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Experimental taxonomy exposes ontogenetic variability and elucidates the taxonomic value of claw configuration in *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela)

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Abstract

In this paper we describe a new apochelan species, *Milnesium variefidum* sp. nov. from Scotland and provide novel morphological and molecular data for *Milnesium berladnicorum* Ciobanu *et al*., 2014. The new species differs from the most similar *M. berladnicorum* by the presence of developmental dimorphism in claw configuration, absent or weakly developed cuticular bars under claws I-III, a different arrangement of cuticular pseudoplates, and by differences in the sequences of three nuclear DNA fragments: 18S rRNA (p-distance: 0.6%), 28S rRNA (2.0%), ITS-2 (9.3%), and on mitochondrial gene COI (12.4%). Although ontogenetic claw configuration change was suspected to occur in some *Milnesium* species, we are the first to document it through the combined use of traditional, molecular and experimental methodologies. We discuss the implications of the observed phenomenon for the taxonomy of the genus and propose a new diagnostic key to all *Milnesium* species described up to the end of 2015. We also review other traits used for species differentiation in the genus and offer recommendations to improve the quality of future descriptions as well as suggest a need for integrative redescriptions of the known species. Finally, we propose to suppress *M. dujiangensis* and *M. tardigradum trispinosum* and suggest that *M. alpigenum* and *M. quadrifidum* are valid species that require thorough redescriptions.

Contents

Introduction

Claw morphology in the class Eutardigrada is thought to be conservative and therefore it has been used to reorganise eutardigrade taxonomy at the family level (Pilato, 1969; Bertolani *et al*., 2014). In the family Milnesiidae, the only family in the order Apochela, the general claw morphology is also conserved and remains unique. In contrast to the parachelan taxa all four apochelan genera, *Milnesium* Doyère, 1840, *Limmenius* Horning *et al*., 1978, *Milnesioides* Claxton, 1999, and *Bergtrollus* Dastych, 2011, exhibit the same claw anatomy: the primary branch is completely separated from the secondary branch. The only observed variability of claw morphology within the Milnesiidae concerns the presence or absence of accessory points on the single-branched primary branches and the number of points on the secondary branches, which in the currently known species varies from two to four. The only exception is *Milnesium dujiangensis* Yang, 2003, a species that allegedly lacks the primary branches and in which the secondary branch of anterior claws IV is equipped only with a single point. However, given such exceptional characters and the very poor quality of the original description, this species is commonly considered dubious.

In the 19th and early 20th century, when not only *Milnesium* taxonomy, but tardigrade taxonomy in general was in its infancy, researchers recognised variability in the number of points on the secondary branches in *Milnesium* and considered it to be a diagnostic trait at the species level. As a result, following the description of the nominal *Milnesium tardigradum* Doyère, 1840, two further species were described solely on the basis of different claw configurations: *Milnesium alpigenum* Ehrenberg, 1853 (with three points on all claws) and *Milnesium quadrifidum* Nederström, 1919 (with four points on all claws). However, soon after, these species were suppressed by Marcus (1928) who reconsidered the taxonomic value of the number of points on the secondary branches and concluded that these variations only represent different 'geographic races' of *M. tardigradum*. This view was supported by Ramazzotti (1962) and by Ramazzotti and Maucci (1983) and only very recently, at the XII International Symposium on Tardigrada (Portugal, July 2012), was the validity of the two species brought back to the debate by Marley (2012). However, given the poor original descriptions, they are still awaiting a proper restoration of their taxonomic status. The opinion that claw configuration is unstable and therefore not useful as a taxonomic trait in *Milnesium* was also supported by Dastych (1984) who found various developmental aberrations in *Milnesium* specimens from the Antarctic. This view, combined with the concept that tardigrades are cosmopolitan, led to a severe underestimation of the number of *Milnesium* species worldwide (Michalczyk *et al*., 2012a, b).

Nevertheless, variation in other traits, such as cuticular sculpture and feeding apparatus morphology, was appreciated by some researches and the genus ceased to be monospecific again as new *Milnesium* species started to appear in the literature in the early 1990s (Binda and Pilato, 1990; Maucci, 1991). These species, as well as further new species described in the following decade, seemed to exhibit stable and distinct claw configurations. Michalczyk *et al*. (2012a, b) attempted to revise the taxonomic value of the claw configuration and their analysis seemed to confirm that in principle (*i.e*. except for occasional developmental aberrations) claw configuration is a stable character that can be used for species differentiation. The abnormalities are usually easy to identify as they occur only in a small fraction of individuals and aberrant spurs are typically smaller than regular points. Thus, given the importance of claw configuration in species differentiation, in order to aid comparisons of species with different claw configurations, Michalczyk *et al*. (2012a, b) introduced a denotation system in which the number of points on the secondary branches on all legs is given as a short string of numbers, *e.g*. [2-3]-[3-2] for *M. tardigradum* (see Material and methods for details).

The great majority of the thirty *Milnesium* descriptions published until the end of 2015 report single claw configurations within type populations. However, there are two intriguing exceptions, *M. tetralamellatum* Pilato and Binda, 1991 and *M. barbadosense* Meyer and Hinton, 2012, in which more than one claw configuration was described. In the type population of the first species Pilato and Binda (1991) identified two groups of specimens, one with a [2-2]-[2-2] and the other with a [2-3]-[2-3] claw configuration. Similarly, Meyer and Hinton (2012) reported a [2-3]-[2-3] and a [3-3]-[3-3] configuration for the type population of *M. barbadosense*. Meyer and Hinton (2012) also noted that the specimens with the lower number of spurs were generally smaller than those with three spurs on all claws. These two exceptions provoke the question as to whether they are examples of intra- or inter-specific variability. On one hand, claw morphology change during development is known to occur in *Milnesium* males, in which immature individuals have unmodified claws whereas in sexually mature males the secondary claws on the first pair of legs develop into robust hooks (Marcus, 1928; Rebecchi and Nelson, 1998; Ciobanu *et al*., 2015). On the other hand, it is not unusual for multiple congeneric taxa to inhabit a single microhabitat (*e.g*. Kaczmarek *et al*., 2011), therefore in such a case different claw configurations could simply represent different species, erroneously classified as a single species (according to a personal communication from Giovanni Pilato, in the case of *M. tetralamellatum* specimens with a [2-2]-[2-2] configuration may represent a different species as they exhibit cuticular sculpturing compared to smooth [2-3]-[2-3] specimens). In principle, this conundrum could be solved by following the development of individuals animals and/or DNA sequencing of specimens exhibiting different claw configurations. However, to the best of our knowledge, no such research has been undertaken.

While examining a lichen sample collected in Scotland, we came across a population of *Milnesium* that was otherwise uniform, except for exhibiting two distinct claw configurations. To test whether this apparent dimorphism is a result of developmental variability or the presence of more than one species in the sample, we applied both an experimental and a molecular approach

to its resolution. As a result of our investigation, in this paper we describe the first ever definite evidence of a developmental claw configuration change in a species of *Milnesium* and discuss its consequences for the taxonomy of the genus. We have identified the species, in which we observed the claw configuration change, as a species new to science and provide its integrative description. We also review the literature and identify other potential species in which claw configuration change could occur and underline the importance of avoiding species descriptions based on a limited number of individuals, especially if type specimens represent only a fraction of expected body size range, *i.e.* when there was a risk that only juvenile or only adult stages were represented. Moreover, given the above findings and the fact that since the last diagnostic key to the genus was published (Michalczyk *et al*., 2012a, b) ten new species have been described, we provide an amended and updated key to the genus *Milnesium*.

Material and methods

Sample processing

The lichen sample (*Xanthoria sp.*) containing the new species was collected from a Mountain Ash (*Sorbus aucuparia*) tree growing near to the village of Insch, Scotland, UK (57°20'31''N, 02°37'08''W; 130 m asl) in August 2014 by Brian Blagden. The sample was collected and examined for tardigrades using standard methods (Dastych, 1980) with modifications described in Stec *et al*. (2015). A total of 67 individuals and 3 exuviae with 15 eggs of the new species were extracted from the sample. Individuals were split into three groups: 55 animals were mounted on microscope slides in Hoyer's medium, a further 6 individuals were prepared for SEM, and the final 6 specimens were processed for DNA sequencing. All exuviae with eggs were placed in the *in vitro* culture (see below for details).

Microscopy and imaging

Specimens for light microscopy were mounted on microscope slides in a small drop of Hoyer's medium prepared according to Morek *et al*. (in press) and secured with a cover slip. Slides were then placed in an incubator and dried for five days at 60 °C. Dried slides were sealed with a transparent nail polish and examined under a *Nikon Eclipse 50i* phase contrast light microscope (PCM) associated with a *Nikon Digital Sight DS-L2* digital camera. In order to obtain clean and extended specimens for SEM, tardigrades were processed according to the protocol by Stec *et al*. (2015). In short, specimens were first subjected to a 60 °C water bath for 30 min to obtain fully extended animals, next to a water/ethanol and an ethanol/acetone series, then to CO_2 critical point drying, and finally sputter coated with a thin layer of gold. Specimens were examined under high vacuum in a *Versa 3D DualBeam* Scanning Electron Microscope at the ATOMIN facility of the Jagiellonian University, Kraków, Poland.

All figures were assembled in *Corel Photo-Paint X6*, ver. 16.4.1.1281. For deep structures that could not be fully focused in a single photograph, a series of 2-10 images were taken every *ca*. 0.25 μm and then assembled into a single deep-focus image.

Morphometrics

All measurements are given in micrometres [μm]. Individuals and their traits were measured only if their orientation was suitable. Body length was measured from the anterior extremity to the end of the body, excluding the hind legs. Claw and buccal tube measurements were made according to Tumanov (2006), and additional buccal tube widths and ratios were determined based on Michalczyk *et al*. (2012a). The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube, expressed as a percentage (Pilato 1981). In the text, the *pt* ratio is always given in *italics*. Morphometric data were handled using the 'Apochela' ver. 1.2 template available from the Tardigrada Register, www.tardigrada.net/register (Michalczyk and Kaczmarek, 2013). Configuration of the number of claw points on secondary branches (claw configuration) is given according to Michalczyk *et al*. (2012b), *i.e.* as a string of bracketed numbers that represent the number of points on the secondary branches on **e**xternal and **i**nternal claws I-III, and on **a**nterior and **p**osterior claws IV: [**e**-**i**]-[**a**-**p**]. Given that non-parametric tests require fewer assumptions than parametric tests, the statistical significance of differences in body and buccal tube lengths between juveniles and adults of the new species were estimated with the Mann-Whitney *U*-test, using IBM SPSS Statistics 22.0 software.

Raw data underlying the description of *Milnesium variefidum* sp. nov. are deposited in the Tardigrada Register (Michalczyk and Kaczmarek, 2013) under www.tardigrada.net/register/0020.htm.

Genotyping

As noted above, six individuals of the new species were processed for DNA extraction, amplification and sequencing. Before extraction, each individual was examined under the PCM in order to establish its claw configuration. Two animals exhibited a [2-2]-[2-2] claw configuration and the remaining four were [2-3]-[2-2], thus we were able to compare the DNA sequences of animals with both claw configuration types (see Fig. 1). The DNA was extracted from individual animals following a Chelex® 100 resin (Bio-Rad) extraction method by Casquet *et al*. (2012) with modifications described in detail in Stec *et al*. (2015). We sequenced four DNA fragments differing in mutation rates (from the most conservative): the small ribosome subunit (18S rRNA, nDNA), the large ribosome subunit (28S rRNA, nDNA), the cytochrome oxidase subunit I (COI, mtDNA), and the internal transcribed spacer (ITS-2, nDNA). All fragments were amplified and sequenced according to the protocols described in Stec *et al*. (2015), primers and origi-

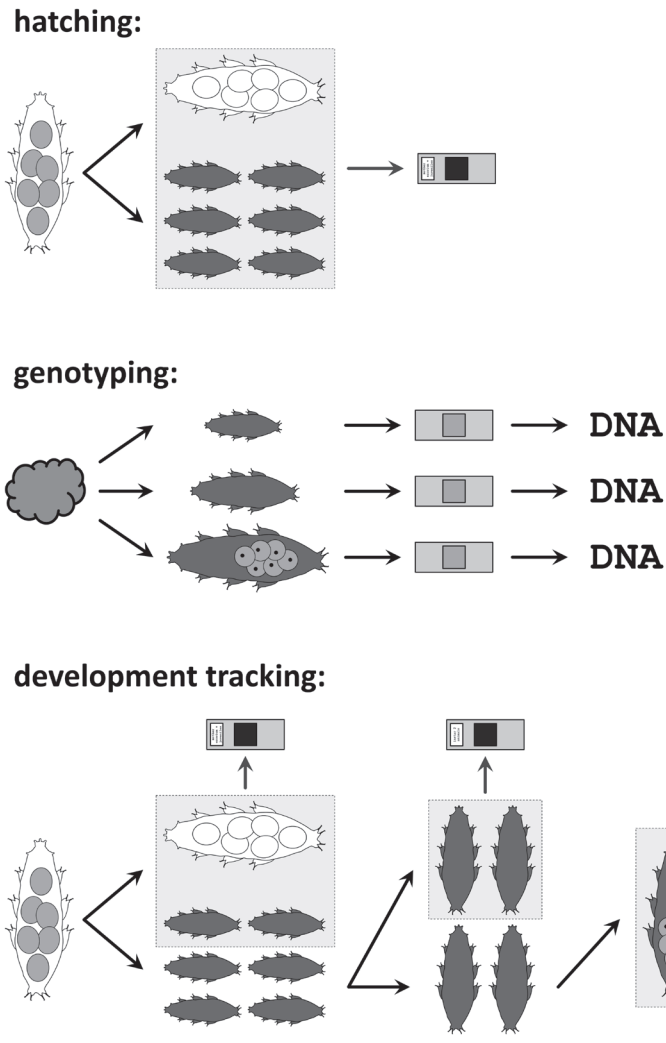


Fig. 1. A schematic illustration of three protocols that allow the identification of developmental variability in claw configuration in the genus *Milnesium*. In the most basic approach, an exuvia with eggs is placed in a small Petri dish filled with distilled water and kept at room temperature. After **hatching**, the juveniles and the exuvia with empty egg shells are mounted on a single permanent slide and the claw configuration of the mother and the offspring is compared. When no exuviae with eggs or live animals are found, **genotyping** can be employed to establish whether individuals exhibiting different claw configurations represent a single or multiple species. In such a case, animals are first individually mounted on temporary water slides, their claw configuration is noted down and documented photographically (photogenophores) under a light microscope. Then, the genomic DNA is isolated individually from the animals and the COI or ITS-2 fragment (or any highly variable DNA barcode) is sequenced and compared between the individuals. Unlike the first two methods, **development tracking** not only tests for developmental variability but it also allows the identification of the life stage at which the transition from one claw configuration to the other occurs. Soon after hatching, the exuvia with empty eggs and a subset of juveniles are mounted permanently on a microscope slide and the remaining offspring are transferred individually to small Petri dishes lined with agar and filled with water and fodder (*e.g.* rotifers) in order to allow offspring growth. Animals are checked daily and if found moulting, a subset of second instars is mounted on a permanent slide. The remaining portion is allowed to moult again and the third instar (*i.e.* reproductively mature) individuals are also mounted permanently on a microscope slide. Finally, claw configurations of animals on the three slides are compared. All three protocols can be also used to test for developmental variability of any morphological and morphometric trait in any tardigrade species.

nal references for specific PCR programmes are listed in Table 1. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of the Jagiellonian University, Kraków, Poland. Sequences were processed in *BioEdit* ver. 7.2.5 (Hall 1999) and submitted to GenBank.

For comparisons, sequences of *Milnesium* spp. from GenBank were used. First, the identity of all four obtained sequences was verified using the Basic Local Alignment Search Tool (BLAST; Altschul *et al*., 1990). Then, after assuring that our sequences were unique, of all *Milnesium* spp. sequences deposited in GenBank, only those of good quality and length were used for more detailed comparisons with the sequences for *M. variefidum* sp. nov. Currently, there are only four *Milnesium* ITS-2 sequences deposited in GenBank, and all were used for comparisons: JF951049 (Michalczyk *et al*., 2012a), GQ403681-2 (Schill *et al*., 2009), and HM150648 (Wełnicz *et al*., 2010). For the COI, seven of eight available sequences were compared with our material: FJ435810 (Guil and Giribet, 2008), JN664950 (Michalczyk *et al*., 2012a), EU244603-4 (Schill, 2007), and KP013598, 601 and 613 (Velasco-Castrillon *et al*., 2014; unpublished). To calculate molecular distances for the 28S rRNA, we used eight of the twelve deposited sequences: JX888541 and JX888585-7 (Adams *et al*., 2012; unpublished), FJ435779-80 (Guil and Giribet, 2008), and KC138808-9 (Zawierucha, 2012; unpublished). Finally, for the 18S rRNA we compared 18 of 32 available sequences: MTU49909 (Aguinaldo *et al*., 1997; unpublished), GQ925683, 685-8 and 692-7 (Chen *et al*., 2009; unpublished), FJ435749-50 (Guil and Giribet, 2012), AY582120 (Jørgensen and Kristensen, 2004), EU266922-3 (Sands *et al*., 2008), and HM187581 (Wełnicz *et al*., 2010). Additionally, sequences of *M. berladnicorum* obtained in the present study were included in the analyses (18S rRNA: KT951660, 28S rRNA: KT951661, ITS-2: KT951662, COI: KT951659, see below for details).

Sequences were aligned with the ClustalW Multiple Alignment tool (Thompson *et al*., 1994) implemented in BioEdit. The aligned sequences were then trimmed to 385 (ITS-2), 605 (COI), 742 (28S rRNA), and 1073 (18S rRNA) bp, respectively. Pairwise distances were calculated using PAUP* ver. 4.0b10 (Swofford, 2002).

In vitro culture

In order to follow the development of individual animals (Fig. 1), exuviae with eggs isolated from the lichen sample were placed individually in wells of a 24-well plastic plate (incubator), each filled with 1 ml of medium made of a mixture of distilled and spring water 'Żywiec Zdrój' (3:1). Eggs were incubated at 16 °C in complete darkness, and checked daily for hatched juveniles. Once all eggs from a given exuvia hatched, the exuvia and a subset of juveniles were mounted on a microscope slide in Hoyer's medium and the remaining offspring were transferred individually to a culture plate. Wells in the culture plate were lined with a thin layer of 2% agar (165 μl per well) and topped with a 1 ml of medium enriched in fodder composed of *Lecane inermis* Bryce, 1982 rotifers and small amounts of freshwater algae (*Chlorococcum* sp. and *Chlorella* sp.; 1:1, Sciento, UK). Offspring in the culture plates were observed daily and after each of the first two moults a subset of several individuals were mounted on microscope slides. Thanks to this experimental design, we were able to compare the claw configuration of the mother, and the first three instars of offspring (see Fig. 1).

Table 1. Primers and references for specific protocols for amplification of the four DNA fragments used in the present study.

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source	PCR programme source
18S rRNA	SSU01_F	forward	AACCTGGTTGATCCTGCCAGT	Sands *et al*. (2008)	Zeller (2010)
	SSU82_R	reverse	TGATCCTTCTGCAGGTTCACCTAC	Sands *et al*. (2008)	
28S rRNA	28SF0001	forward	ACCCVCYNAATTTAAGCATAT	Mironov *et al*. (2012)	Mironov *et al*. (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov *et al*. (2012)	
ITS-2	ITS3	forward	GCATCGATGAAGAACGCAGC	White *et al*. (1990)	Wełnicz *et al*. (2011)
	ITS4	reverse	TCCTCCGCTTATTGATATGC	White *et al*. (1990)	
COI	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*. (1994)	Michalczyk *et al*. (2012a)
	HCOoutout	reverse	GTAAATATATGRTGDGCTC	Prendini *et al*. (2005)	

Table 2. A comparison of body and buccal tube lengths between juveniles ([2-2]-[2-2] claw configuration) and adults ([2-3]-[2-2] configuration) of *Milnesium variefidum* sp. nov. found in a lichen sample from Insch, Scotland. Juveniles are statistically significantly smaller than adults in both traits (Z = test statistic, p = statistical significance, r = effect size).

Statistic	Body length (μm)		Buccal tube length (μm)	
	Juveniles (n=9)	Adults (n=26)	Juveniles (n=9)	Adults (n=26)
Minimum	217	366	23.0	30.6
Maximum	348	763	25.7	50.0
Mean	291	529	24.6	40.8
Standard Deviation	42	120	1.0	6.1
U-test	*Z=4.416, p<0.001, r=0.75*		*Z=4.416, p<0.001, r=0.75*	

Literature review and comparative material

Data on body length ranges and taxonomic traits used for the construction of the diagnostic key to the genus were extracted from original descriptions and redescriptions of all known *Milnesium* species and subspecies described up to the end of 2015 (*i.e*. Doyère, 1840; Ehrenberg, 1853; Nederström, 1919; Rahm, 1931; Ramazzotti, 1962; Binda and Pilato, 1990; Maucci, 1991; Pilato and Binda, 1991; Pilato *et al*., 2002; Yang, 2003; Kaczmarek *et al*., 2004; Tumanov, 2006; Kaczmarek and Michalczyk, 2007; Wallendorf and Miller, 2009; Meyer and Hinton, 2010, 2012; Kaczmarek *et al*., 2012; Michalczyk *et al*., 2012a; Meyer *et al*., 2013; Bartels *et al*., 2014; Ciobanu *et al*., 2014; Ciobanu *et al*., 2015; Londoño *et al*., 2015; Meyer, 2015; Roszkowska *et al*., 2015).

No morphometric data were provided in the original descriptions of *M. alpigenum* and *M. quadrifidum* (Ehrenberg, 1853 and Nederström, 1919, respectively) and no redescriptions of these species are available, making it difficult to place these two species in the diagnostic key. Fortunately, *M. quadrifidum* is currently the only known *Milnesium* species with four points on secondary branches, thus it can be distinguished from other species by its unique claw configuration. *Milnesium alpigenum*, however, falls into the most species-rich group with three points on all secondary branches, thus additional traits are required in order to differentiate this species from other congeners with the same claw configuration. In order to overcome the lack of type or neotype data for this species, we have measured fifteen individuals from a parthenogenetic lab strain kindly provided by Dr. R.O. Schill (Stuttgart University, Germany). The strain has been founded with individuals isolated from a moss sample collected from Tübingen, Bebenhausen in Germany (48°33'42''N, 09°03'48''E, 377 m asl) in 2002 by Dr. R.O. Schill and maintained in lab ever since. Given that this strain differs morphometrically from all other [3-3]-[3-3] *Milnesium* species and because the collection site lies only about 300 km from the *locus typicus* of *M. alpigenum*, for the purpose of this work we have assumed that the strain represents a population of *M.* cf. *alpigenum*. In order to obtain as much phenotypic variation as possible (Kosztyła *et al*., in press), we reared individuals under three temperature regimes (8, 16 and 24 °C). Moreover, to ensure the coverage of a wide body size range, we measured three individuals of each of the first five instars (*i.e*. 3 temperature regimes × 5 instars, represented equally). The morphometric data for this strain of *M.* cf. *alpigenum* are presented in Table 3.

Given that the new species is most similar to *Milnesium berladnicorum* Ciobanu *et al*., 2014, and as the original description of the species did not mention the presence of cuticular pseudoplates or include molecular barcodes, we obtained the type series and a lichen sample from the type locality of *M. berladnicorum*, courtesy of Dr. Łukasz Kaczmarek (Adam Mickiewicz University, Poland) and Daniel Ciobanu (Alexandru Ioan Cuza University, Romania). The sample was collected from Bârlad, Romania (46°14'41''N, 27°40'19''E; 80 m asl), in April 2014 by D. Ciobanu, and processed by us as described above. A total of 13 individuals (4 juveniles and 9 adults) were found and subsequently split into two groups: 3 animals (1 juvenile and 2 adults) were mounted on microscope slides in Hoyer's medium, 3 adults were fixed for SEM, and 7 individuals (3 juveniles and 4 adults) were processed for DNA sequencing (using protocols described above). The sequences of *M. berladnicorum* obtained in the present study were uploaded to GenBank (see above for accession numbers).

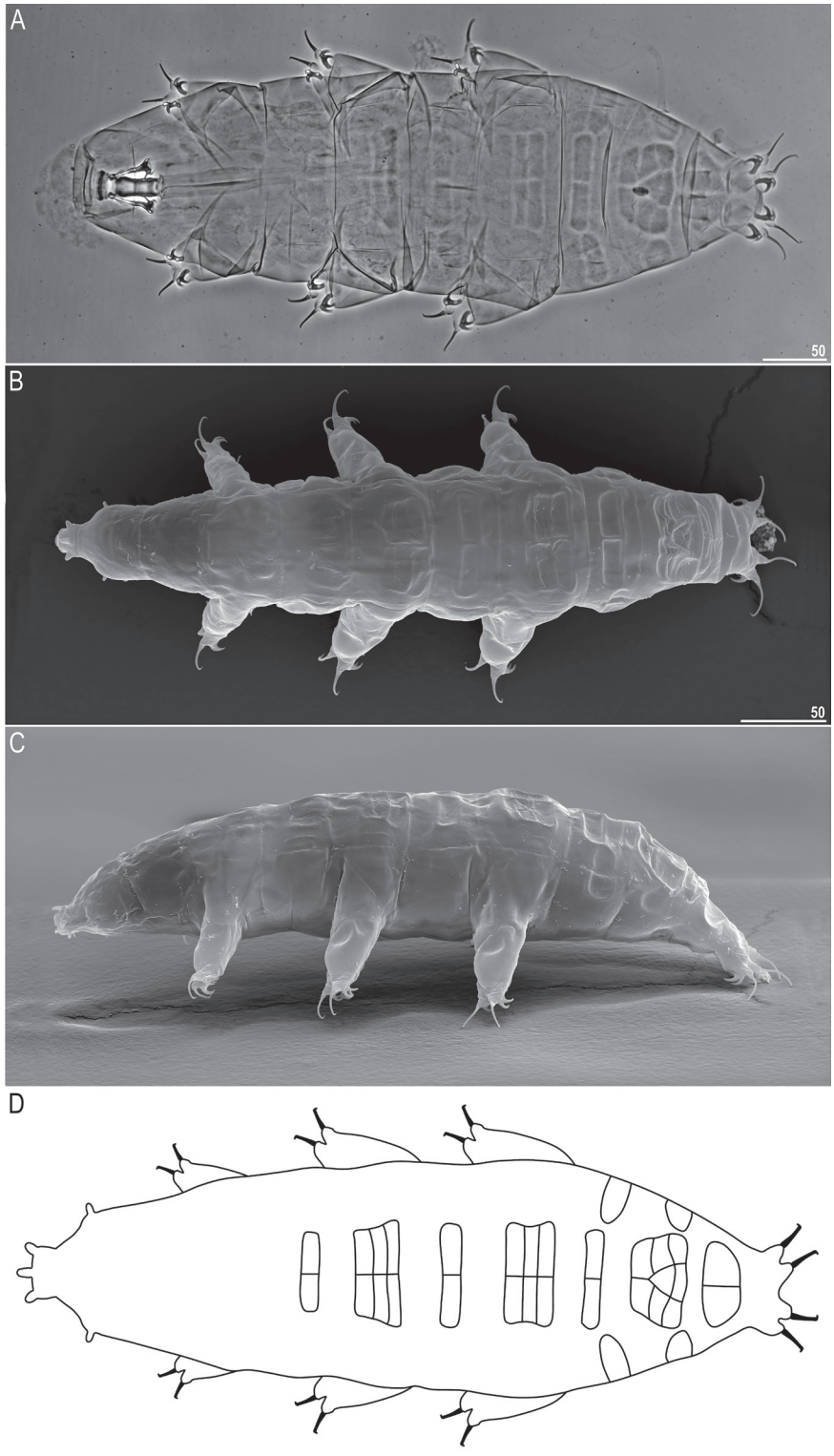


Fig. 2. *Milnesium variefidum* sp. nov.: habitus and dorsal pseudoplates: A – dorsal view (holotype, PCM); B – dorsal view (paratype, SEM); C – lateral view (paratype, SEM); D – semi-schematic drawing of the dorsal pseudoplates based on PCM and SEM observations.

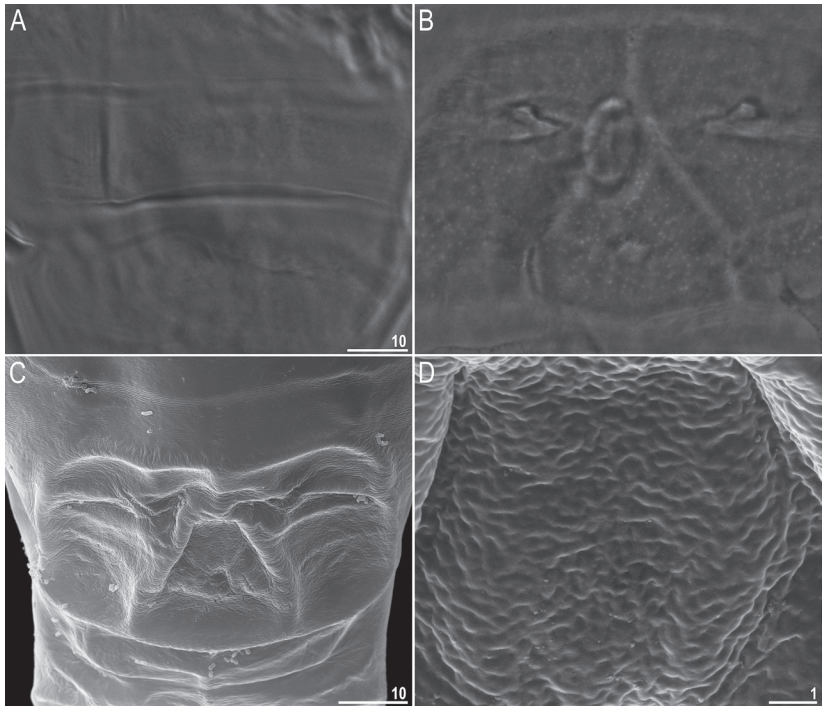


Fig. 3. Milnesium variefidum sp. nov.: caudo-dorsal cuticle: A – juvenile cuticle without visible pseudoplates or pseudopores (paratype, PCM); B – adult cuticle with clearly visible pseudoplates and pseudopores (holotype, PCM); C – adult cuticle with caudo-dorsal pseudoplates (paratype, SEM); D – a magnified fragment showing irregular wrinkled sculpturing on a caudo-dorsal pseudoplate (paratype, SEM). Scale on Fig. B same as on Fig. A.

Results

Taxonomic account of the new species

Phylum Tardigrada Doyère, 1840
Class Eutardigrada Richters, 1926
Order Apochela Schuster *et al*., 1980
Family Milnesiidae Ramazzotti, 1962
Genus *Milnesium* Doyère, 1840
Milnesium variefidum sp. nov.
(Table 4, Figs 2-5)

Description of Milnesium variefidum *sp. nov.* Animals (morphometrics in Table 4): Body yellowish before fixation and transparent afterwards, eyes present in 32% of fixed specimens (Fig. 2). In juveniles, cuticle appears smooth and only occasionally with poorly outlined pseudoplates and single very faint pseudopores in the caudo-dorsal part of the body (Fig. 3A). In adults, however, the dorsal cuticle is covered with small, faint and scattered pseudopores (Fig. 3B), visible better on the caudal cuticle, especially on pseudoplates. Pseudopores are so small that they do not cause the cuticle above them to collapse, thus they are not identifiable under SEM (Fig. 3C-D). The adult dorsum is also covered with pseudoplates (delineated geometric areas of cuticle), Figs 2, 3A, C. The most anterior identifiable pseudoplate is placed immediately before the segment with legs II, the most posterior pseudoplate is on the terminal segment, just above legs IV (Fig. 2). The segment between legs I and II and the segment between legs II and III are each covered with a single rectangular paired pseudoplate. Segments with legs II and III are both covered with a triple rectangular paired pseudoplate. The first segment posterior to legs III is covered with a single rectangular paired pseudoplate and two smaller dorso-lateral pseudoplates. The second segment posterior to legs III is covered by a complex of ten pseudoplates, eight clustered on the dorsum and two placed dorso-laterally. Finally, on the segment immediately above legs IV there is a paired pseudoplate.

Six peribuccal papillae (ventral papilla smallest) and six peribuccal lamellae (of unequal size, 4+2) around the mouth opening present (Figs 4A-B). External surface of peribuccal lamellae and a band of cuticle between lamellae and papillae covered with minute granulation (*ca*. 0.1 μm in diameter), visible only in SEM (Fig. 4B). Two cephalic papillae positioned laterally (Fig. 4A). Buccal apparatus of the *Milnesium* type (Figs 4C-D). Buccal tube narrow and long (standard width on average 27% of the length), and funnel-shaped, wider anteriorly (posterior diameter on average 85% of the anterior diameter). Pharyngeal

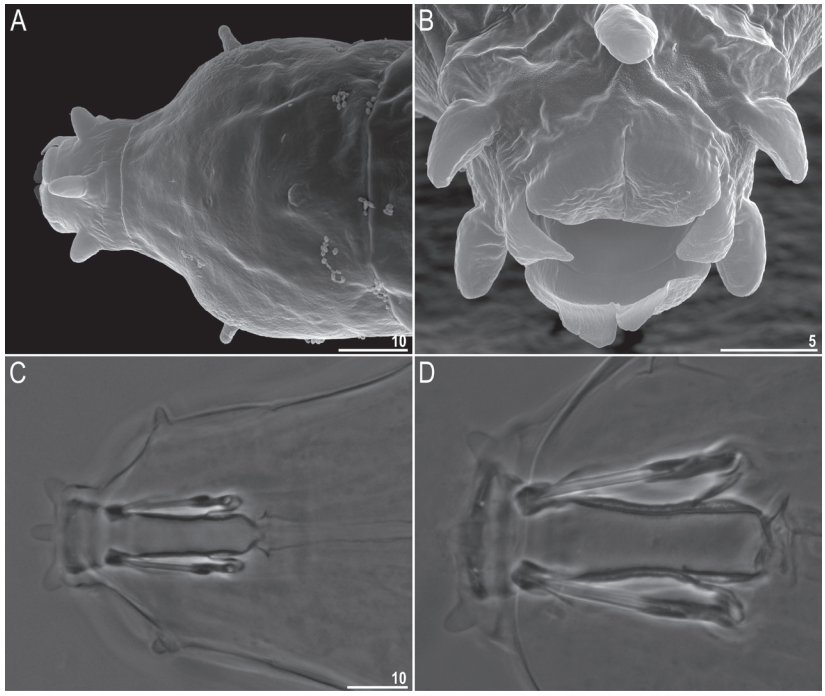


Fig. 4. Milnesium variefidum sp. nov.: head and buccal apparatus: A – a dorsal view of the head with cephalic and peribuccal papillae (paratype, SEM); B – mouth opening surrounded by six peribuccal papillae and six unequal (four large and two small; 4+2) peribuccal lamellae (paratype, SEM); C-D – buccal apparatus (C – juvenile, D – adult, both paratypes, PCM). Scale on Fig. D same as on Fig. C.

bulb elongated, pear-shaped and without placoids or septulum.

Claws of the *Milnesium* type, slender (Fig. 5). Primary branches on all legs with small, but distinct accessory points detaching from the branch at its greatest curvature. Secondary branches with rounded basal thickenings. In juveniles (*i.e.* first two instars), all secondary branches on all legs with two points (claw configuration: [2-2]-[2-2], Fig. 5A,C); in adults internal secondary branches I-III with three points and the remaining branches with two points (claw configuration: [2-3]-[2-2], Figs 5B,D). In *ca.* 50% individuals (both juveniles and adults) single cuticular bars under claws I-III are absent (Fig. 5A). In the remaining half of the type series, bars are very faint (Fig. 5B).

No males were found among the total of 67 specimens.

Eggs: Oval, smooth and deposited in exuvia as in all other known *Milnesium* species.

DNA sequences: All six sequenced individuals exhibited a single haplotype in 18S rRNA (GenBank accession number: KT951664), 28S rRNA (KT951665) and COI (KT951663), but two haplotypes were present in ITS-2 (*haplotype 1* (KT951667) with a C and a T in positions 229 and 304, respectively; *haplotype 2* (KT951666) with an A and a C in these positions). Importantly, both ITS-2 haplotypes were present in each of the claw configuration morphotypes.

The type DNA sequences for *M. variefidum* sp. nov. are provided in supplementary Table S1.

Type locality. 57°20’31’’N, 02°37’08’’W; 130 m asl: United Kingdom, Scotland, near Insch; lichen on a Mountain Ash (*Sorbus aucuparia*) tree. Coll. Brian Blagden.

Etymology. The name of the new species refers to the ontogenetic claw configuration variability, fully documented for the first time in the genus *Milnesium* in the present study.

Type depositories. The type series is preserved at the Department of Entomology, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland. The type series comprises: the holotype (slide GB.001.47) and 47 paratypes (9 juveniles and 38 adults on 33 slides: GB.001.01, 04, 19-29, 34, 39-46, 48-49, 54, 59-60, 67, 69-74).

Claw configuration in Milnesium variefidum *sp. nov.*
Both the molecular and the experimental (*in vitro* culturing) analyses independently confirmed that *Milnesium* individuals isolated from our Scottish lichen sample

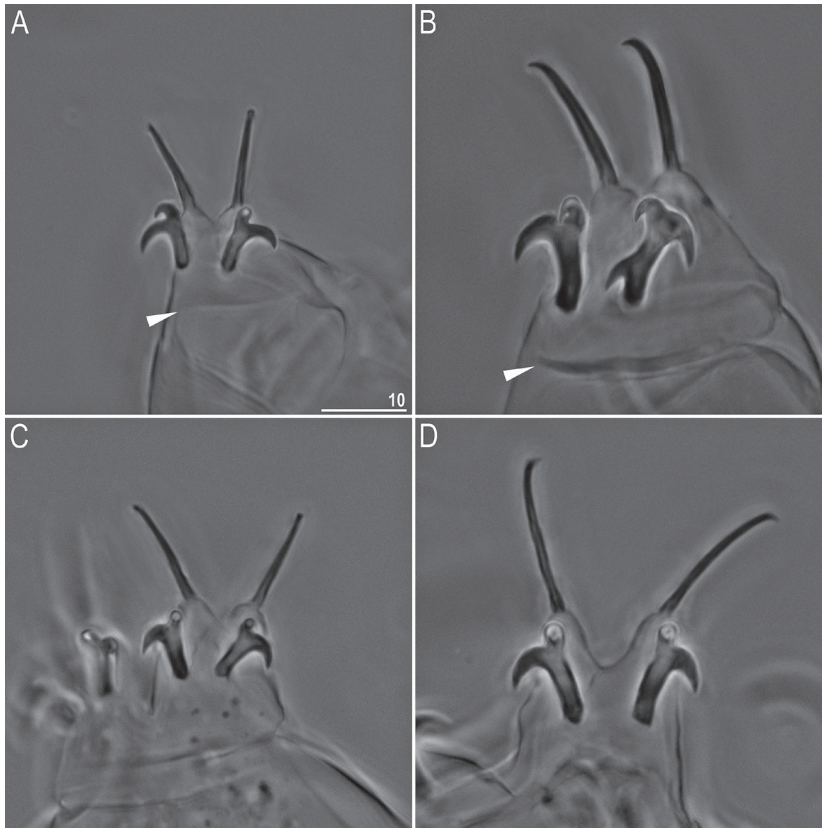


Fig. 5. Milnesium variefidum sp. nov.: claws: A-B – claws III (A – juvenile, arrowhead shows no bar; B – adult, arrowhead indicates a faint bar); C-D – claws IV (C – juvenile, D – adult); all paratypes, PCM. Scale on Figs B-C same as on Fig. A.

belong to a single species. Animals with the [2-**2**]-[2-2] and the [2-**3**]-[2-2] claw configuration represented the same haplotypes across all four sequenced DNA fragments. Moreover, juveniles that hatched from eggs laid into [2-**3**]-[2-2] exuviae exhibited the [2-**2**]-[2-2] claw configuration. By following individual development of juveniles that hatched in the lab, we established that the first two instars consistently had the [2-**2**]-[2-2] configuration and that from the third instar onwards the claw configuration was always [2-**3**]-[2-2]. The third instar was also the first life stage in which we observed oocytes, thus we have concluded that claw configuration change, in *M. variefidum* sp. nov., correlates with reproductive maturity. Juveniles and adults differed significantly both in terms of body and buccal tube lengths, moreover, length ranges did not overlap (see Table 2 for statistics).

Phenotypic differential diagnosis

Milnesium variefidum sp. nov., with a claw configuration [2-2/3]-[2-2], is similar to four species, two with a [2-3]-[2-2] and two with a [2-2]-[2-2] claw configuration. However, the new species differs from all these four species by the appearance of the cuticular bars under claws I-III (on average, the bars are absent in half of the specimens and, if they are present, they are always faint; in contrast, in all four *Milnesium* species with which we compare the new species, the bars are always present and well defined). Moreover, *M. variefidum* sp. nov. differs in further traits specifically from:

M. berladnicorum Ciobanu *et al*., 2014 (known only from the type locality in Romania) by the claw development mode (developmental claw dimorphism in the new species *vs*. a single claw configuration in *M. berladnicorum*), the arrangement of cuticular pseudoplates (a single paired pseudoplate between legs I and II, a triple paired pseudoplate at the level of legs II, a triple paired pseudoplate at the level of legs III, and four lateral pseudoplates between legs III and IV in the new species *vs*. no pseudoplate between legs I and II, a single paired pseudoplate at the level of legs II, a double paired pseudoplate at the level of legs III, and two lateral pseudoplates between legs III and IV in *M. berladnicorum*, compare Figs 2D and 6A), and by fine sculpturing of pseudoplates (irregular wrinkles in the new species *vs*.

a regular reticulum-like design in *M. berladnicorum*; trait detectable only under SEM, see Figs 3D and 6C).

All type specimens of *M. berladnicorum*, including animals with buccal tube 23.0 µm long, have a [2-3]-[2-2] claw configuration, which suggests that the species does not undergo claw configuration change in course of development, however only development tracking can verify this assumption. Even though the pseudoplates were not noted in the original description of *M. berladnicorum*, we were able to observe them clearly on adult type specimens. Given that adults of both species have very similar pseudopores (Figs 3B and 6D), special care must be taken when identifying either of the two species as the only difference is the appearance of the cuticular bars under claws I-III (compare Figs 5A-B and 6E-F) and the arrangement of pseudoplates (compare Figs 2D and 6A). According to the original description, *M. berladnicorum* has six equally sized peribuccal lamellae, but our SEM analysis has shown that *M. berladnicorum* exhibits the same lamellae configuration as the new species, *i.e.* 4+2 (see Figs 4B and 6B). Despite the striking morphological and morphometric similarities between the two species, they are genetically distinct (see below for details).

M. almatyense Tumanov, 2006 (known only from the type locality in Kazakhstan) by the cuticular sculpture (scarce and faint pseudopores in the new species *vs.* smooth cuticle in *M. almatyense*). Given that only large (most likely mature) specimens constitute the type series of *M. almatyense*, it remains to be established whether juveniles of this species exhibit a different claw configuration than adults (*i.e.* [2-3]-[2-2]).

M. katarzynae Kaczmarek *et al.*, 2004 (known from the type locality in China and also from Costa Rica and Colombia; Kaczmarek *et al.*, 2014; Caicedo *et al.*, 2014) by: the cuticular sculpture (scarce and faint pseudopores in the new species *vs.* dense and distinct pseudopores forming a reticulum in *M. katarzynae*) and higher *pt* values of the external secondary branches of claws IV (*33.2-46.2* in the new species *vs. 26.7-28.3* in *M. katarzynae*). Given that only small (possibly juvenile) specimens constitute the type series of *M. katarzynae*, it remains to be established whether adults of this species exhibit a different claw configuration than juveniles (*i.e.* [2-2]-[2-2]).

M. kogui Londoño *et al.*, 2015 (known only from the type locality in Colombia) by: the cuticular sculpture (scarce and faint pseudopores in the new species *vs.* smooth cuticle in *M. kogui*) and higher *pt* values of the primary branches of all claws I-III (*35.3-53.3* in the new species *vs. 29.3-34.2* in *M. kogui*). Given that only small (possibly juvenile) specimens constitute the type series of *M. kogui*, it remains to be established whether adults in this species exhibit a different claw configuration than juveniles (*i.e.* [2-2]-[2-2]).

Genotypic differential diagnosis

All sequences obtained for *M. variefidum* sp. nov. are unique and distinct from all *Milnesium* sequences currently deposited in GenBank as well as from the sequences of *M. berladnicorum* acquired in the present study. The p-distances between the new species and *Milnesium* spp. from GenBank vary between 19.0% and 25.0% for ITS-2, from 13.8% to 28.3% for COI, from 3.5% to 8.7% for 28S rRNA, and from 0.6% to 2.7% for 18S rRNA. The p-distances between the new species and *M. berladnicorum* are as follows: 12.4% (COI), 9.3-9.7% (ITS-2, *haplotype 1* and *2*, respectively), 2.0% (28S rRNA), and 0.6% (18S rRNA). Thus, except for 18S rRNA, *M. berladnicorum* is genetically most similar to the new species among available *Milnesium* sequences. As expected, distances for more conservative regions (*i.e.* 28S and 18S rRNA) are much lower than those for more variable sites (*i.e.* ITS-2 and COI). Importantly, however, p-distances for COI are well above the 3% threshold proposed for species delineation (Cesari *et al.*, 2009, but see also Michalczyk *et al.*, 2012a).

Discussion

Claw configuration variability in the genus Milnesium

Thanks to the combined use of experimental and molecular methodology we were able to demonstrate the first definitive evidence for claw configuration change during individual development. Until now, it has been assumed that each *Milnesium* species exhibits the same claw configuration in all life stages (Michalczyk *et al.*, 2012a), except for claws I in mature males (reviewed in Rebecchi and Nelson, 1998) and occasional developmental aberrations (reviewed in Michalczyk *et al.*, 2012a). However, there is an example in the *Milnesium* literature providing indirect evidence that at least one species, *M. barbadosense* Meyer and Hinton, 2012, may also undergo a claw configuration change during its development. However, the authors of that descriptions only mentioned that they found more than one claw configuration in their populations, without further analyses that would allow an unambiguous

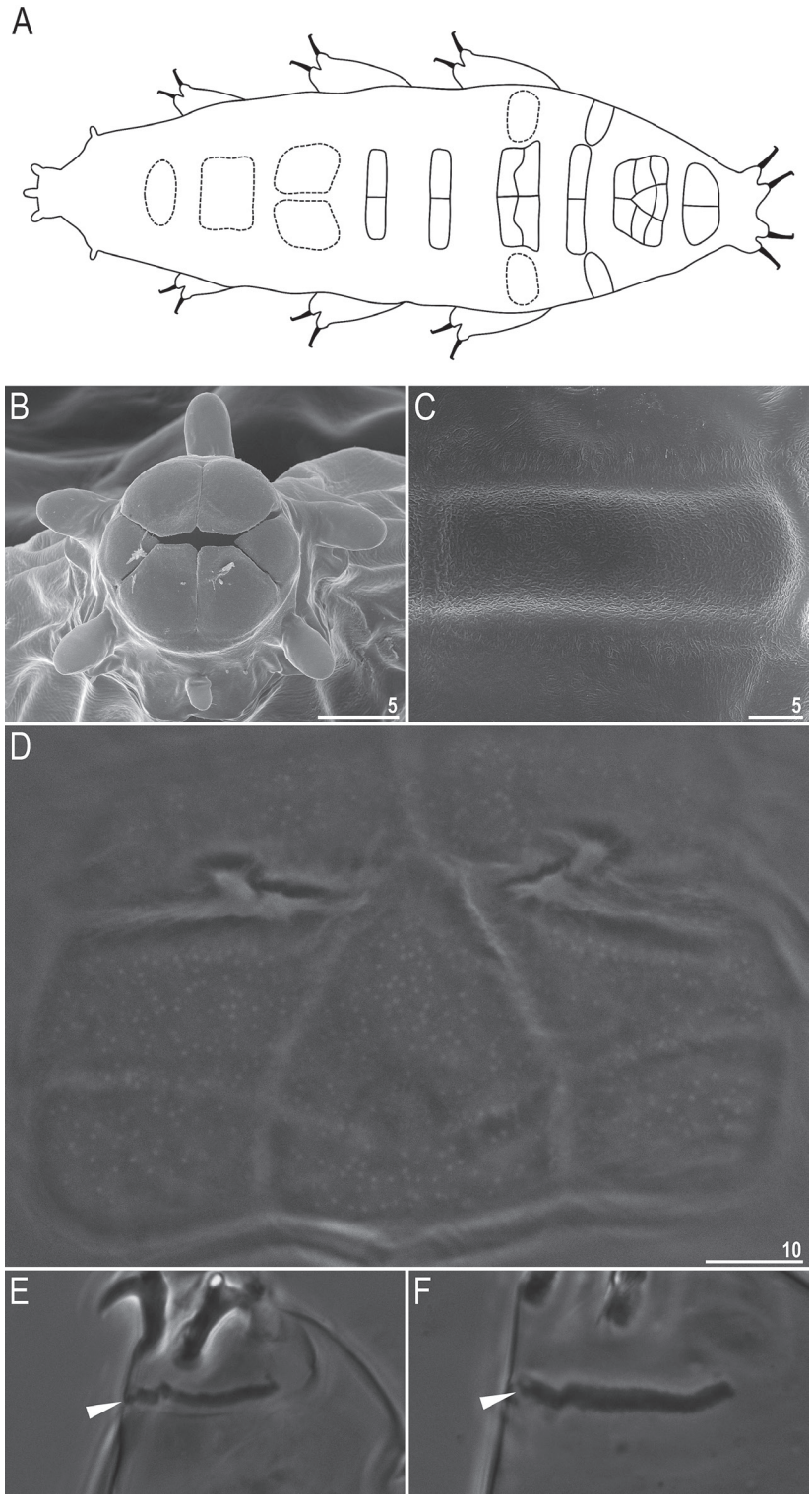


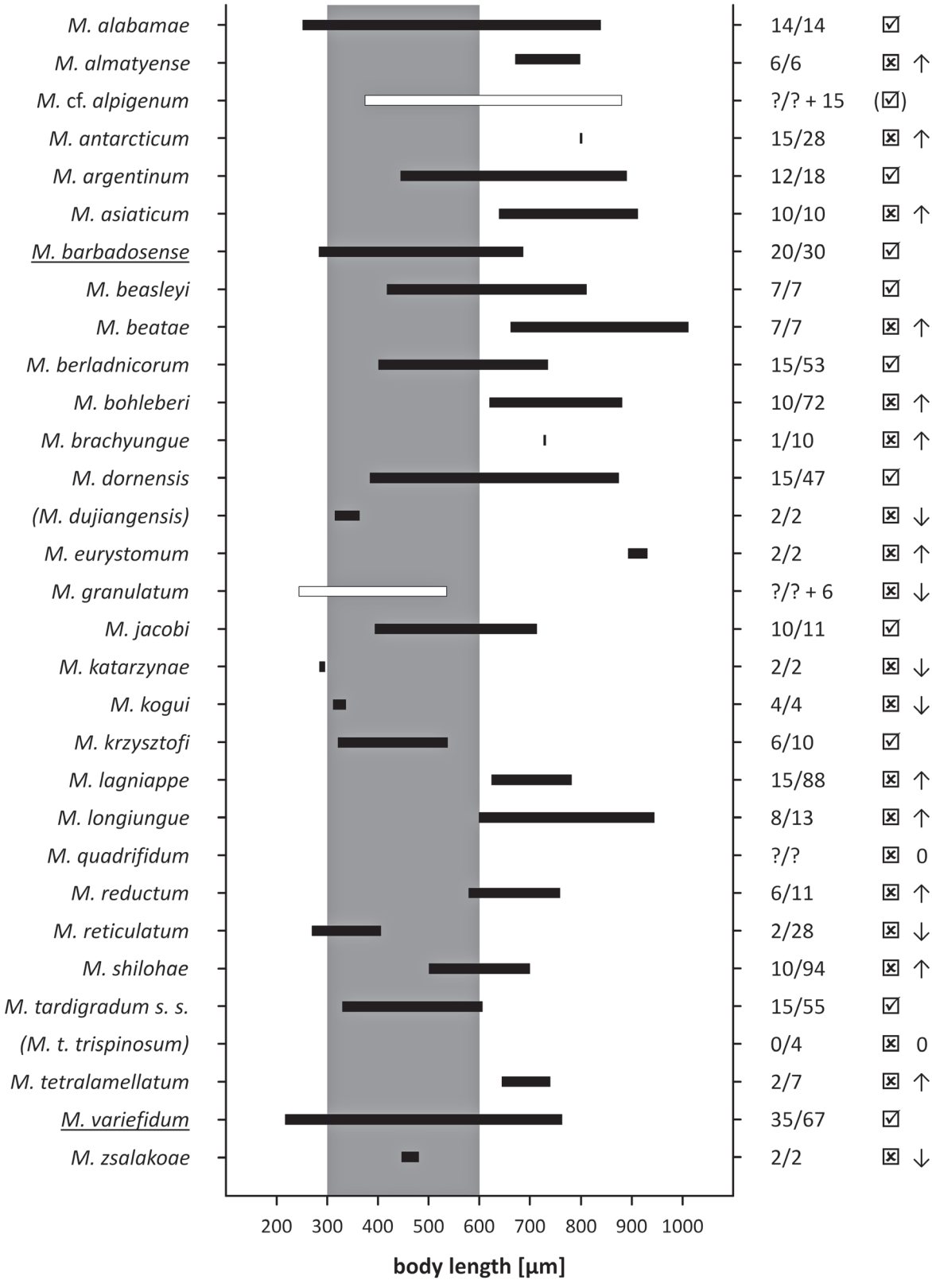
Fig. 6. Milnesium berladnicorum Ciobanu *et al*., 2014 (specimens from the type locality and paratypes): A – semi-schematic drawing of the dorsal pseudoplates based on PCM and SEM observations (pseudoplates with dashed boundaries are visible only under SEM and those with solid boundaries are identifiable both under PCM and SEM; compare with Fig. 2D); B – peribuccal lamellae (SEM); C – fine reticulum-like sculpturing on a dorsal pseudoplate (SEM); D – caudo-dorsal pseudoplates with pseudopores (PCM, compare with Fig. 7); E-F – evident bars (arrowheads) under claws III: E – juvenile (compare with Fig. 5A); F – adult (compare with Fig. 5B). Scale on Figs E-F same as on Fig. D.

identification of the two forms as representing a single species and pinpointing the life stage at which the change occurs, if indeed both forms belonged to the same species. Thus, currently there is only one species with a confirmed claw configuration developmental variability (*M. variefidum* sp. nov.) and one species (*M. barbadosense*) in which there is indirect evidence for such variability. However, it is not currently possible to assess the proportion of species within the genus that truly possess either a single or multiple claw configuration. The key problem is that the majority of species type series descriptions seem to be based on unrepresentative samples (see Fig. 7). In fact, only for 11 species (36%) do the type and neotype series and/or non-typical records represent body size ranges that are likely to both comprise juvenile and adult life stages (Fig. 7). In the remaining 19 species (61%), described body size ranges seem to encompass individuals that are either too small (6 spp./19%) or too large (11 spp./36%) to represent both juveniles and adults, or are not known at all (2 spp./6%), see Fig. 7. Also, it would be unjustified to assume that all species within the genus *Milnesium* exhibit the same minimal and maximal body size. Thus, in order to estimate the proportion of species in which representative body size ranges have been described, we assumed a body length window in which the transition from juveniles to adults is likely to occur (Fig. 7). This window, corresponding to *ca.* 300 μm in body length, is an educated guess based on our observations of various species. We have assumed that if the described body length range overlaps with at least 50% of our putative transition zone, then it is likely that juvenile and adult instars were measured. These assumptions need to be verified by a more systematic approach, nevertheless our basic analysis allowed us to identify the species in which the measured specimens are definitely not ontogenetically representative (Fig. 7). Importantly, among the species with representative body size ranges (11 spp.), the majority exhibit only a single claw configuration regardless of the life stage (10 spp./91%) and only for one (9%) is there indirect evidence suggesting claw configuration may change during the animal's life. Extrapolating this proportionally to the remaining 18 valid species, we should expect to find claw configuration developmental variability in an additional two species. The likely candidates are species in which only very small or very large specimens were measured, *i.e. M. katarzynae*, *M. kogui*, *M. reticulatum* and *M. almatyense*, *M. antarcticum*, *M. asiaticum*, *M. bohleberi*, *M. lagniappe*, *M. longiungue*, *M. reductum*, *M. shilohae*, respectively. That some of these species may in fact undergo claw configuration change is suggested by the fact that two of the species with possibly underestimated body size (*M. katarzynae* and *M. kogui*) exhibit claw configurations with few points ([2-2]-[2-2]), which gives the potential for an increase in the number of points in larger, mature animals. Conversely, four species described with very large body size (*M. antarcticum*, *M. asiaticum M. bohleberi*, and *M. longiungue*) exhibit a [3-3]-[3-3] claw configuration, which may suggest that juvenile instars could be equipped with fewer points. Also, the possibility that young animals may exhibit more points than adults should not be discarded.

Therefore, we would like to stress the importance of describing species using a representative sample size as otherwise erroneous conclusions about the claw configuration may be drawn, which, in turn, may lead to taxonomic inflation and later to synonymisations when more comprehensive data are available. Tardigrade researchers are usually limited by the sample they have and it is very often difficult to obtain additional material (Stec *et al*., in press). However, it is not unusual to find at least one exuvia with eggs among individuals extracted from a sample. If an exuvia with eggs is placed in distilled water at room temperature, juveniles should hatch within a week or two. Then, mounting both juveniles and the maternal exuvia on a permanent slide is a very simple yet powerful method of testing for claw configuration developmental variability (Fig. 1). Alternatively, when no exuviae are found, but more than one claw configuration is present among collected individuals, DNA sequences of COI or ITS-2 extracted from both types of specimens can be compared (Fig. 1). Although these two methods are sufficient to test for developmental variability, they are not able to determine the instar at which the claw configuration changes. In order to achieve this, the development of single animals needs to be followed (Fig. 1). Development tracking requires more time and effort than the other two methods, but it is the most comprehensive approach.

Diagnostic traits in Milnesium

In general, eutardigrades exhibit few taxonomically useful morphological traits. However, the genus *Milnesium* is one of the genera that are exceptionally poor in such traits as two sources of potentially important morphological variation, placoids in the pharynx and egg ornamentation, are absent. Below we provide a list


M. alabamae
M. almatyense
M. cf. alpigenum
M. antarcticum
M. argentinum
M. asiaticum
M. barbadosense
M. beasleyi
M. beatae
M. berladnicorum
M. bohleberi
M. brachyungue
M. dornensis
(M. dujiangensis)
M. eurystomum
M. granulatum
M. jacobi
M. katarzynae
M. kogui
M. krzysztofi
M. lagniappe
M. longiungue
M. quadrifidum
M. reductum
M. reticulatum
M. shilohae
M. tardigradum s. s.
(M. t. trispinosum)
M. tetralamellatum
M. variefidum
M. zsalakoae
14/14 ☑
6/6 ☒ ↑
?/? + 15 (☑)
15/28 ☒ ↑
12/18 ☑
10/10 ☒ ↑
20/30 ☑
7/7 ☑
7/7 ☒ ↑
15/53 ☑
10/72 ☒ ↑
1/10 ☒ ↑
15/47 ☑
2/2 ☒ ↓
2/2 ☒ ↑
?/? + 6 ☒ ↓
10/11 ☑
2/2 ☒ ↓
4/4 ☒ ↓
6/10 ☑
15/88 ☒ ↑
8/13 ☒ ↑
?/? ☒ 0
6/11 ☒ ↑
2/28 ☒ ↓
10/94 ☒ ↑
15/55 ☑
0/4 ☒ 0
2/7 ☒ ↑
35/67 ☑
2/2 ☒ ↓
200 300 400 500 600 700 800 900 1000
body length [µm]

of morphological traits used by authors to describe *Milnesium* species and we annotate them with comments based on the analysis of the literature as well as on our own observations and experience. The states of all traits for all known species of the genus are listed in Table 5, which is an updated and corrected version of Table 1 in Michalczyk *et al*. (2012a).

1. *Cuticle sculpturing*. The dorso-lateral cuticle can be smooth (19 species, *i.e*. 61% of described *Milnesium* taxa), pseudoporous (7 spp./23%) or reticulated (5 spp./16%). The pseudopores and reticulum are both the effect of empty areas within the cuticle. When these intra-cuticular cavities are small, they do not cause the cuticle above them to collapse and they appear under PCM as tiny light spots with blurry edges (*e.g*. in *M. variefidum* sp. nov.). If the cavities are larger, the cuticle above them collapses and forms minute cuticular depressions that appear under PCM as light spots with blurry edges (*e.g*. in *M. beasleyi* Kaczmarek *et al*., 2012). However, when cavities are large, the depressions in the cuticle are deep, large and densely arranged, and their rims form a continuous reticulum (*e.g*. in *M. krzysztofi* Kaczmarek and Michalczyk, 2007). Therefore, the distinction between the two types of cuticular sculpturing may not be obvious in some cases (*e.g*. in *M. katarzynae* Kaczmarek *et al*., 2004). It is also important to note that pseudopores can be difficult to identify, especially with poor quality microscopes without an optical contrast. Moreover, this trait has been described only recently (for the first time in *M. beasleyi*) and earlier authors could have overlooked it in some species. Thus, it is possible that in some of the older species, in which the cuticle was described as smooth, it is in fact pseudoporous. Finally, cuticular sculpturing may be subject to developmental variation as shown, for example, in *M. variefidum* sp. nov. where pseudopores are almost undetectable in juveniles and visible in adults (see Figs 3A-B). In all known species the ventral cuticle is smooth.
2. *Cuticular structures*: The dorsal cuticle can be covered with gibbosities (1 sp./3%), have spines (1 sp./3%) or possess pseudoplates, whereas legs I-III can be equipped with transverse cuticular bars. The taxonomic value of pseudoplates (see Figs 2D and 6A) and bars under claws I-III is not known (see Figs 5A-B and 6E-F). However, the description of *M. variefidum* sp. nov. shows that both pseudoplates and bars under claws I-III can potentially be taxonomically useful. Marley (2012) hypothesised that the three cuticular spines in *M. tardigradum trispinosum* are in fact cuticular folds (such as those observed, for example, in *M. krzysztofi*), but this hypothesis needs to be verified with type or neotype material.
3. *Peribuccal lamellae number and relative size*. There can be either six ('6') or four ('4') peribuccal lamellae of equal size, or six lamellae with four being distinctly larger than the remaining two ('4+2'). This can be viewed as a sequence of states, starting with the putative ancestral six symmetrical lamellae ('6'), then with the two lateral lamellae becoming smaller and the two ventral and two dorsal lamellae becoming larger ('4+2'), and finally with the lateral lamellae reduced and the ventral and dorsal lamellae covering the entire mouth opening ('4'). Peribuccal lamellae are very difficult to observe under LM. In fact, definite observations can be made only under SEM and occasionally under LM, in exceptionally well relaxed and properly oriented specimens (*Milnesium* individuals very often retract their mouth parts when placed in mounting media; see Morek *et al*. (in press) for a mounting protocol that minimises retraction issues). Whereas the presence of only four lamellae is usually unambiguous

◀ *Fig. 7.* A graphic comparison of body length ranges of type populations of the known *Milnesium* Doyère, 1840 species. Black horizontal bars represent length ranges provided in original descriptions whereas white bars represent length ranges for non-type specimens. There are no data, either type or non-type, for two species, *M. quadrifidum* and *M. tardigradum trispinosum*. The shaded grey vertical rectangle indicates the size range at which second moulting, *i.e*. the transition from the juvenile to the adult stage, is most likely to occur. Underlined are species names in which more than one claw configuration was found and dubious species are in brackets. Values next to the right vertical axis represent the number of measured specimens and after the slash, the number of individuals found (*i.e*. that were potentially available for measurements). Question marks indicate no data on the number of measured and found individuals. In those species for which non-typical material was analysed, the number of measured individuals is given after the plus symbol. Boxes next to the sample size values indicate whether the body length range for a given species was in our opinion wide enough to encompass both juvenile and adult animals (ticked = yes, crossed = not). In case of descriptions with an insufficient number of measured specimens (*i.e*. crossed boxes), the arrow indicates whether the upper or the lower part of the expected (hypothetical) body length range was measured (an upward arrow = juveniles were likely to be missed, a downward arrow = adults could have been missed).

(although see *M. lagniappe* Meyer *et al*., 2013), a confident distinction between the 6 and the 4+2 states is rarely possible without SEM. In fact, LM observations can be misleading, which can be exemplarily illustrated with *M. berladnicorum*. The original description, based solely on LM imaging, states that this species has six peribuccal lamellae of equal size whereas our SEM analysis revealed that *M. berladnicorum* is equipped with four large and two small lamellae. Moreover, the majority of descriptions of species with more than four lamellae simply state that there are six lamellae present, without specifying whether they are all equal in size or not. Thus, it is not surprising that in the majority of species the exact state of this trait is unknown. There are only three species (10%) in which six equal lamellae were identified (all under LM), two (6%) with the confirmed 4+2 configuration (both under SEM), and also two (6%) with four lamellae. In the great majority of species (17 spp./55%) the number of lamellae was described as 'six', without providing information on their relative size, thus the configuration in these species cannot be currently determined and has to be denoted as '6 or 4+2' until redescribed. In one species (3%) the number of peribuccal lamellae could be 4+2 or 4 and in the remaining six species (19%) there are no data on this trait (see Table 5). Therefore, with the currently very incomplete knowledge on the number and size of lamellae among *Milnesium* species (known only in 7 spp./23%) and the difficulties in determining the state, this character has a very limited taxonomic value and should be used with caution when erecting species similar to species in which the lamellae configuration is unknown. Hence, we strongly advise that new *Milnesium* species should always be analysed under SEM in addition to LM preparations in order to describe peribuccal lamellae unambiguously.

4. *Primary branches*. Except for one species (3%), all *Milnesium* taxa are equipped with primary branches (30 spp./97%). The exception, *M. dujiangensis* Yang, 2003, is extremely poorly described, thus the absence of primary branches in this species has to be confirmed either by the re-examination of the type material or by establishing a neotype series.
5. *Accessory points*. The great majority of *Milnesium* species have primary branches tipped with accessory points (24 spp./77%) and only 5 species (16%) have been described as being devoid of these structures (data for 2 spp./6% are unavailable). However, none of the species in which accessory points were not observed were analysed with SEM; thus, it is possible that some of them could possess minute points that are difficult to detect under LM.
6. *Secondary claw configuration*. Given that currently there are eight distinct claw configurations known in the genus, the number and position of claw points on the secondary branches is the most variable and taxonomically important morphological trait. Unambiguously, the most common is the [3-3]-[3-3] type (15 spp./48%), then [2-3]-[3-2] (6 spp./19%), [2-2]-[2-2] (4 spp./13%), [2-3]-[2-2] (3 spp./10%), [2-3]-[2-3] (2 spp./6%), [2-3]-[3-3], [2-2]-[1-2] and [4-4]-[4-4] (each with 1 spp./3%). Please note that percentages do not sum up to a 100%, because in three species two claw configurations have been described and in one species the configuration is not known. Except for one species, *M. shilohae* from Hawaii, all *Milnesium* taxa exhibit symmetrical spurs on external and internal and on anterior and posterior branches. Since the [3-3]-[3-3] configuration is most common in *Milnesium* and it is also the only configuration found in the remaining genera of the family Milnesiidae, it seems a very likely candidate for the ancestral state, with all other configurations being derivatives. Michalczyk *et al*. (2012a, b) proposed the denotation system in which the number of points on claws I-III and then on claws IV are given as a sting of bracketed numbers. This, however, does not account for modified secondary branches of claws I in mature males. Given that the number of points on the secondary branches on the remaining claws remain unchanged and are the same in males and females, we propose to slightly modify the system so it encompasses both sexes. Specifically, the claw configuration should be read from claws III and IV only. As shown above, in some species the claw configuration is subject to developmental variability. Thus, in order to prevent taxonomic inflation and future synonymisations, we strongly advice describing *Milnesium* species only when both juvenile and adult instars are available.

In addition to the six traits discussed above, when describing new *Milnesium* species, researchers have also mentioned the presence or absence of eyes and described the size of lunulae under the secondary branches. However, the taxonomic value of these traits has never been evaluated. Therefore, until both intra- and inter-specific variability in these traits is systematically assessed, we recommend no new species should be described based solely on them. Taking into consideration

the number of described states of the six available morphological characters (*i.e.* respectively 3, 3, 3, 2, 2, 8), theoretically there are as many as 864 distinct combinations. However, the states are not independent of each other. Also, the frequency of states is non-random and highly skewed towards some of them (*e.g.* smooth cuticle, the presence of accessory points, the [3-3]-[3-3] claw configuration). Thus, in reality, combinations of morphological traits appear repeatedly and some species have been erected solely on the basis of morphometric characters of the buccal tube and claws. Given that the number of such species is likely to increase, we strongly suggest new taxa are only described when the sample size and composition allows *(i)* a description of both juveniles and adults, *(ii)* a proper morphometry (*i.e.* a number of individuals representing as many life stages as possible), *(iii)* SEM observations (especially of the peribuccal lamellae), and ideally also *(iv)* an acquisition of DNA sequences.

Composition of the genus Milnesium *and quality of species descriptions*

Compared to the last available *Milnesium* species list (Michalczyk *et al*., 2012a), the list presented in this paper adds 13 species – two suppressed by Marcus (1928) that were also omitted in Michalczyk *et al*. (2012a) but are reinstituted in this study and 11 species described since the publication of the former list, including *M. variefidum* sp. nov. – which amounts to 30 species and one subspecies (Table 5). However, one species and one subspecies (*M. dujiangensis* and *M. tardigradum trispinosum*, respectively) are designated as *nomina dubia* in this paper (see below for the detailed justification). Therefore, at the end of 2015, the total number of valid species in the genus *Milnesium* is 29.

The quality of species descriptions has varied significantly with time, typically becoming much more detailed recently, although not always do the contemporary descriptions meet modern taxonomic standards. In order to better identify the needs for redescriptions, we divided all known *Milnesium* species into five classes of decreasing description quality, based on the fulfilment of the following four principal criteria: *(a)* good verbal description accompanied by appropriate illustrations, *(b)* coverage of body size that potentially encompasses both juveniles and adults, *(c)* unambiguous determination of the peribuccal lamellae configuration, *(d)* at least one DNA sequence, and *(e)* development tracking:

1. *Species with full descriptions*. All five data criteria are met for species in this group (*a+b+c+d+e*). Currently, there is only one such species (3%): *M. variefidum* sp. nov.
2. *Species with very good descriptions*. Descriptions in this group meet three of the five criteria, either *(a+b+c)*, or (*a+b+d*) but always lack development tracking. There are only three species (10%) falling under this category: *M. barbadosense*, *M. dornensis* and *M. tardigradum*. The descriptions of the first two species lack DNA barcodes and development tracking. Also, it must be noted that the configuration of the peribuccal lamellae was determined only on the basis of LM observations. Thus, a definite confirmation by SEM analysis of the lamellae state as well as DNA barcodes and development tracking, ideally made using type populations, are needed to upgrade these to first class descriptions. The redescription of *M. tardigradum* by Michalczyk *et al*. (2012a) provided DNA barcodes but it lacks SEM photomicrographs and development tracking. Although in all three species the reported body size range seems sufficient to encompass both juveniles and adults (criterion *b*), only development tracking can ultimately verify the hypothesis that these taxa do not exhibit developmental variability in claw configuration.
3. *Species with good descriptions*. Descriptions in this category contain only two of the four necessary standards, *i.e.* the first and either the second (*a+b*) or the third (*a+c*) and they always lack the fourth and the fifth criterion. In total, there are seven species (23%) in this category, three (10%) with known peribuccal lamellae configurations (determined under LM only) but without sufficient body size ranges (*M. bohleberi*, *M. reticulatum*, *M. tetralamellatum*) and five (17%) with sufficient body size ranges but lacking knowledge of the peribuccal lamellae configuration (*M. alabamae*, *M. argentinum*, *M. beasleyi*, *M. berladnicorum*, *M. jacobi*). In this study, we have unambiguously established the lamellae state (*i.e.* 4+2, Fig. 6B) and provided DNA barcodes for *M. berladnicorum*, thus now only development tracking is required to upgrade this species to the first class description. Obtaining the missing data for the remaining species is vital.
4. *Species with inadequate descriptions*. Descriptions in this group meet only the first criterion (*a*), which means that they need considerable redescriptions. Worryingly, this category of description is the largest (15 spp./48%): *M. almatyense*, *M. antarcticum*, *M. asiaticum*, *M. beatae*, *M. brachyungue*, *M. eurystomum*, *M. granulatum*, *M. katarzynae*, *M. kogui*,

M. krzysztofi, *M. lagniappe*, *M. longiungue*, *M. reductum*, *M. shilohae*, and *M. zsalakoae*. These species require urgent complementary redescriptions, in some cases extending to the verification of the cuticular appearance.

5. *Species requiring complete redescriptions*. Descriptions in this last class are out of date and/or of extremely poor quality. Four species (13%) fall under this category: *M. alpigenum*, *M. dujiangensis*, *M. quadrifidum* and *M. tardigradum trispinosum*. Although in this paper we provide the first morphometric data for a population of what we have identified as *M.* cf. *alpigenum* and by virtue of this could upgrade this species by one class, a proper redescription, ideally based on material from the locus typicus, is definitely needed. The remaining three species are thought to exhibit unique traits, *i.e.* cuticular spines, a lack of primary branches and a [4-4]-[4-4] claw configuration. The first two traits are questionable and in the case of *M. quadrifidum* it is possible that there is more than one species that exhibits the [4-4]-[4-4] claw configuration, thus, until these species are properly redescribed, no new species that exhibit similar traits should be described. For the time being we propose to designate *M. dujiangensis* and *M. tardigradum trispinosum* as *nomina dubia*, which was also suggested by Marley (2012). Given that *Milnesium* specimens with the [4-4]-[4-4] configuration have been observed by several researchers (*e.g.* Marcus, 1928 or our personal observations), *M. quadrifidum* should remain valid, but it requires a thorough redescription.

Overall, the great majority of valid *Milnesium* species (28 spp./97%) have descriptions that are incomplete to various degrees. The lack of vital data for these species has serious consequences for the taxonomy of the genus and further, for virtually any studies that rely on accurate species identification. On one hand, in the case of cautious researchers, poor descriptions of known species may hold back the descriptions of new species and therefore lead to an underestimation of the magnitude of biodiversity. On the other hand, incomplete or poor descriptions may result in taxonomic inflation when less careful taxonomists observe traits not described in the original descriptions, assume that they are not present in the existing species and use them to establish (false) new species. Therefore, we think that prioritising towards redescriptions rather than describing new taxa would greatly help in stabilising and systematising the taxonomy of *Milnesium*. Also, including SEM imagery and DNA sequences in new descriptions will greatly improve the taxonomy of the genus. Interestingly, at the moment there are over 4,500 DNA *Milnesium* sequences deposited in the GenBank. Unfortunately, however, with all but several specific names being most certainly assigned erroneously, which prevents their use for phylogenetic analyses. This is important, because in contrast to the order Parachela, phyletic relationships within the order Apochela, and therefore also within the family Milnesiidae, have not yet been addressed. In fact, being the sister group to Parachela, the Apochela has been used in eutardigrade phylogenetic analyses only as an outgroup for parachelan taxa (*e.g.* see Bertolani *et al.*, 2014). However, with the steeply growing number of *Milnesium* species, the need for phylogeny reconstruction of the Apochela is also increasing. Currently, the order Parachela comprises fifty seven genera, whereas there are only four in the order Apochela (Bertolani *et al.*, 2014). Therefore, it is plausible that *Milnesium* may hold multiple evolutionary lineages that could be defined as separate genera. Nevertheless, given the very low number of available morphological traits that are easily identifiable, the erection of new milnesiid genera is likely to be challenging.

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Appendix

Diagnostic key to the genus Milnesium

The most recent diagnostic key to the genus *Milnesium* (by Michalczyk *et al*., 2012b) used cuticle appearance as the main differentiating trait. Consequently, *Milnesium* species fell into two groups: with smooth cuticle (the *tardigradum* group) and with sculptured cuticle (the *granulatum* group). However, given the constantly increasing number of species in the genus, this simple division is no longer practical. Therefore, we propose a key that uses claw configuration as the main branching trait. This way species cluster in smaller groups, within which species identification is faster and more straightforward.

Important notes:
- Given that the claw configuration in *M. tardigradum trispinosum* remains unknown, this species had to be placed at the beginning of the key, above the division by claw configuration type.
- Since in two species, *M. barbadosense* and *M. variefidum* sp. nov., more than one claw configuration was described, each of these species appears in the key in two places and the appropriate life stage (*i.e.* juvenile or adult) is indicated.
- Because the description of *M. alpigenum* is very basic and it does not contain any morphometric data and the type material no longer exists, this species requires a redescription based on a new material, preferably from the locus typicus in the Alps. However, in order to provisionally include this species in the key, we measured a number of specimens kindly provided by Ralph Schill (Stuttgart University, Germany) that are very likely to be *M. alpigenum* (see Table 3 for morphometrics). Nevertheless, when a redescription of *M. alpigenum* is available, the morphometrics used for the sake of this key will have be verified against the neotype material and amended if necessary.
- As in mature *Milnesium* males secondary branches of claws I are modified into single-point hooks, when identifying males the configuration of external and internal claws should be determined using claws III instead of claws I-III.
- The wildcard (*) represents more than one possible claw state.

1. Three cuticular spines on the dorso-caudal cuticle present ..... ***M. tardigradum trispinosum*** Rahm, 1931 *nomen dubium*
–. No cuticular spines on the dorso-caudal cuticle **2**

2(1). Secondary branches of external claws I-III with two points, *i.e*. claw configuration [2-*]-[*-*] **3**
–. Secondary branches of external claws I-III with three or four points, *i.e*. claw configuration [3-*]-[*-*] or [4-*]-[*-*] **18**

3(2). Secondary branches of internal claws I-III with two points, claw configuration [2-2]-[*-*] **4**
–. Secondary branches of internal claws I-III with three points, claw configuration [2-3]-[*-*] **7**

4(3). Secondary branches of anterior claws IV with one point, claw configuration [2-2]-[1-2] ..... ***M. dujiangensis*** Yang, 2003 *nomen dubium*
–. Secondary branches of anterior claws IV with two points, claw configuration [2-2]-[2-2] **5**

5(4). Dorsal cuticle with distinct and dense pseudopores ..... ***M. katarzynae*** Kaczmarek *et al*., 2004
–. Dorsal cuticle smooth or with faint and scarce pseudopores **6**

6(5). The *pt* values of the primary branches of claws I-III lower than 38%, cuticle always smooth ..... ***M. kogui*** Londoño *et al*., 2015
–. The *pt* values of the primary branches of claws I-III higher than 38%, cuticle may possess small and faint pseudopores ..... ***M. variefidum*** sp. nov. (juvenile)

7(3). Secondary branches of anterior claws IV with two points, claw configuration [2-3]-[2-*] **8**
–. Secondary branches of anterior claws IV with three points, claw configuration [2-3]-[3-*] **12**

8(7). Secondary branches of posterior claws IV with two points, claw configuration [2-3]-[2-2] **9**
–. Secondary branches of posterior claws IV with three points, claw configuration [2-3]-[2-3] **11**

9(8). Dorsal cuticle smooth ..... ***M. almatyense*** Tumanov, 2006
–. Dorsal cuticle with pseudopores **10**

10(9). Cuticular bars under claws I-III always present and well developed, a double paired pseudoplate at the level of legs III ..... ***M. berladnicorum*** Ciobanu *et al*., 2014
–. Cuticular bars under claws I-III absent or when present always poorly developed, a triple paired pseudoplate at the level of legs III ..... ***M. variefidum*** sp. nov. (adult)

11(8). Four peribuccal lamellae around the mouth opening ..... ***M. tetralamellatum*** Pilato and Binda, 1991
–. Six peribuccal lamellae around the mouth opening .... ***M. barbadosense*** Meyer and Hinton, 2012 (juvenile)

12(7). Secondary branches of posterior claws IV with two points, claw configuration [2-3]-[3-2] **13**
–. Secondary branches of posterior claws IV with three points, claw configuration [2-3]-[3-3] ..... ***M. jacobi*** Meyer and Hinton, 2010

13(12). Cuticle smooth..... **14**
–. Cuticle sculptured **15**

14(13). Primary branches with accessory points ..... ***M. tardigradum*** Doyère, 1840
–. Primary branches without accessory points ..... ***M. reductum*** Tumanov, 2006

15(13). Four peribuccal lamellae around the mouth opening..... **16**
–. Six peribuccal lamellae around the mouth opening **17**

16(15). Gibbosities on the dorsal cuticle ..... ***M. reticulatum*** Pilato *et al*., 2002
–. Cuticle without gibbosities ..... ***M. lagniappe*** Meyer *et al*., 2013

17(15). Pseudopores on the dorsal cuticle over 0.5 μm in diameter, densely arranged and forming a faint reticular pattern ..... ***M. krzysztofi*** Kaczmarek and Michalczyk, 2007
–. Pseudopores on the dorsal cuticle below 0.5 μm in diameter, scattered and not forming a reticular pattern ..... ***M. beasleyi*** Kaczmarek *et al*., 2012

18(2). Secondary branches of all claws with three points, *i.e*. claw configuration [3-3]-[3-3] **19**
–. Secondary branches of all claws with four points, *i.e*. claw configuration [4-4]-[4-4] ..... ***M. quadrifidum*** Nederström, 1919

19(18). Cuticle sculptured **20**
–. Cuticle smooth **24**

20(19). Primary branches without accessory points ..... ***M. alabamae*** Wallendorf and Miller, 2009
–. Primary branches with accessory points **21**

21(20). Dorso-caudal cuticle covered with a reticular pattern ..... ***M. granulatum*** Ramazzotti, 1962
–. Dorso-caudal cuticle covered with small and sparsely distributed pseudopores **22**

22(21). Buccal tube standard width to length ratio below 35% ..... ***M. argentinum*** Roszkowska *et al*., 2015
–. Buccal tube standard width to length ratio above 35% **23**

23(22). Buccal tube funnel-shaped, posterior to anterior width ratio below 77% ..... ***M. beatae*** Roszkowska *et al*., 2015
–. Buccal tube cylindrical, posterior to anterior width ratio above 77% ..... ***M. dornensis*** Ciobanu *et al*., 2015

24(19). Primary branches without accessory points **25**
–. Primary branches with accessory points **26**

25(24). The *pt* of the posterior primary branch IV length lower than *93%* ..... ***M. longiungue*** Tumanov, 2006
–. The *pt* of the posterior primary branch IV length higher than *93%* ... ***M. zsalakoae*** Meyer and Hinton, 2010

26(24). Spurs on anterior secondary branches considerably longer than spurs on posterior secondary branches ..... ***M. shilohae*** Meyer, 2015
–. Spurs on anterior and posterior secondary branches of similar lengths **27**

27(26). The *pt* of the posterior primary branch IV lower than *35%* ..... ***M. brachyungue*** Binda and Pilato, 1990
–. The *pt* of the posterior primary branch IV higher than *35%* **28**

28(27). Buccal tube standard width to length ratio above 50% **29**
–. Buccal tube standard width to length ratio below 50% **30**

29(28). Posterior primary branch IV longer than 33 μm ..... ***M. eurystomum*** Maucci, 1991
–. Posterior primary branch IV shorter than 33 μm ..... ***M. bohleberi*** Bartels *et al*., 2014

30(28). Eyes absent in live animals ..... ***M. barbadosense*** Meyer and Hinton, 2013 (adult)
–. Eyes present in live animals **31**

31(30). The *pt* of the primary branch IV higher than *60%* ..... ***M. asiaticum*** Tumanov, 2006
–. The *pt* of the primary branch IV lower than *60%* **32**

32(31). The *pt* of the stylet support insertion point lower than *69%*, buccal tube shorter than 57 μm, buccal standard tube width lower than 22 μm, posterior primary branch IV shorter than 31 μm (values based on a population from Tübingen) ..... ***M.* cf. *alpigenum*** Ehrenberg, 1853
–. The *pt* of the stylet support insertion point higher than *69%*, buccal tube longer than 57 μm, buccal standard tube width higher than 22 μm, posterior primary branch IV longer than 31 μm ..... ***M. antarcticum*** Tumanov, 2006

Table 3. Measurements (in µm) and the *pt* values of selected morphological structures of 15 specimens of *Milnesium* cf. *alpigenum* Ehrenberg, 1853 from Tübingen, Germany, mounted in Hoyer's medium. Three individuals of each of the first five instars were measured; each instar was represented by animals cultured at 8, 16 and 24 °C. Note that sample size for specific traits varied since not all traits were measureable in all individuals.

CHARACTER	N	RANGE µm	RANGE *pt*	MEAN µm	MEAN *pt*	SD µm	SD *pt*
Body length	15	367 – 877	*1382 – 1820*	645	*1573*	175	*118*
Peribuccal papillae length	11	4.4 – 10.6	*15.0 – 22.6*	6.7	*17.5*	2.0	*2.1*
Lateral papillae length	12	3.4 – 9.5	*12.4 – 21.0*	6.5	*16.4*	2.3	*2.6*
Buccal tube							
Length	15	25.5 – 49.5	–	40.5	–	8.9	–
Stylet support insertion point	15	17.3 – 32.2	*61.8 – 68.8*	26.5	*65.7*	5.5	*2.2*
Anterior width	14	9.1 – 20.1	*30.4 – 41.2*	14.5	*36.1*	3.9	*3.3*
Standard width	15	7.5 – 19.8	*26.7 – 41.1*	13.0	*31.8*	3.9	*4.3*
Posterior width	15	7.0 – 20.4	*25.5 – 42.3*	13.5	*32.7*	4.2	*4.6*
Standard width/length ratio	15	27% – 41%	–	32%	–	4%	–
Posterior/anterior width ratio	14	74% – 109%	–	89%	–	11%	–
Claw 1 lengths							
External primary branch	15	11.6 – 23.7	*40.4 – 49.2*	17.6	*43.4*	4.0	*2.7*
External base + secondary branch	15	7.4 – 17.5	*26.9 – 36.2*	13.2	*32.3*	3.6	*3.1*
External spur	8	2.9 – 6.4	*9.3 – 13.1*	4.7	*11.4*	1.4	*1.3*
External branches length ratio	14	57% – 85%	–	75%	–	7%	–
Internal primary branch	15	10.1 – 25.5	*39.6 – 52.9*	18.0	*44.2*	4.5	*3.1*
Internal base + secondary branch	15	8.4 – 18.1	*27.8 – 37.6*	13.1	*32.2*	3.2	*2.4*
Internal spur	13	2.8 – 7.7	*10.2 – 16.5*	6.0	*14.4*	1.9	*2.0*
Internal branches length ratio	15	64% – 87%	–	73%	–	6%	–
Claw 2 lengths							
External primary branch	15	11.4 – 26.6	*43.5 – 55.2*	19.5	*47.9*	5.0	*3.3*
External base + secondary branch	15	8.8 – 17.7	*27.2 – 38.6*	13.6	*33.5*	3.3	*2.9*
External spur	12	2.9 – 6.6	*10.5 – 14.3*	5.0	*12.7*	1.4	*1.2*
External branches length ratio	15	63% – 77%	–	70%	–	5%	–
Internal primary branch	15	12.1 – 25.8	*39.9 – 53.5*	18.9	*46.8*	4.2	*3.2*
Internal base + secondary branch	15	8.1 – 17.6	*30.6 – 36.2*	13.5	*33.2*	3.4	*1.8*
Internal spur	15	3.2 – 9.2	*12.4 – 20.3*	6.7	*16.3*	1.9	*2.7*
Internal branches length ratio	15	65% – 83%	–	71%	–	6%	–
Claw 3 lengths							
External primary branch	15	11.8 – 25.6	*42.9 – 54.5*	19.7	*48.5*	4.7	*3.0*
External base + secondary branch	15	8.4 – 18.2	*29.9 – 37.3*	13.7	*33.6*	3.3	*2.5*
External spur	12	3.1 – 7.8	*8.9 – 16.9*	5.1	*12.5*	1.5	*2.4*
External branches length ratio	15	63% – 76%	–	69%	–	4%	–
Internal primary branch	15	10.6 – 25.3	*39.6 – 52.5*	18.6	*45.7*	4.6	*3.7*
Internal base + secondary branch	14	8.4 – 18.0	*30.7 – 38.9*	13.6	*33.8*	3.6	*2.3*
Internal spur	15	2.7 – 9.2	*9.8 – 20.3*	6.6	*16.1*	1.9	*2.8*
Internal branches length ratio	14	64% – 83%	–	74%	–	6%	–
Claw 4 lengths							
Anterior primary branch	15	13.0 – 29.0	*48.5 – 60.2*	22.4	*54.9*	5.7	*3.1*
Anterior base + secondary branch	15	9.4 – 22.4	*32.7 – 46.5*	15.4	*37.7*	4.4	*3.9*
Anterior spur	13	3.9 – 9.9	*14.1 – 21.2*	7.2	*17.7*	2.0	*2.2*
Anterior branches length ratio	15	60% – 77%	–	69%	–	5%	–
Posterior primary branch	15	13.9 – 30.3	*51.9 – 62.9*	23.9	*58.6*	5.7	*3.1*
Posterior base + secondary branch	15	9.5 – 21.8	*34.5 – 44.7*	15.9	*38.9*	4.3	*3.2*
Posterior spur	12	3.6 – 11.2	*10.3 – 23.2*	5.6	*13.8*	2.1	*3.6*
Posterior branches length ratio	15	58% – 73%	–	66%	–	4%	–

Table 4. Measurements (in µm) and the *pt* values of selected morphological structures of 15 specimens of *Milnesium variefidum* sp. nov. from Insch, Scotland, mounted in Hoyer's medium. Individuals were chosen to represent the entire body length ratio, with as equal representation of all available life stages as possible. Note that sample size for specific traits varied since not all traits were measureable in all individuals.

Character	N	Range µm	Range *pt*	Mean µm	Mean *pt*	SD µm	SD *pt*	Holotype µm	Holotype *pt*
Body length	15	217 – 760	*908 – 1556*	483	*1262*	163	*183*	686	*1556*
Peribuccal papillae length	7	0.0 – 7.2	*11.8 – 18.1*	4.3	*13.8*	2.3	*2.3*	?	*?*
Lateral papillae length	12	3.3 – 9.1	*12.9 – 19.4*	5.8	*15.0*	2.0	*2.1*	7.7	*17.5*
Buccal tube									
Length	15	23.9 – 49.9	–	37.5	–	8.9	–	44.1	–
Stylet support insertion point	15	16.3 – 35.6	*67.4 – 74.6*	26.7	*70.8*	6.7	*2.1*	31.2	*70.7*
Anterior width	13	7.0 – 20.8	*25.5 – 41.7*	12.9	*33.0*	4.7	*5.4*	15.3	*34.7*
Standard width	15	5.9 – 13.7	*22.1 – 33.8*	9.9	*26.2*	2.8	*3.2*	12.6	*28.6*
Posterior width	15	6.2 – 15.3	*23.2 – 38.1*	10.5	*27.7*	3.1	*3.9*	13.5	*30.6*
Standard width/length ratio	15	22% – 34%	–	26%	–	3%	–	29%	–
Posterior/anterior width ratio	13	62% – 99%	–	85%	–	10%	–	88%	–
Claw 1 lengths									
External primary branch	15	10.4 – 20.8	*33.7 – 44.7*	15.0	*40.0*	3.8	*3.1*	19.7	*44.7*
External base + secondary branch	15	7.6 – 16.1	*27.7 – 35.8*	12.0	*31.8*	3.1	*2.1*	15.8	*35.8*
External branches length ratio	13	73% – 88%	–	80%	–	4%	–	?	–
Internal primary branch	15	9.6 – 20.5	*32.8 – 45.1*	14.5	*38.9*	3.3	*3.5*	19.1	*43.3*
Internal base + secondary branch	15	7.6 – 16.2	*28.2 – 35.4*	11.9	*31.7*	2.9	*1.9*	15.6	*35.4*
Internal spur	7	2.0 – 4.5	*5.6 – 10.0*	3.4	*8.2*	0.9	*1.6*	4.4	*10.0*
Internal branches length ratio	14	73% – 96%	–	82%	–	6%	–	?	–
Claw 2 lengths									
External primary branch	14	10.1 – 25.4	*38.0 – 50.9*	16.5	*43.7*	4.4	*3.4*	20.3	*46.0*
External base + secondary branch	14	6.6 – 19.8	*27.6 – 39.7*	12.9	*33.8*	3.9	*2.9*	16.9	*38.3*
External branches length ratio	14	65% – 83%	–	78%	–	5%	–	83%	–
Internal primary branch	14	10.2 – 22.5	*33.9 – 45.6*	15.7	*40.6*	3.9	*3.1*	20.1	*45.6*
Internal base + secondary branch	15	6.2 – 19.0	*25.9 – 38.1*	12.1	*32.0*	3.6	*3.4*	15.9	*36.1*
Internal spur	9	2.6 – 5.6	*6.5 – 13.9*	3.9	*9.8*	1.0	*2.1*	3.8	*8.6*
Internal branches length ratio	15	65% – 83%	–	71%	–	6%	–	67%	–
Claw 3 lengths									
External primary branch	14	11.6 – 25.1	*38.8 – 50.3*	17.1	*44.4*	4.0	*3.5*	21.0	*47.6*
External base + secondary branch	14	6.2 – 19.1	*25.9 – 39.0*	12.6	*33.3*	4.0	*3.5*	17.2	*39.0*
External branches length ratio	15	63% – 76%	–	69%	–	4%	–	63%	–
Internal primary branch	14	10.5 – 21.1	*34.2 – 47.2*	15.7	*41.0*	3.6	*3.9*	20.8	*47.2*
Internal base + secondary branch	15	6.1 – 17.3	*25.5 – 37.2*	12.1	*32.0*	3.4	*2.9*	16.4	*37.2*
Internal spur	10	2.3 – 5.6	*7.3 – 16.6*	4.0	*9.9*	1.0	*2.7*	3.8	*8.6*
Internal branches length ratio	14	64% – 83%	–	74%	–	6%	–	79%	–
Claw 4 lengths									
Anterior primary branch	15	12.4 – 27.4	*46.6 – 61.0*	19.6	*52.1*	5.2	*4.2*	26.9	*61.0*
Anterior base + secondary branch	15	7.9 – 18.6	*30.6 – 42.2*	13.3	*35.2*	3.9	*3.1*	18.6	*42.2*
Anterior branches length ratio	15	60% – 77%	–	69%	–	5%	–	68%	–
Posterior primary branch	14	13.0 – 27.5	*35.9 – 62.4*	19.3	*53.1*	4.6	*6.6*	27.5	*62.4*
Posterior base + secondary branch	14	7.1 – 19.4	*27.5 – 41.0*	13.5	*36.2*	4.0	*3.5*	18.1	*41.0*
Posterior branches length ratio	13	50% – 75%	–	66%	–	6%	–	66%	–

Table 5. An alphabetic list of *Milnesium* Doyère, 1840 taxa described up to the end of 2015 (including two dubious species), with their type localities and a summary of their key taxonomic traits. Given that the number of peribuccal lamellae is not always possible to determine under light microscope, the state of this trait is problematic in many species of the genus. A question mark (?) indicates that the number and size of peribuccal lamellae is unknown; 'SEM' and 'LM' in brackets indicate that the state has been confirmed either by a scanning electron microscopy or by an unambiguous light observation, respectively; a number in square brackets indicates the state as described in the original description if later analyses identified a different lamellae state.

Species	Locus typicus (country, continent)	Cuticle surface	Cuticular gibbosities	Dorsal spines	Peribuccal lamellae	Primary branches	Accessory points	Claw configuration
M. alabamae Wallendorf and Miller, 2009	Alabama, USA, North America	reticulated	absent	absent	6 or 4+2	present	absent	[3-3]-[3-3]
M. almatyense Tumanov, 2006	Kazakhstan, Asia	smooth	absent	absent	6 or 4+2	present	present	[2-3]-[2-2]
M. alpigenum Ehrenberg, 1853	Switzerland, Europe	smooth	absent	absent	?	present	present	[3-3]-[3-3]
M. antarcticum Tumanov, 2006	King George Island, Antarctica	smooth	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. argentinum Roszkowska, Ostrowska and Kaczmarek, 2015	Argentina, South America	pseudo-porous	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. asiaticum Tumanov, 2006	Kirghizia, Asia	smooth	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. barbadosense Meyer and Hinton, 2012	Barbados, North Atlantic	smooth	absent	absent	6 (LM)	present	present	[2-3]-[2-3] [3-3]-[3-3]
M. beasleyi Kaczmarek, Jakubowska and Michalczyk, 2012	Turkey, Asia	pseudo-porous	absent	absent	6 or 4+2	present	present	[2-3]-[3-2]
M. beatae Roszkowska, Ostrowska and Kaczmarek, 2015	Argentina, South America	pseudo-porous	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. berladnicorum Ciobanu, Zawierucha, Moglan and Kaczmarek, 2014	Romania, Europe	pseudo-porous	absent	absent	4+2 (SEM) [6] (LM)	present	present	[2-3]-[2-2]
M. bohleberi Bartels, Nelson, Kaczmarek and Michalczyk, 2014	North Carolina, USA, North America	smooth	absent	absent	6 (LM)	present	present	[3-3]-[3-3]
M. brachyungue Binda and Pilato, 1990	Chile, South America	smooth	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. dornensis Ciobanu, Roszkowska and Kaczmarek, 2015	Romania, Europe	pseudo-porous	absent	absent	6 (LM)	present	present	[3-3]-[3-3]
M. dujiangensis Yang, 2003 *nomen dubium*	Sichuan Province, China, Asia	smooth	absent	absent	?	absent	absent	[2-2]-[1-2]
M. eurystomum Maucci, 1991	Greenland, Denmark, Arctic	smooth	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. granulatum Ramazzotti, 1962	Chile, South America	reticulated	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. jacobi Meyer and Hinton, 2010	Texas, USA, North America	smooth	absent	absent	6 or 4+2	present	present	[2-3]-[3-3]
M. katarzynae Kaczmarek, Michalczyk and Beasley, 2004	Sichuan Province, China, Asia	reticulated	absent	absent	?	present	present	[2-2]-[2-2]
M. kogui Londoño, Daza, Caicedo, Quiroga and Kaczmarek, 2015	Colombia, South America	smooth	absent	absent	6 or 4+2	present	present	[2-2]-[2-2]
M. krzysztofi Kaczmarek and Michalczyk, 2007	Costa Rica, Central America	reticulated	absent	absent	?	present	present	[2-3]-[3-2]

Table 5. cont.

Species	Locus typicus (country, continent)	Cuticle surface	Cuticular gibbosities	Dorsal spines	Peribuccal lamellae	Primary branches	Accessory points	Claw configuration
M. lagniappe Meyer, Hinton and Dupré, 2013	Louisiana, USA, North America	pseudo-porous	absent	absent	4 or 4+2	present	present	[2-3]-[3-2]
M. longiungue Tumanov, 2006	India, Asia	smooth	absent	absent	6 or 4+2	present	absent	[3-3]-[3-3]
M. quadrifidum Nederström, 1919	Finland, Europe	smooth?	absent	absent	?	present	?	[4-4]-[4-4]
M. reductum Tumanov, 2006	Kirghizia, Asia	smooth	absent	absent	6 or 4+2	present	absent	[2-3]-[3-2]
M. reticulatum Pilato, Binda and Lisi, 2002	Seychelles, Africa	reticulated	present	absent	4 (LM)	present	present	[2-3]-[3-2]
M. shilohae Meyer, 2015	Hawaii, USA, Pacific Ocean	smooth	absent	absent	6 or 4+2	present	present	[3-3]-[3-3]
M. tardigradum Doyère, 1840	Germany, Europe	smooth	absent	absent	6 or 4+2	present	present	[2-3]-[3-2]
M. tardigradum trispinosum Rahm, 1931 *nomen dubium*	Chile, South America	smooth?	absent	present	?	present	?	[?-?]-[?-?]
M. tetralamellatum Pilato and Binda, 1991	Tanzania, Africa	smooth	absent	absent	4 (LM)	present	present	[2-3]-[2-3]
M. variefidum sp. nov.	United Kingdom, Europe	pseudo-porous	absent	absent	4+2 (SEM)	present	present	[2-2]-[2-2] [2-3]-[2-2]
M. zsalakoae Meyer and Hinton, 2010	Arizona and New Mexico, USA, North America	smooth	absent	absent	6 or 4+2	present	absent	[3-3]-[3-3]