Genotyping

The DNA was extracted from individual specimens (for sample size see Table 1) following the Chelex® 100 resin (Bio-Rad) extraction method by Casquet et al. (2012), with modifications by Stec et al. (2015). Before the DNA extraction, the specimens were mounted on temporary water slides in order to identify the morphotype (CC) and associate it with the genotype. The four standard molecular markers were sequenced, three nuclear: the small ribosomal subunit (18S rRNA), large ribosomal subunit (28S rRNA), Internal Transcribed Spacer 2 (ITS-2); and one mitochondrial, Cytochrome Oxidase C subunit I (*COI*). The PCR protocols followed Stec et al. (2015), primers and PCR programmes with relevant references are listed in table 2. The obtained chromatograms were checked manually in BioEdit ver. 7.2.5 (Hall 1999). In addition, the *COI* sequences were translated into amino acids using MEGA 7 (Kumar et al. 2016) to ensure that no pseudogenes were amplified. All sequences are deposited in GenBank (accession numbers are listed in Table 1).

RESULTS

Morphological species delineation

In the first step, permanent slides made from dead animals (Fig. 1, step 1) found in the sample were analysed, revealing the presence of four distinct CCs: [2-2]-[2-2], [2-3]-[2-2], [2-3]-[3-2] (including a single specimen with an additional spur on claws III), and [3-3]-[3-3]. Based on the latest key to the genus *Milnesium* (Morek et al. 2016a), the species were initially identified

Table 1. The details of the *Milnesium* species found in the analysed sample, with indication of the claw configuration present in each species. Data/analysis types: PCM - imaging and morphometry in light microsopy, DNA - DNA extraction and sequencing, DEV - developmental tracking, SEM - imaging in scanning electron miscroscopy. The GenBank accession numbers are provided below the species names

Claw configuration	Species	Number of specimens analysed			
		PCM	DNA	DEV	SEM
[2-2]-[2-2]: hatchlings and juveniles [2-3]-[2-2]: adults	*Milnesium variefidum* 18S rRNA: MK484080 28S rRNA: MK483988 ITS-2: MK484014 *COI*: MK492293	18	2	9	0
[3-3]-[3-3]: hatchlings [2-3]-[3-2]: juveniles and adults	*Milnesium pseudotardigradum* sp. nov. 18S rRNA: MK484088 28S rRNA: MK483997 ITS-2: MK484022 *COI*: MK492297	108	5	93	20
[3-3]-[3-3]: all life stages	*Milnesium* sp. nov. 18S rRNA: MK484089 ITS-2: MK484023	10	3	9	0

as *M.* cf. *variefidum* Morek et al. 2016a ([2-2]-[2-2] and [2-3]-[2-2]), *M.* cf. *tardigradum* ([3-3]-[3-3] and [2-3]-[3-2]), and a putative new species with a [3-3]-[3-3] CC. However, this morphological identification was only preliminary and served as a starting point for further genetic and developmental analyses, which allowed for an integrative species delineation and identification.

The alive specimens were either destined for DNA extraction and sequencing (Fig. 1, step 4), with prior examination of the CC on temporary water slides, or used to establish isogenic cultures (Fig. 1, step 3). In the long term, only cultures founded with specimens with adult [2-3]-[3-2] CC were stable culture and provided sufficient numbers of animals for all planned analyses.

Genetic delineation

Individuals with a [2-2]-[2-2] CC and a [2-3]-[2-2] CC shared the same haplotypes in all four markers, unequivocally indicating they represent a single species. The comparison of these DNA sequences with all available *Milnesium* sequences showed the closest affinity to the type sequences for *M. variefidum* (*p*-distances: 0.0% in 18S rRNA and 28S rRNA, 2.6–3.0% in ITS-2, and 3.6% in *COI*). Thus, genetic data confirmed the initial morphological identification of [2-2]-[2-2] CC individuals as hatchlings and juveniles, and [2-3]-[2-2] CC animals as adults of *M. variefidum*.

Similarly, some small specimens with a [3-3]-[3-3] and all medium or large individuals with a [2-3]-[3-2] CC exhibited the same haplotypes in each of the four sequenced markers, signifying that they represent a single species. The comparisons with all available *Milnesium* sequences indicated that despite morphological and ontogenetic similarities to *M. tardigradum*, these individuals in fact represent a species new to science. The comparison of these DNA sequences with all available *Milnesium* sequences showed the closest affinity to *M. tardigradum* (*p*-distances: 1.6–2.1% in 18S rRNA; 3.8–4.1% in 28S rRNA, 12.5–22.1%; in ITS-2; and 12.6–14.0% in *COI*). Since we were able to obtain sufficient numbers of individuals for our integrative analyses, we formally describe this species as *M. pseudotardigradum* sp. nov. below.

Finally, some small and all large specimens with a [3-3]-[3-3] CC shared the same haplotypes in both 18S rRNA and ITS-2 (28S rRNA and *COI* failed to sequence), indicating that they represent a single species. The comparisons with all available *Milnesium* sequences indicated that the individuals represent a species new to science. The comparison of these DNA sequences with all available *Milnesium* sequences showed the closest affinity to another undescribed species designated in Morek and Michalczyk (in press) as "*Milnesium* sp. nov. 5 GB.096" (*p*-distances: 1.0% in 18S rRNA; 3.9%; in ITS-2). Since we were not able to obtain sufficient numbers of individuals for our integrative analyses, we decided to postpone the description of this species until more specimens are available.

Developmental variability

Developmental tracking revealed that specimens with a [2-2]-[2-2] and a [2-3]-[2-2] CC represent the same species that exhibits late positive CC change. Some of the hatchlings with a [3-3]-[3-3] CC moulted into [2-3]-[3-2] juveniles, *i.e.*, they exhibited early negative CC change. Finally, some specimens had a stable [3-3]-[3-3] CC from hatchlings through juveniles to adults. Thus, this analysis indicated the presence of three species in the sample, two with ontogenetic CC change and one with a stable CC.

When mounting large specimens with a [2-3]-[3-2] CC from the culture (Fig. 1, step 1) on microscope slides, we spotted that in the largest animals (presumably after the 3rd moult), additional spurs may appear, especially on claws II and III. Thus, we extended the developmental tracking to older life stages in order

Table 2. Primers and references for specific protocols for amplification of the four DNA fragments sequenced in the study

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source	PCR programme
18S rRNA	18S_Tar_Ff1	forward	AGGCGAAACCGCGAATGGCTC	Stec et al. (2017)	Zeller (2010)
	18S_Tar_Rr1	reverse	GCCGCAGGCTCCACTCCTGG		
28S rRNA	28S_Eutar_F	forward	ACCCGCTGAACTTAAGCATAT	Gąsiorek et al.(2018)	Mironov et al. (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)	
ITS-2	ITS2_Eutar_Ff	forward	GCATCGATGAAGAACGCAGC	Stec et al. (2018)	Stec et al. (2018)
	ITS2_Eutar_Rr	reverse	TCCTCCGCTTATTGATATGC		
COI	COI_Mil.tar_Ff	forward	TATTTTATTTTTGGTATTTGATGTGC	Morek et al. (2019a)	Morek et al. (2019a)
	COI_Mil.tar_Rr	reverse	CCTCCCCCTGCAGGATC		

to test whether the additional spurs were aberrations present only in some specimens or whether it was a stable pattern characterising older animals. The tracking revealed first that they are truly additional spurs, because they exhibit typical morphology, *i.e.*, they are of a typical shape and similar size, being only slightly smaller than the regular ones, in contrast to aberrations, which are shorter and less curved or even straight (Kaczmarek et al. 2012; Morek et al. 2019a). Secondly, the additional spurs are very common in the 4th instar onwards, at least one additional spur is present in 86% specimens, mostly on claws II–IV (Fig. 2). Most commonly, specimens are equipped with two additional spurs (19%), although single specimens with zero and eight spurs (*i.e.*, resulting in a [3-3]-[3-3] CC) were observed (see also Description of females below).

TAXONOMIC ACCOUNT

Phylum: Tardigrada Doyère, 1840
Class Apotardigrada Guil, Jørgensen and Kristensen, 2018
Order Apochela Schuster et al., 1980
Family Milnesiidae Ramazzotti, 1962
Genus *Milnesium* Doyère, 1840

***Milnesium pseudotardigradum* sp. nov.**
(Figs. 2-6, Table 3)
urn:lsid:zoobank.org:act:0BE101DC-62F5-44FE-BEF1-06FDA9B4B157

Integrative description

Females (morphometrics and holotype measurements in Table 3): *Milnesium* of moderate length, up to 862 µm (Fig. 3A, B), yellowish. Eyes present in alive specimens, but dissolved in 80% of specimens (86/108 of the type series) mounted in Hoyer's medium. Cuticle smooth without pseudopores or pseudoplates both in PCM (Fig. 3E) and SEM (Fig. 3F). The six rather short peribuccal papillae present around the mouth opening, with the ventral one being the smallest. Mouth opening with six triangular peribuccal lamellae of unequal size, *i.e.*, the two lateral lamellae are significantly smaller than the pair of dorsal and lateral lamellae, the 4+2 configuration (Fig. 3D), which is detectable only under SEM. Two short lateral papillae present. Buccal tube funnel shape and of moderate width (Fig. 3C). Claws typical for this genus, primary branches with tiny, but well-visible accessory points (Fig. 4C, D, H). Typically internal and anterior secondary branches equipped with a basal spur, *i.e.*, with a [2-3]-[3-2] CC (Fig. 4C, D, G). In the majority of specimens from the 4th life stages onwards (86% of the type series) additional spurs on external and/or posterior secondary branches may be present (Fig. 4E, F). These additional spurs are always smaller than the regular spurs (Fig. 4H), but they are characterised by a regular, normal shape, indicating they are not aberrations. The number of extra spurs in a single animal ranges from one to eight resulting in such cases in specimens with a [3-3]-[3-3] CC. This means that the number of spurs can be uneven and different on left and right pairs of claws. Cuticular bars under claws I–III are always present.

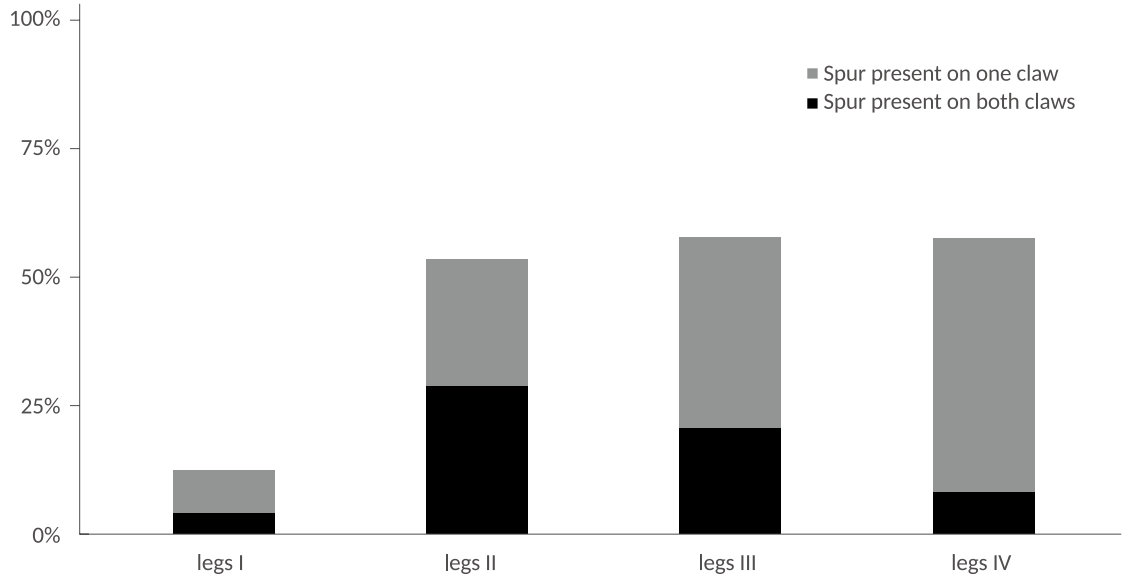

Fig. 2. The graph showing the frequencies of number of additional spurs in 4th+ specimens of *Milnesium pseudotardigradum* sp. nov. on internal/posterior claws of each pair of legs.

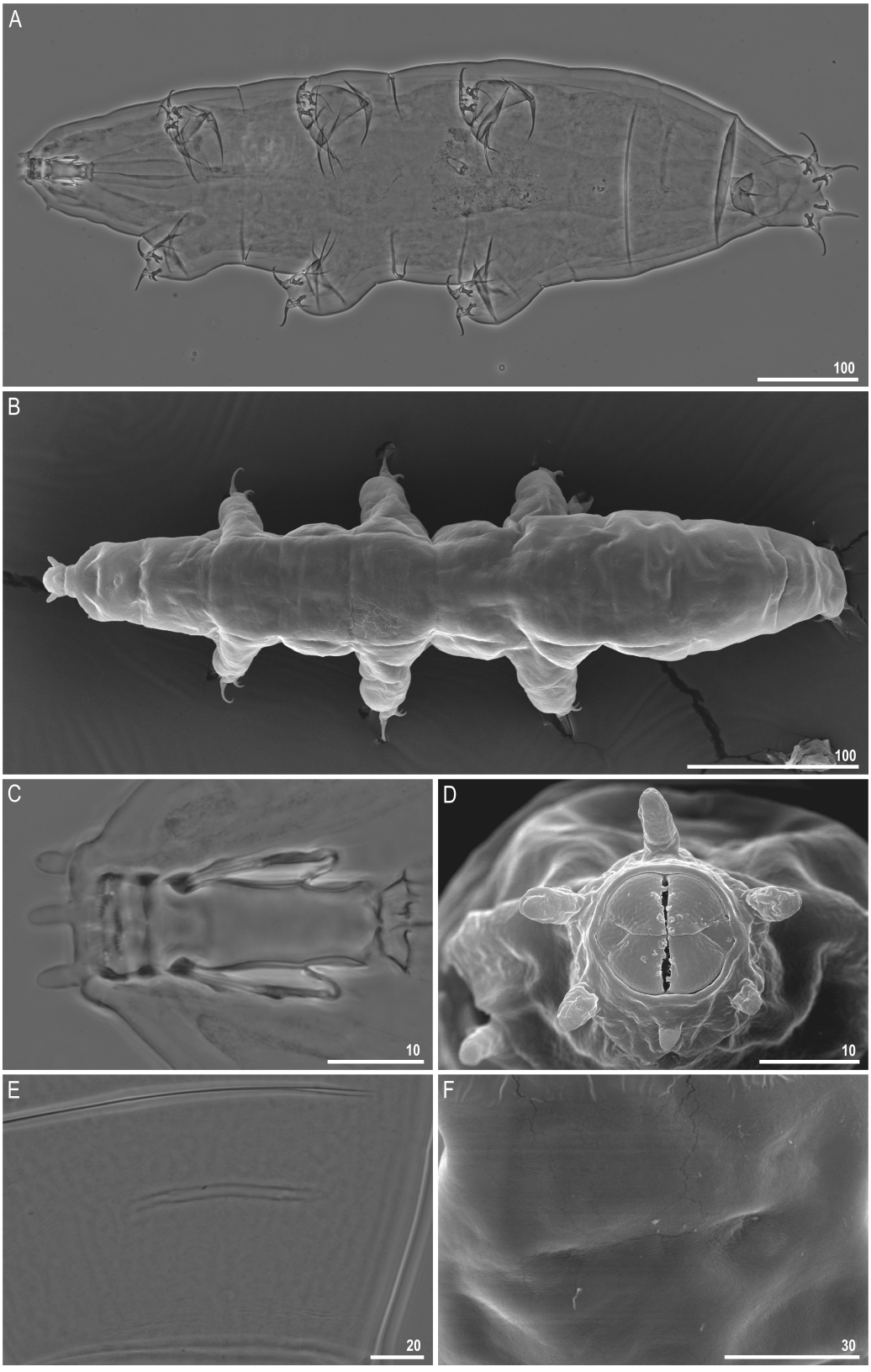

Fig. 3. *Milnesium pseudotardigradum* sp. nov. A, habitus, ventral view (holotype, PCM). B, habitus, dorsal view (paratype, SEM). C, Buccal apparatus (holotype, PCM). D, six peribuccal lamellae of unequal size, *i.e.*, 4 + 2 configuration (paratype, SEM). E, smooth dorsal cuticle (holotype, PCM). F, smooth dorsal cuticle (paratype, SEM). All the scale bars in μm.

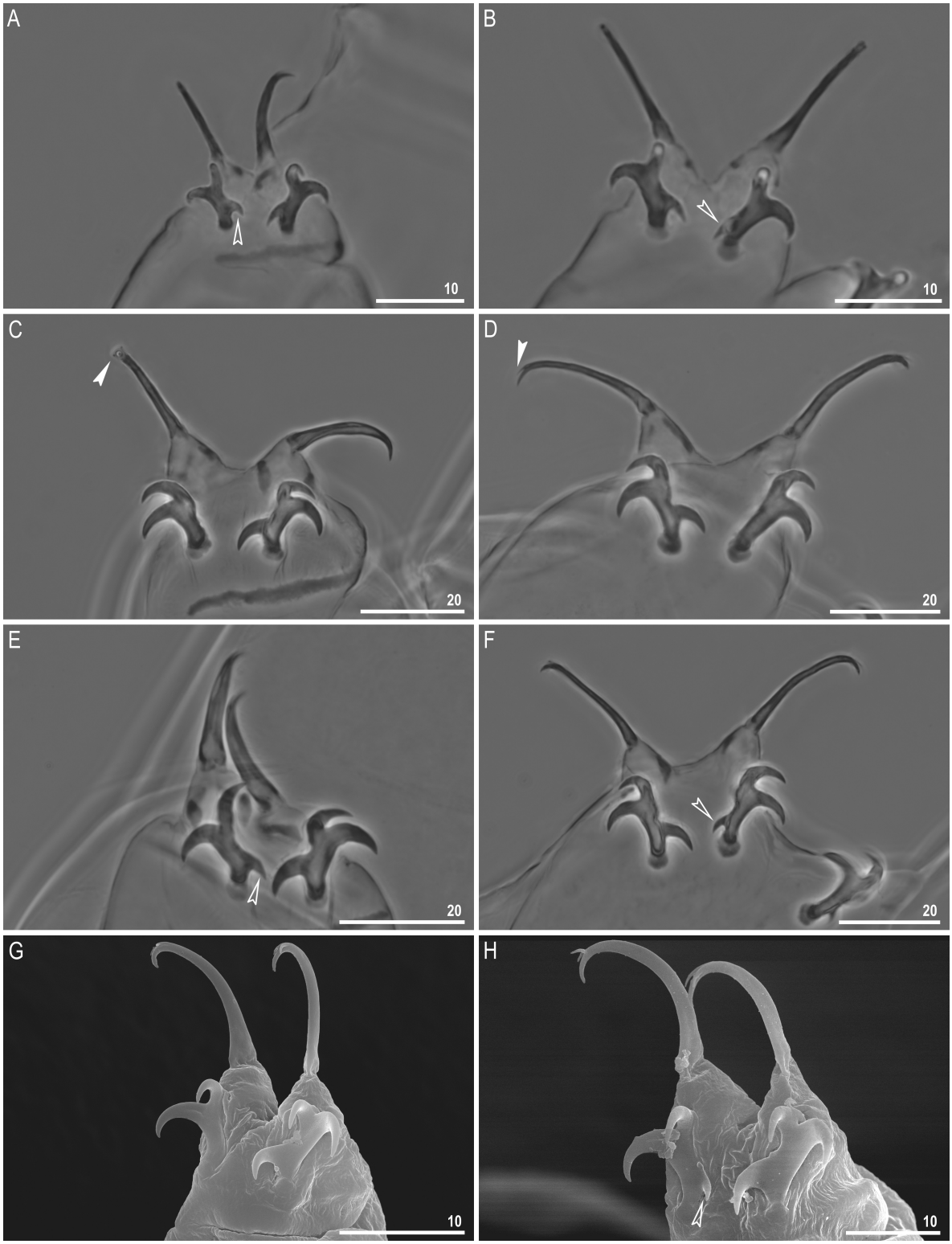

Fig. 4. *Milnesium pseudotardigradum* sp. nov. A, claws III of hatchling with [3-3]-[3-3] CC (paratype, PCM), the empty arrow indicate the external spur, which disappear after the first moult. B, claws IV of hatchling with [3-3]-[3-3] CC (paratype, PCM)), the empty arrow indicate the posterior spur, which disappear after the first moult. C, claws III of adult (paratype, PCM), the arrow indicates the accessory points. D, claws IV of adult (holotype, PCM), the arrow indicates the accessory points. E, claws III of adult (paratype, PCM) with additional spur indicated by empty arrow. F, claws IV of adult (paratype, PCM) with additional spur indicated by empty arrow. G, claws III of adult (paratype, SEM). H, claws IV of adult (paratype, SEM) with additional spur indicated by empty arrow. All the scale bars in μm.

Table 3. Measurements (in μm) and the *pt* values of selected morphological structures of 108 specimens of *Milnesium pseudotardigradum* sp. nov. from type locality, Reykjavík Botanic Garden, Iceland, mounted in Hoyer's medium. Individuals were chosen to represent the entire body length range, with as equal representation of all available life stages as possible

CHARACTER	N	RANGE		MEAN		SD	
		μm	*pt*	μm	*pt*	μm	*pt*
Body length	106	290–862	*1312–2092*	639	*1791*	155	*166*
Peribuccal papillae length	82	3.8–10.3	*13.7–24.8*	7.2	*19.9*	1.8	*2.5*
Lateral papillae length	94	3.3–7.9	*11.0–21.8*	5.5	*15.6*	1.2	*1.8*
Buccal tube							
Length	108	21.6–45.4	–	35.4		7.2	
Stylet support insertion point	104	15.2–30.3	*61.8–75.9*	23.6	*67.2*	4.5	*2.9*
Anterior width	99	8.2–19.4	*32.5–49.4*	15.0	*42.2*	3.4	*3.5*
Standard width	98	7.0–20.3	*25.5–50.8*	13.4	*37.5*	3.9	*5.7*
Posterior width	99	6.5–20.6	*26.5–52.0*	13.2	*37.0*	3.8	*6.1*
Standard width/ length ratio	98	25%–51%	–	38%		7%	
Posterior/anterior width ratio	98	65%–121%	–	87%		14%	
Claw 1 heights							
External primary branch	87	10.0–20.3	*36.6–53.7*	15.6	*44.1*	3.1	*3.0*
External base + secondary branch	65	5.8–15.7	*26.2–37.4*	11.5	*32.8*	2.6	*2.4*
External spur	11	2.5–3.3	*11.0–14.1*	2.9	*12.7*	0.9	*1.0*
External branches length ratio	58	57%–80%	–	75%		11%	
Internal primary branch	88	9.9–19.4	*34.9–51.2*	14.7	*42.8*	3.0	*3.2*
Internal base + secondary branch	92	7.2–15.6	*26.0–37.2*	11.3	*32.6*	2.4	*2.6*
Internal spur	91	2.6–9.0	*10.7–22.0*	6.3	*17.4*	1.6	*2.1*
Internal branches length ratio	77	61%–91%	–	76%		10%	
Claw 2 heights							
External primary branch	89	10.5–21.3	*40.1–58.2*	17.1	*48.8*	3.3	*3.4*
External base + secondary branch	79	7.1–15.3	*29.1–41.0*	12.2	*35.3*	2.6	*2.7*
External spur	19	2.3–6.7	*10.4–18.8*	4.3	*13.7*	1.5	*2.1*
External branches length ratio	68	60%–81%	–	72%		10%	
Internal primary branch	90	10.1–21.0	*39.1–55.6*	16.5	*47.4*	3.4	*3.6*
Internal base + secondary branch	82	7.1–15.5	*25.8–51.9*	11.6	*34.1*	2.6	*3.5*
Internal spur	99	3.2–9.6	*14.1–23.4*	7.0	*19.5*	1.7	*1.9*
Internal branches length ratio	68	59%–81%	–	72%		10%	
Claw 3 heights							
External primary branch	93	10.8–23.0	*40.1–57.5*	17.6	*49.8*	3.4	*3.3*
External base + secondary branch	74	6.9–15.9	*28.0–41.6*	12.2	*35.5*	2.7	*2.8*
External spur	22	2.9–7.4	*7.6–19.9*	4.5	*13.6*	1.5	*3.2*
External branches length ratio	65	63%–78%	–	71%		9%	
Internal primary branch	81	10.5–21.2	*39.9–56.6*	17.1	*48.3*	3.4	*3.9*
Internal base + secondary branch	63	6.2–14.4	*28.1–42.0*	11.6	*34.1*	2.5	*3.0*
Internal spur	93	3.4–9.4	*15.0–23.5*	6.9	*19.5*	1.5	*1.8*
Internal branches length ratio	48	61%–82%	–	71%		11%	
Claw 4 heights							
Anterior primary branch	107	10.2–25.5	*43.5–71.9*	21.1	*59.6*	4.2	*6.0*
Anterior base + secondary branch	100	7.5–18.0	*31.9–45.4*	13.9	*38.9*	3.0	*3.5*
Anterior spur	91	3.1–9.3	*12.9–23.1*	6.8	*18.9*	1.6	*2.1*
Anterior branches length ratio	100	53%–83%	–	66%		8%	
Posterior primary branch	103	11.1–27.2	*46.4–76.0*	22.5	*63.7*	4.5	*5.9*
Posterior base + secondary branch	102	7.0–20.4	*31.7–51.0*	14.9	*41.8*	3.3	*4.1*
Posterior spur	18	2.2–5.7	*5.3–20.3*	3.7	*12.6*	1.2	*3.7*
Posterior branches length ratio	98	55%–77%	–	66%		8%	

Males: No males were found in the sample, and the culture confirmed that the type population is (at least facultatively) parthenogenetic.

Juveniles: Morphologically identical to adults, with the exception that juveniles never exhibit the additional spurs on external and posterior secondary branches of claws, *i.e.*, the CC is always [2-3]-[3-2] (Fig. 4C, D). After fixation in Hoyer's medium eyes dissolved in 22/25 (88%) of the juveniles.

Hatchlings: Morphologically similar to adults and juveniles, apart from the claws, which are characterised by the presence of spurs on each secondary branch *i.e.*, the CC is always [3-3]-[3-3] (Fig. 4A, B). After fixation in Hoyer's medium eyes dissolved in 9/11 (82%) of the hatchlings.

Ontogenetic variability (Fig. 5): The species exhibits ontogenetic variability in CC. Hatchlings have a [3-3]-[3-3] CC, whereas older life stages, from juveniles onwards exhibit a [2-3]-[3-2] CC, thus the species is characterised by an early negative CC change. Moreover, the new species also exhibits a unique feature, never observed in any *Milnesium* species before: the irregular appearance of additional spurs on the internal and posterior claws in adults from the 4th life stage (second adult instar) onwards (the summary of changes is depicted in figure 4). The additional spurs are more often present on claws II–IV than on claws I (exact frequencies are presented in figure 5. No variability in cuticle sculpture, presence of cuticular bars or eyes was detected.

Eggs: Smooth, oval, slightly yellowish and laid within exuviae, up to 10 in a single clutch found in the culture.

DNA markers: All four markers were characterised by single haplotypes, the sequences were of the following lengths: 18S rRNA: 1032 bp (GenBank: MK484088), 28S rRNA: 786 bp (MK483997), ITS-2: 545 bp (MK484022), and *COI*: 580 bp (MK492297).

Type locality: 64°08'23"N, 21°52'09"W; 14 m asl: Iceland, Reykjavík Botanic Garden, lichen from stone.

Etymology: The name of the species highlights the similarity between the new species and *M. tardigradum*.

Type repositories: The series consists of the holotype (IS.008.74) and 107 paratypes (IS.008.03–30; 53–56; 70–73; 75–141). All the slides are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland.

Adult and juvenile phenotypic differential diagnosis

Milnesium pseudotardigradum sp. nov. is one of eight described species of *Milnesium* characterised by a [2-3]-[3-2] adult CC. Since in the culture we found only a single specimens with a [3-3]-[3-3] CC (< 1% of all analysed specimens), the differential diagnosis for species with adult [3-3]-[3-3] CC is not provided. The new species differs from all species with a [2-3]-[3-2] CC by the irregular appearance of external and posterior spurs from the 4th instars onwards (see above). In addition, *M. pseudotardigradum* sp. nov.

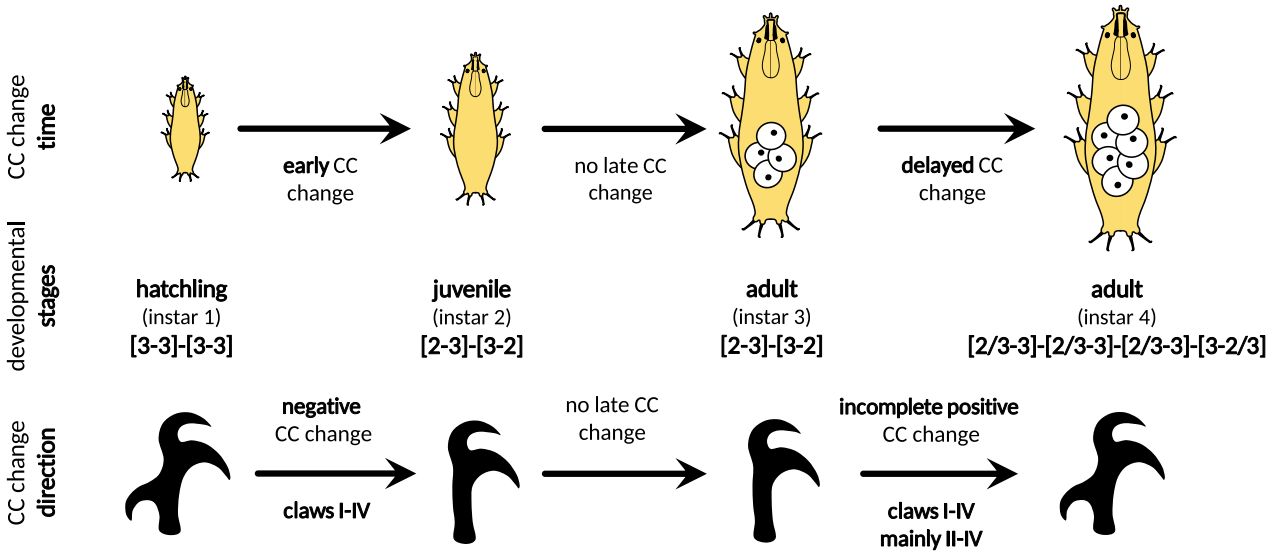

Fig. 5. The scheme illustrating the developmental variability in *Milnesium pseudotardigradum* sp. nov. Note that this species undergoes the developmental change twice—between hatchling and juvenile stage—early negative CC change, and between adult stages, which is presented for the first time, and which we call the delayed CC change. The second change is incomplete, *i.e.*, not all spurs on external and posterior secondary branches appear.

differs specifically from:

M. barbadosense Meyer and Hinton, 2012, known from type locality in Barbados, by relatively longer internal spurs on claws I–III, the difference is most visible in claws III (*15.0–23.5* in the new species *vs 3.6–13.2* in *M. barbadosense*).

M. beasleyi Kaczmarek, Jakubowska and Michalczyk, 2012, known only from type locality in Turkey, by the appearance of cuticle (smooth dorsal cuticle in the new species *vs* well-visible pseudopores in *M. beasleyi* on the entire dorsum) and by relatively longer internal spurs on claws III (*14.1–23.4* in the new species *vs 7.7–14.3* in *M. beasleyi*).

M. krzysztofi Kaczmarek and Michalczyk, 2007, reported from Costa Rica and Peru (Kaczmarek et al. 2014), by the appearance of cuticle (smooth dorsal cuticle in the new species *vs* reticulated in *M. krzysztofi*) and by relatively longer internal spurs on claws III (*15.0–23.5* in in the new species *vs 10.8* in *M. krzysztofi*).

M. lagniappe Meyer, Hinton and Dupré, 2013, known from the United States, by the appearance of cuticle (smooth dorsal cuticle in the new species *vs* pseudoporous in *M. lagniappe*) and by a smaller relative buccal tube standard width (*25.5–50.8* in in the new species *vs 63.4–77.9* in *M. lagniappe*).

M. reductum Tumanov, 2006, known from Kyrgyzstan and China (Yang 2007), by the presence of accessory points on claw primary branches.

M. reticulatum Pilato, Binda and Lisi, 2002 known from the type locality on Seychelles, by the absence of gibbosities and cuticular reticulation on the dorsum.

M. tardigradum Doyère, 1840, reported from integratively identified European localities (Michalczyk et al. 2012a; Morek et al. 2019a) and a single locality in Asia (Morek and Michalczyk, in press), by statistically higher *pt* values for claw spur heights: Claw I internal spur (*10.3–22.0*, on average *17.3* in the new species *vs 8.70–22.05*, on average *14.7* in *M. tardigradum*; t_{260} = 8.283, $p < 0.001$); claw II internal spur (*14.1–23.4*, on average *19.5* in the new species vs *9.6–22.5*, on average *16.6* in *M. tardigradum*; t_{279} = 9.292, $p < 0.001$), claw III internal spur (*15.0–23.5*, on average *19.5* in the new species *vs 9.6–24.5*, on average *16.4* in *M. tardigradum*; t_{268} = 9.951, $p < 0.001$), claw IV anterior spur (*12.9–23.1*, on average *18.9* in the new species *vs 10.7–21.8*, on average *16.0* in *M. tardigradum*; t_{257} = 9.323, $p < 0.001$).

M. tetralamellatum Pilato and Binda, 1991, known from Tanzania, by a higher number of peribuccal lamellae (six in the new species *vs* four in *M. tetralamellatum*) and by a more posterior position of stylets support insertion points (*61.8–75.9* in in the new species *vs 61.9–64.37* in *M. tetralamellatum*).

M. vorax Pilato, Sabella and Lisi, 2016, reported from Sicily, by a lower *pt* of the buccal tube standard width (*25.5–49.4* in in the new species *vs 59.5–60.3* in *M. vorax*).

Hatchling phenotypic differential diagnosis

Milnesium pseudotardigradum sp. nov. hatchlings differ from the described species with a [3-3]-[3-3] CC by:

The position of stylet support insertion point: *68.4–75.9* in hatchlings of the new species *vs 63.6–66.9* in *M. asiaticum* Tumanov, 2006, *64.3–68.1* in *M. dornensis* Ciobanu, Roszkowska and Kaczmarek, 2015, *59.1–66.7* in *M. longiungue* Tumanov, 2006, *63.0–65.9* in *M. minutum* Pilato and Lisi, 2016, *58.0–60.5* in *M. sandrae* Pilato and Lisi, 2016, *75.5–77.5* in *M. shilohae* Meyer, 2015, *66.6–68.2* in *M. swansoni* Young, Chappell, Miller and Lowman, 2016, *63.6–70.0* in *M. tardigradum* hatchlings, *52.3* in *M. tumanovi* Pilato, Sabella and Lisi, 2016, *62.0–65.1* in *M. validum* Pilato, Sabella, D'Urso and Lisi, 2017, and *62.0–64.8* in *M. zsalakoae* Meyer and Hinton, 2010.

The *pt* of the buccal tube anterior width: *32.5–46.1* in hatchlings of the new species *vs 66.7–70.3* in *M. beatae* Roszkowska, Ostrowska and Kaczmarek, 2015, *63.4–74.6* in *M. bohleberi* Bartels, Nelson, Kaczmarek and Michalczyk, 2014, *56.2–75.5* in *M. burgessi* Schlabach, Donaldson, Hobelman, Miller and Lowman, 2018, and *47.5–58.3* in *M. shilohae*.

The *pt* of the of primary branches heights of all claws: *41.1–60.4* in hatchlings of the new species *vs 22.9–33.1* in *M. brachyungue* Binda and Pilato, 1990.

The *pt* of the claw I external base + secondary branch height: *26.2–34.26* in hatchlings of the new species *vs 16.3–20.0* in *M. antarcticum* Tumanov, 2006, *22.0–25.2* in *M. argentinum* Roszkowska, Ostrowska and Kaczmarek, 2015, *22.9* in *M. brachyungue*, *38.8–55.2* in *M. burgessi*, and *35.0–36.6* in *M. swansoni* Young, Chappell, Miller and Lowman, 2016.

The posterior/anterior buccal tube width ratio: 68%–100% in hatchlings of the new species *vs* 62%–65% in *M. eurystomum* Maucci, 1991.

Buccal tube length: 21.6–24.2 µm in hatchlings of the new species *vs* 32.3–67.5 µm in *M. alpigenum*, Ehrenberg, 1853, 24.4–46.1 µm in *M. dornensis*, 25.8–56.0 µm in *M. inceptum* Morek et al. 2019b, 25.7–26.4 µm in *M. minutum*, 28.6–36.3 µm in *M. sandrae*, 38.4–50.3 µm in *M. shilohae*, 44.1–71.0 µm in *M. swansoni*, and 44.1–71.0 µm in *M. validum*.

Molecular differential diagnosis

The ranges of uncorrected *p*-distances between the new species and available sequences for other

congeners are as follows:

18S rRNA: 1.5%–3.8% (2.7% on average), with the most similar being *M. tardigradum*, (MK484076 from Russia Morek and Michalczyk, in press) and the least similar being *M.* cf. *tardigradum* (GQ925697, Chen et al., unpublished).

28S rRNA: 2.4%–9.4% (6.8% on average), with the most similar being *M. variefidum* (MK483988, found in the same lichen sample, Morek and Michalczyk, in press) and the least similar being an unidentified species from Madagascar (MK483999, Morek and Michalczyk, in press).

ITS-2: 7.7%–23.9% (16.6% on average with the most similar being *M. tardigradum*, (MK484011 from Russia, Morek and Michalczyk, in press) and the least similar being an unidentified species from Australia (MK484018, Morek and Michalczyk, in press).

COI: 11.4%–21.3% (16.6% on average), with the most similar being *M. variefidum* (MK492293, from the same sample, Morek and Michalczyk, in press), whereas the least similar being an unidentified species from Australia (MK492295, Morek and Michalczyk, in press).

The phylogenetic position of *M. pseudotardigradum* sp. nov., as well as the remaining two analysed species, is shown in figure 6.

DISCUSSION

The identification of species and the acquisition of taxonomic data for *Milnesium* species may pose a serious challenge when dealing with samples where multiple species with different CCs are present. Only a combination of various techniques, including DNA barcoding and culturing, allows for the collection of reliable data and confident interpretations (see such successful approaches in other animal groups: Shih et al. 2017; Chen et al. 2018; Jung et al. 2018; Hosoishi and Ogata 2019). In return, the collected data for further analysis are richer and no diversity is omitted due to difficulties in handling the material. The current study also shows that sole classic morphological data are starting to be insufficient in *Milnesium* taxonomy. Without the DNA sequencing and extensive culturing, it would have been impossible to differentiate the new species from *M. tardigradum*. In the sample, only a single specimen of *M. pseudotardigradum* sp. nov. with additional spurs was present, which would be insufficient to reveal that this is the key morphological trait differentiating the two species rather than an aberration Thus, in fact, it was the considerable distances in DNA markers that opened the investigation that resulted in establishing that individuals initially identified as *M. tardigradum* represent a morphologically very similar, yet a distinct species. Since the morphometric data for this pair of taxa overlap, and a statistical analysis of multiple specimens is required to tell them apart, the two species may be considered pseudocryptic. Recently, a similar pair of pseudocryptic species was identified: *M. alpigenum* (Ehrenberg, 1853) and *M. inceptum* (Morek et al. 2019b), with the exception that those species, despite striking morphological and morphometric similarities,

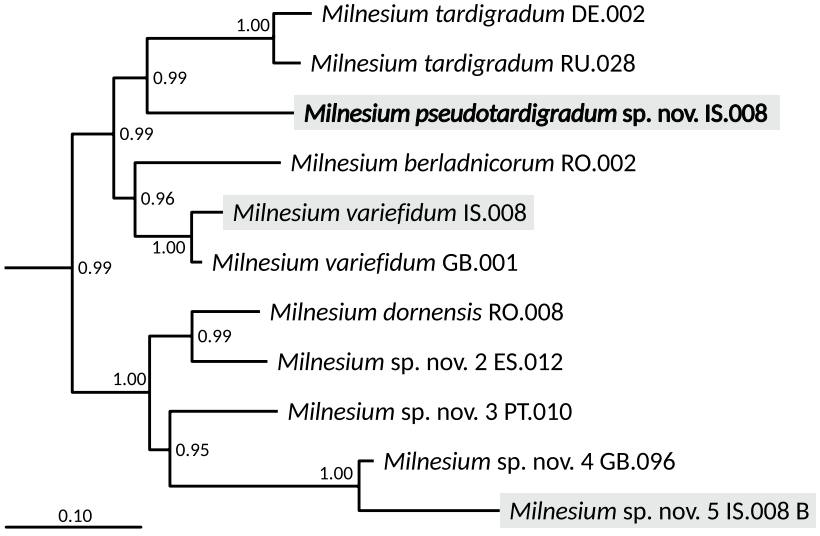

Fig. 6. Fragment of the Bayesian phylogenetic tree based on concatenated 18S rRNA + 28S rRNA + ITS-2 + *COI* nucleotide sequences obtained by Morek and Michalczyk (in press), showing the positions of the three *Milnesium* species analysed in the study (shaded in grey). Values at nodes represent Posterior Probability (PP) supports and scale bar shows the number of substitutions per site. For the entire tree as well as the details of the methods please see the reference.

are not immediate kin. These discoveries of hidden diversity indicate that *Milnesium* is most likely much more species-rich than it has been assumed, which is in line with findings stemming from a recent phylogenetic analysis of the genus (Morek and Michalczyk, in press), where in a survey of only 34 *Milnesium* populations, 25 species were detected, including as many as 17 new taxa.

The discovery of *M. pseudotardigradum* sp. nov. can be seen as another strong argument against the assumption that *M. tardigradum* has a cosmopolitan distribution. The redescription from 2012 by Michalczyk et al. explicitly showed that this species has a limited intraspecific morphological variability and the previous records from all around the world should be treated with extreme caution and preferably verified with DNA sequencing. The study presented herein shows that the sole morphological data may not be sufficient to confidently identify specimens as *M. tardigradum*, even if they can be delimited as such using available taxonomic keys. Therefore, there is a high risk of misidentified records, especially when based on single or few specimens. Last but not least, it is important to note that the present study provides a genetically verified record of *M. variefidum* outside its type locality, which is one of a very few such examples for *Milnesium* species. Thus, the *Milnesium* fauna of Iceland has now records of three recognised species (*M. variefidum*, *M. pseudotardigradum* sp. nov. and *Milnesium* cf. *quadrifidum*) and two undescribed taxa (*Milnesium* sp. nov. 5 IS.008 B and *Milnesium* sp. nov. 6 IS.037 in Morek and Michalczyk, in press), but importantly the presence of *M. tardigradum* on the island was not confirmed.

The most important trait of *M. pseudotardigradum* sp. nov. is the unique pattern of claw configuration change during ontogeny. Not only does the new species exhibit the early negative CC change, but it is also characterised by additional changes that occur between adult instars (Fig. 5), which makes it the first discovered species with a double ontogenetic CC change. Moreover, the second change is incomplete, which means that the shift is present in a large fraction of individuals (86%), but not in all of them. It is also important to note, that the additional spurs may appear only on single branches, making the left and right claws of a single pair of legs asymmetrical. However, the high frequency of additional spurs and their typical size and shape indicate they are not aberrations but a genuine character. This phenomenon is demonstrated for the first time in *Milnesium* and is the only morphological trait that differentiates the new species form *M. tardigradum*. In a wider perspective, this further highlights the importance of analysing all the life stages of a given species, with as many specimens as possible. This new, unexpected phenomenon makes the taxonomy of *Milnesium* even more challenging, therefore, it is crucial to describe and redescribe species with as accurate assessment of developmental variability as possible, ideally coupled with DNA markers. In fact, the characterisation of developmental variability and obtaining DNA sequences for already described species is very much needed, as the lack of such data poses a serious obstacle in *Milnesium* taxonomy.

CONCLUSIONS

Our study presents a pipeline that allows for the identification of species in challenging samples containing *Milnesium* species characterised by multiple CCs. The utilisation of integrative taxonomy allowed us to collect detailed datasets for each species/morphotype and led to the discovery of a species new to science, *M. pseudotardigradum* sp. nov., which is characterised by a double CC change during the ontogeny, a novel, unique pattern of developmental variability in the genus *Milnesium*. The extreme similarities between the new species and *M. tardigradum* add to the doubts regarding the putative cosmopolitan distribution of the latter taxon.

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Authors' contributions: WM and ŁM conceived the study. BS examined the sample, provided the measurements and photographs of the new species, ran statistical analyses, and drafted the manuscript. WM examined the sample, collected molecular data, prepared the remaining figures and drafted the manuscript. ŁM supervised the entire process and drafted the manuscript. All the authors read and approved the final manuscript.

Competing interests: The authors declare that they have no competing interests.

Availability of data and materials: The slides and SEM stubs are deposited at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland. All the obtained sequences were deposited in the GenBank.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable

REFERENCES

Bartels PJ, Nelson DR, Kaczmarek Ł, Michalczyk Ł. 2014. The genus *Milnesium* (Tardigrada: Eutardigrada: Milnesiidae) in the Great Smoky Mountains National Park (North Carolina and Tennessee, USA), with the description of *Milnesium bohleberi* sp. nov. Zootaxa **3826(2):**356–368. doi:10.11646/zootaxa.3826.2.5.

Binda MG, Pilato G. 1990. Tardigradi di Terra del Fuoco e Magallanes. I. *Milnesium brachyungue*, nuova specie di Tardigrado Milnesiidae. Animalia **17:**105–110.

Buda J, Olszanowski Z, Wierzgoń M, Zawierucha K. 2018. Tardigrades and oribatid mites in bryophytes from geothermally active lava fields (Krafla, Iceland) and the description of *Pilatobius islandicus* sp. nov. (Eutardigrada). Polish Polar Research **39(3):**425–453. doi:10.24425/118755.

Casquet J, Thebaud C, Gillespie RG. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Mol Ecol Resour **12:**136–141. doi:10.1111/j.1755-0998.2011.03073.x.

Chen SL, Shih HT. 2018. A review of the harvestman genus *Metadentobunus* (Opiliones: Sclerosomatidae: Gagrellinae) with a description of a new species from Taiwan. Zool Stud **57:**57. doi:10.6620/ZS.2018.57-57.

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(4):**542–564. doi:10.11646/zootaxa.3941.4.4.

de Coninck LAP. 1939. Wetenschappelijke resultaten der studiereis van Prof. Dr. P. Van Oye op Ijsland (Scientific results of Prof. Dr. P. Van Oye's expedition in Iceland). VI. Tardigrades. Biologisch Jaarboek **4:**190–218.

Degma P, Bertolani R, Guidetti R. 2007–2019. Actual checklist of Tardigrada species (2009–2019, 35rd Edition: 30-06-2018). Available from http://www.tardigrada.modena.unimo.it/miscellanea/Actual%20checklist%20of%20Tardigrada.pdf. Accessed 31 July 2019.

Degma P, Guidetti R. 2007. Notes to the current checklist of Tardigrada. Zootaxa **1579:**41–53. doi:10.11646/zootaxa.1579.1.2.

Doyère LMF. 1840. Memoire sur les Tardigrades. I. Annales des Sciences Naturelles, Paris, Series 2 **14:**269–362.

Ehrenberg CG. 1853. Diagnoses novarum formarum. Verhandlungen der Königlich Preussische Akademie der Wissenschaften zu Berlin **8:**526–533.

Gąsiorek P, Stec D, Zawierucha K, Kristensen RM, Michalczyk Ł. 2018. Revision of *Testechiniscus* Kristensen, 1987 (Heterotardigrada: Echiniscidae) refutes the polar-temperate distribution of the genus. Zootaxa **4472(2):**261–297. doi:10.11646/zootaxa.4472.2.3.

Guidetti R, Bertolani R. 2005. Tardigrade taxonomy: an updated check list of the taxa and a list of characters for their identification. Zootaxa **845:**1–46. doi:10.11646/zootaxa.845.1.1.

Guidetti R, Rebecchi L, Bertolani R, Jönsson KI, Kristensen RM, Cesari M. 2016. Morphological and molecular analyses on *Richtersius* (Eutardigrada) diversity reveal its new systematic position and lead to the establishment of a new genus and a new family within Macrobiotoidea. Zool J Linn Soc-Lond **178:**834–845. doi:10.1111/zoj.12428.

Guil N, Jørgensen A, Kristensen RM. 2018. An upgraded comprehensive multilocus phylogeny of the Tardigrada tree of life. Zool Scr **48(1):**120–137. doi:10.1111/zsc.12321.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acid S **41:**95–98.

Hosoishi S, Ogata K. 2019. Cryptic diversity of the widespread Asian ant *Crematogaster rothneyi* (Hymenoptera: Formicidae) inferred from morphological and genetic evidence. Zool Stud **58:**11. doi:10.6620/ZS.2019.58-11.

Jung J, Jung J, Kim W. 2018. Subdividing the common intertidal hermit crab *Pagurus minutus* Hess, 1865 (Decapoda: Anomura: Paguridae) based on molecular, morphological and coloration analyses. Zool Stud **57:**61. doi:10.6620/ZS.2018.57-61.

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. doi:10.11646/zootaxa.3589.1.3.

Kaczmarek Ł, Michalczyk Ł. 2007. A new species of Tardigrada (Eutardigrada: Milnesiidae): *Milnesium krzysztofi* from Costa Rica (Central America). New Zeal J Zool **34:**297–302. doi:10.1080/03014220709510088.

Kaczmarek Ł, Michalczyk Ł, McInnes SJ. 2014. Annotated zoogeography of non-marine Tardigrada. Part I: Central America. Zootaxa **3763:**001–062. doi:10.11646/zootaxa.3763.1.1.

Kaczmarek Ł, Michalczyk Ł, McInnes SJ. 2016. Annotated zoogeography of non-marine Tardigrada. Part III: North America and Greenland. Zootaxa **4203:**001–249. doi:10.11646/zootaxa.4203.1.1.

Kosztyła P, Stec D, Morek W, Gąsiorek P, Zawierucha K, Michno K, Ufir K, Małek D, Hlebowicz K, Laska A, Dudziak M, Frohme M, Prokop ZM, Kaczmarek Ł, Michalczyk Ł. 2016. Experimental taxonomy confirms the environmental stability of morphometric traits in a taxonomically challenging group of microinvertebrates. Zool J Linn Soc-Lond **178:**765–775. doi:10.1111/zoj.12409.

Kumar S, Stecher G, Taura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol **33:**1870–1874. doi:10.1093/molbev/msw054.

Maucci W. 1991. Tre nuove specie di Eutardigradi della Groenlandia Meridionale. Bollettino del Museo Civico di Storia Naturale di Verona **15:**279–289.

Maucci W. 1996. Tardigrada of the Arctic tundra with descriptions of two new species. Zool J Linn Soc-Lond **116:**185–204. doi:10.1111/j.1096-3642.1996.tb02343.x.

Meyer HA. 2015. Water bears (Phylum Tardigrada) of Oceania, with the description of a new species of *Milnesium*. New Zeal J Zool **42:**173–186. doi:10.1080/03014223.2015.1062402.

Meyer HA, Hinton JG. 2010. *Milnesium zsalakoae* and *M. jacobi*, two new species of Tardigrada (Eutardigrada: Apochela: Milnesiidae) from the southwestern United States. P Biol Soc Wash **123:**113–120. doi:10.2988/09-29.1.

Meyer HA, Hinton JG. 2012. Terrestrial Tardigrada of the Island of Barbados in the West Indies, with the description of *Milnesium barbadosense* sp. n. (Eutardigrada: Apochela: Milnesiidae). Caribb J Sci **46:**194–202. doi:10.18475/cjos.v46i2.a8.

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 Naturalist **73:**295–301. doi:10.3398/064.073.0305.

Michalczyk Ł, Kaczmarek Ł. 2013. The Tardigrada Register: a comprehensive online data repository for tardigrade taxonomy. J Limnol **7:**175–181. doi:10.4081/jlimnol.2013.s1.e22.

Michalczyk Ł, Wełnicz W, Frohme M, Kaczmarek Ł. 2012a. Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. Zootaxa **3154:**1–20. doi:10.11646/zootaxa.3154.1.1.

Michalczyk Ł, Wełnicz W, Frohme M, Kaczmarek Ł. 2012b. Corrigenda of *Zootaxa 3154: 1–20*. Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. Zootaxa **3393:**66–68. doi:10.11646/zootaxa.3393.1.6.

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. doi:10.11646/zootaxa.3253.1.2.

Morek W, Gąsiorek P, Stec D, Bladgen B, Michalczyk Ł. 2016a. Experimental taxonomy exposes ontogenetic variability and elucidates the taxonomic value of claw configuration in *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela). Contrib Zool **85:**173–200. doi:10.1163/18759866-08502003.

Morek W, Michalczyk Ł. in press. First extensive multilocus phylogeny of the genus *Milnesium* (Tardigrada) reveals no congruence between genetic markers and morphological traits. Zool J Linn Soc-Lond. doi:10.1093/zoolinnean/zlz040.

Morek W, Stec D, Gąsiorek P, Schill RO, Kaczmarek Ł, Michalczyk Ł. 2016b. An experimental test of eutardigrade preparation methods for light microscopy. Zool J Linn Soc-Lond **178:**785–793. doi:10.1111/zoj.12457.

Morek W, Stec D, Gąsiorek P, Surmacz B, Michalczyk Ł. 2019a. *Milnesium tardigradum* Doyère, 1840: The first integrative study of interpopulation variability in a tardigrade species. J Zool Syst Evol Res **57:**1–23. doi:10.1111/jzs.12233.

Morek W, Suzuki AC, Schill RO, Georgiev D, Yankova M, Marley NJ, Michalczyk Ł. 2019b. Redescription of *Milnesium alpigenum* Ehrenberg, 1853 (Tardigrada: Apochela) and a description of *Milnesium inceptum* sp. nov., a tardigrade laboratory model. Zootaxa **4586:**35–64. doi:10.11646/zootaxa.4586.1.2.

Morgan CI. 1980. A systematic survey of tardigrada from Iceland. Acta Naturalia Islandica **27:**1–25.

Nelson DR, Guidetti R, Rebecchi L. 2015. Phylum Tardigrada. Ecology and General Biology (fourth ed.), Thorp and Covich's Freshwater Invertebrates, vol. 1, Elsevier, Oxford (2015), pp. 347–380. doi:10.1016/B978-0-12-385026-3.00017-6.

Pilato G. 1981. Analisi di nuovi caratteri nello studio degli Eutardigradi. Animalia **8:**51–57.

Pilato G, Binda MG. 1991. *Milnesium tetralamellatum*, new species of Milnesiidae from Africa (Eutardigrada). Trop Zool **4:**103–106. doi:10.1080/03946975.1991.10539480.

Pilato G, Binda MG, Lisi O. 2002. Notes on tardigrades of the Seychelles with the description of two new species. Bollettino dell'Accademia Gioenia Scienze Naturali **35:**503–517. doi:10.1080/11250000409356569.

Pilato G, Lisi O. 2016. *Milnesium minutum* and *Milnesium sandrae*, two new species of Milnesiidae (Tardigrada, Eutardigrada, Apochela). ZooKeys **580:**1–12. doi:10.3897/zookeys.580.6603.

Pilato G, Sabella G, D'Urso V, Lisi O. 2017. Two new species of Eutardigrada from Victoria Land, Antarctica. Zootaxa **4317(3):**541–558. doi:10.11646/zootaxa.4317.3.6.

Pilato G, Sabella G, Lisi O. 2016. Two new species of *Milnesium* (Tardigrada: Milnesiidae). Zootaxa **4132(4):**575–587. doi:10.11646/zootaxa.4132.4.9.

Ramazzotti G. 1962. Tardigradi del Cile–con descrizione di quattro nuove specie e di una nuova varietà. Atti della Società Italiana di Scienze Naturali e del Museo Civico di Storia Naturale in Milano **101:**275–287.

Roszkowska M, Ostrowska M, Kaczmarek Ł. 2015. The genus *Milnesium* Doyère, 1840 (Tardigrada) in South America with descriptions of two new species from Argentina and discussion of the feeding behaviour in the family Milnesiidae. Zool Stud **54:**12. doi:10.1186/s40555-014-0082-7.

Schlabach 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. Transactions of the Kansas Academy of Science **121:**39–48. doi:10.1660/062.121.0204.

Schuster RO, Nelson DR, Grigarick AA, Christenberry D. 1980. Systematic criteria of the Eutardigrada. T Am Microsc Soc **99:**284–303. doi:10.2307/3226004.

Shih HT, Cai Y, Niwa N, Nakahara Y. 2017. A new species of land-locked freshwater shrimp of the genus *Neocaridina* (Decapoda: Caridea: Atyidae) from Iki Island, Kyushu, Japan. Zool Stud **56:**30. doi:10.6620/ZS.2017.56-30.

Stec D, Gąsiorek P, Morek W, Kosztyła P, Zawierucha K, Michno K, Kaczmarek Ł, Prokop ZM, Michalczyk Ł. 2016. Estimating optimal sample size for tardigrade morphometry. Zool J Linn Soc-Lond **178:**776–784. doi:10.1111/zoj.12404.

Stec D, Morek W, Gąsiorek P, Michalczyk Ł. 2018. Unmasking hidden species diversity within the Ramazzottius oberhaeuseri complex, with an integrative redescription of the nominal species for the family Ramazzottiidae (Tardigrada: Eutardigrada: Parachela). Syst Biodivers **16(4):**357–376. doi:10.1080/14772000.2018.1424267.

Stec D, Smolak R, Kaczmarek Ł, Michalczyk Ł. 2015. An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: hufelandi group) from Kenya. Zootaxa **4052(2):**501–526. doi:10.11646/zootaxa.4052.5.1.

Stec D, Zawierucha K, Michalczyk Ł. 2017. An integrative description of *Ramazzottius subanomalus* (Biserov, 1985) (Tardigrada) from Poland. Zootaxa **4300:**403–420. doi:10.11646/zootaxa.4300.3.4.

Tumanov DV. 2006. Five new species of the genus *Milnesium* (Tardigrada, Eutardigrada, Milnesiidae). Zootaxa **1122:**1–23. doi:10.5281/zenodo.171737.

Tuxen SL. 1941. XXI. Tardigrada. Zoology of Iceland **3(24):**1–11.

Yang T. 2007. One New Species and Four Newly Recorded Species of the Class Eutardigrada from China (Apochela: Milnesiidae; Parachela: Macrobiotidae, Hypsibiidae). Acta Zootaxonomica Sinica **32:**84–91.

Young A, Chappell B, Miller W, 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(5):**559–568. doi:10.11646/zootaxa.4072.5.3.

Zeller C. 2010. Untersuchung der Phylogenie von Tardigraden anhand der Genabschnitte 18S rDNA und Cytochrom *c* Oxidase Untereinheit 1 (COX I). Master thesis, Technische Hochschule Wildau, 105 pp.