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Redescription of *Milnesium alpigenum* Ehrenberg, 1853 (Tardigrada: Apochela) and a description of *Milnesium inceptum* sp. nov., a tardigrade laboratory model

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Abstract

Intra- and interspecific variability, being at the very core of alpha taxonomy, has been a long-standing topic of debate among tardigrade taxonomists. Early studies tended to assume that tardigrades exhibit wide intraspecific variation. However, with more careful morphological studies, especially those incorporating molecular tools that allow for an independent verification of species identifications based on phenotypic traits, we now recognise that ranges of tardigrade intraspecific variability are narrower, and that differences between species may be more subtle than previously assumed. The taxonomic history of the genus *Milnesium*, and more specifically that of the nominal species, *M. tardigradum* described by Doyère in 1840, is a good illustration of the evolution of views on intraspecific variability in tardigrades. The assumption of wide intraspecific variability in claw morphology led Marcus (1928) to synonymise two species with different claw configurations, *M. alpigenum* and *M. quadrifidum*, with *M. tardigradum*. Currently claw configuration is recognised as one of the key diagnostic traits in the genus *Milnesium*, and the two species suppressed by Marcus have recently been suggested to be valid. In this study, we clarify the taxonomic status of *M. alpigenum*, a species that for nearly a century was considered invalid. We redescribe *M. alpigenum*, using a population collected from the *locus typicus*, by the means of integrative taxonomy, *i.e.* including light microscopy, scanning electron microscopy, ontogenetic observations, and genetic barcoding. Moreover, the redescription of *M. alpigenum* allowed us to verify the uncertain taxonomic status of two popular laboratory models that were originally considered to be *M. tardigradum*; though one was recently reidentified as *M.* cf. *alpigenum*. Our analysis showed that both laboratory strains, despite being morphologically and morphometrically nearly identical to *M. alpigenum*, in fact represent a new species, *M. inceptum* **sp. nov.** The two species, being disnguishable only by statistical morphometry and/or DNA sequences, are the first example of pseudocryptic species in tardigrades.

Key words: barcoding, cryptic species, integrative taxonomy, *M. tardigradum s.s.,* phylogeny, pseudocryptic species

Introduction

Tardigrades, also known as water bears, are a phylum of microscopic invertebrates that dwell in marine, freshwater and terrestrial ecosystems (Nelson *et al.* 2015). The first formal descriptions of tardigrade species were published in the first half of the XIX century. Among them was *Milnesium tardigradum* Doyère 1840, described by a French zoologist Louis Michel François Doyère. The species was established as the nominal taxon for the genus *Milnesium* Doyère, 1840, family Milnesiidae Ramazzotti, 1962, and the order Apochela Schuster *et al*., 1980. In the following decades, two further species of this genus were described: *M. alpigenum* Ehrenberg, 1853 from Monte Rosa (Italy/Switzerland) and *M. quadrifidum* Nederström, 1919 from Utsjoki, Finland. Both species were differentiated from *M. tardigradum* based on claw morphology (Morek *et al.* 2016a), a trait currently termed the claw configuration (CC). However, soon after *M. quadrifidum* was described, Marcus (1928), in his influential monograph on tardigrade biology, expressed an opinion that claw morphology was only a manifestation of

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intraspecific variability, making it unsuitable for the differentiation of *Milnesium* species. Therefore, he synonymised both *M. alpigenum* and *M. quadrifidum* with *M. tardigradum*. This resulted in a widespread conviction that *M. tardigradum* exhibited considerable morphological variability and throughout the following decades researchers used to classify any *Milnesium* species as *M. tardigradum*, no matter what geographic origin of specimens or morphological variation (*e.g.* see Ramazzotti & Maucci 1983; Dastych 1988).

Thus, with numerous records throughout the globe, *M. tardigradum* inevitably became recognised as cosmopolitan and for decades remained the only species in the genus *Milnesium*. This started to change only at the end of the XX century, when several new *Milnesium* species descriptions, although based on traits other than claw configuration, were published (Ramazzotti, 1962; Binda & Pilato, 1990; Maucci, 1991; Pilato & Binda, 1991). Later, Tumanov (2006) described additional five species and proposed to standardise some of the key morphometric measurements. However, the greatest increase in *Milnesium* species descriptions occurred after the redescription of *M. tardigradum* (Michalczyk *et al.* 2012a, b), when 46% of all known species of the genus were described within only six years (*i.e.* from 2012 to 2017). Based on their observations, Michalczyk *et al.* (2012a, b) assumed that CC is stable at the species level and they emphasised the value of this trait in *Milnesium* spp. differentiation. However, recently Morek *et al.* (2016a), using an experimental approach, discovered that some *Milnesium* species may undergo ontogenetic CC change. In fact, the latest integrative study on intraspecific variability of *M. tardigradum* by Morek *et al*. (2019) showed that the nominal species also exhibited developmental variability in CC, which had gone undetected in the redescription by Michalczyk *et al.* (2012a, b). Importantly, however, the discovery of developmental variability in CC does not undermine the taxonomic value of CC itself, because the developmental pattern seems to be species-specific Morek *et al*. (2019). Moreover, the pattern of CC ontogenetic variability may constitute an additional set of traits for species delimitation in the genus *Milnesium*.

In other words, modern advances in *Milnesium* taxonomy showed that the opinion of Marcus (1928) was incorrect, and *M. alpigenum* and *M. quadrifidum* are good species and are now pending integrative redescriptions (Morek *et al.* 2016a). These redescriptions are particularly important because with the simplistic original descriptions, it is impossible to differentiate *M. alpigenum* and *M. quadrifidum* from congeners that differ solely in quantitative (morphometric) and/or molecular traits. This, in turn, may prevent the identification of new species and lead to underestimation of species diversity and overestimation of species geographic ranges. *Milnesium quadrifidum* is the only known *Milnesium* species with the [4-4]-[4-4] CC. Therefore, describing new species that exhibit this claw configuration and morphology before *M. quadrifidum* is redescribed involves a risk of taxonomic inflation (Morek *et al.* 2016a). On the other hand, *M. alpigenum*, with the [3-3]-[3-3] CC, was the first described member of the largest group of *Milnesium* species defined by the CC. Thus, the redescription should verify whether any of the eighteen reported species with the [3-3]-[3-3] CC and smooth cuticle require synonymising with *M. alpigenum* (Morek *et al.* 2016a). Moreover, the two most studied *Milnesium* laboratory strains, one from Japan (Suzuki 2003) and the other from Germany (Schill *et al.* 2004), fit the description of *M. alpigenum*. Both laboratory strains were originally considered to be "*M. tardigradum*"; though the German strain was recently tentatively re-identified as "*M.* cf. *alpigenum*" (see Michalczyk *et al.* 2012a, Morek *et al.* 2016a, and Morek *et al.* 2019 for details). Thus, it is vital to verify whether the laboratory strains represent *M. alpigenum*, or a new species of the [3-3]-[3-3] CC group (Morek *et al.* 2016a). These laboratory strains have been used in studies on cryptobiosis (*e.g.* Hengherr *et al*., 2008a, b, Hengherr *et al*., 2009a), astrobiology (*e.g.* Jönsson *et al.* 2016), cell biology (*e.g.* Beisser, *et al.* 2012, Schokraie *et al.* 2012, Grohme *et al.* 2013), physiology (*e.g.* Reuner *et al.* 2010a, Förster *et al*. 2012), developmental biology (*e.g.* Suzuki 2003, Suzuki 2006), experimental taxonomy (*e.g.* Kosztyła *et al*. 2016, Morek *et al*. 2016b, Stec *et al*. 2016), and ethology (Shcherbakov *et al.* 2010), thus pinpointing their identity is of great importance.

In this paper, we aim to clarify the taxonomy within the genus *Milnesium* and to verify the taxonomic status of the popular laboratory models. To achieve this, we integratively analysed *Milnesium* individuals, with the [3-3]-[3-3] CC and unsculptured cuticle, collected from the *M. alpigenum* type locality in northern Italy, and compared them with the two *M.* cf. *alpigenum* laboratory cultures (from Japan and Germany). Additionally, we analysed two similar populations, from Switzerland and Bulgaria, to test whether these represented either *M. alpigenum* or the species used to establish the laboratory strains. In either case, these additional populations could extend the genetic and phenotypic variability as well as the geographic range of the species in question.

Materials and methods

Nomenclature. Claw configuration (abbreviated throughout the text as "CC") is denoted according to Michalczyk *et al*. (2012a, b), *i.e.* as a string of bracketed numbers that represent the number of points on the secondary branches on **e**xternal and **i**nternal **c**laws I–III, and on **a**nterior and **p**osterior claws IV: formula [e-i]-[a-p]. The developmental

terminology follows Morek *et al*. (2019), *i.e.* immature individuals: the first instar = hatchling, and the second instar = juvenile; mature individuals from the third instar onwards = adults.

Sampling and specimen isolation. Detailed collection data are provided in Table 1. All moss samples were collected and processed according to standard methods (*e.g.* Stec *et al.* 2015). Tardigrades were cultured following the protocol in Kosztyła *et al*. (2016), *i.e.* they were fed rotifers, *Lecane inermis* (Bryce, 1892), and kept on plastic Petri dishes with scratched bottoms, at a stable temperature (8 or 16°C), and in complete darkness. Individuals isolated from samples and/or cultures were split into 3–4 analysis groups: (i) development tracking, (ii) imaging and morphometry in phase contrast light microscope (PCM), (iii) imaging in scanning electron microscopy (SEM), and (iv) DNA sequencing; see Table 1 for details.

Milnesium alpigenum was originally described from the Monte Rosa massif, thus we sampled this locality in order to find a population that could be designated as the neotype series. Since the original description is very limited and the type material no longer exists, any *Milnesium* with unsculptured cuticle and the [3-3]-[3-3] CC found in the vicinity of the type locality could be designated as neotype *M. alpigenum*. Our colleagues from the Adam Mickiewicz University (Poland) kindly provided us with a moss sample with a candidate population collected in the Monte Rosa massif, from which we isolated five live females (see Table 1 for details). The small number of individuals was insufficient for all the required analyses, so we cultured the five females separately (to control for potential multiple species exhibiting similar morphology and dwelling in a single moss cushion). After the isolines have perpetuated for several generations, producing sufficient numbers of individuals, we sequenced animals from all isolines (see below for details). DNA sequencing confirmed that all five isolines represent a single species, thus all individuals were pooled and used for the planned analyses (Table 1).

Alongside the Italian *M. alpigenum* neotype material, we also analysed a further four *Milnesium* populations, which conformed to the original description of *M. alpigenum*, to test whether they represent *M. alpigenum* or new species (see Table 1 for details). These were:

- a German laboratory strain established by R.O. Schill at the University of Tübingen in 2003 with specimens collected in the Bebenhausen forest and subsequently maintained at the University of Stuttgart (sample code DE.001; "Tübingen strain");
- a Japanese laboratory strain established by A.C. Suzuki at the University of Keio in 2002 with specimens collected in Hiyoshi (sample code JP.010; "Hiyoshi H-1 strain");
- a wild population from Switzerland (sample CH.002);
- a wild population from Bulgaria (sample BG.058).

Microscopy and imaging. A total of 47 individuals of the neotype *M. alpigenum* population (IT.057), 98 from the Tübingen laboratory strain (DE.001), 15 from the Japanese laboratory strain (JP. 010), 9 from the Swiss population (CH.002), and 69 from the Bulgarian population (BG.058) were used for the PCM analysis. All were mounted in Hoyer's medium on microscope slides according to the recipe and protocol described by Morek *et al.* (2016b). Photographs and measurements were taken using *Nikon Eclipse 50i* PCM associated with a *Nikon Digital Sight DS-L2* digital camera (termed here as "a standard class/quality PCM") and with *Olympus BX53* PCM associated with *Olympus DP74* digital camera ("high class/quality PCM"). For structures that could not be focused in a single photograph, a series of up to eight pictures were taken and merged into one deep-focus image using *Corel Photo-Paint X8*.

Additionally, 20 individuals of the neotype population of *M. alpigenum* (IT.057), 15 from the German laboratory strain (DE.001), and 18 from the Bulgarian population (BG.058) were processed for SEM imaging, following the protocol described in Stec *et al.* (2015), and then examined under high vacuum in *Versa 3D DualBeam* SEM at the ATOMIN facility of the Jagiellonian University.

Ontogenetic variability detection. In order to test for ontogenetic variability, developmental tracking according to Morek *et al.* (2016a) was employed. In brief, exuviae with eggs were incubated individually and emerging hatchlings were split into three subsets: (i) mounted on permanent microscope slides in Hoyer's medium

TABLE 1. Information about *Milnesium* populations used in the study. Bolded sample codes indicate type and neotype localities. Analyses performed: (i): culturing and development tracking; (ii): imaging and morphometry in PCM; (iii): imaging in SEM; and (iv): DNA sequencing. Analysis numbers correspond to those used in the text.

Sample code	Locality	Coordinates, altitude	Sample type	Collection date	Collector	Species	Analyses performed
IT.057	Italy, Monte Rosa, lower chair-lift station of Macugnaga	45°58'13"N 07°57'07"E 1370 m asl	moss on roof	27.06.2015	Łukasz Kaczmarek, Milena Roszkowska, Weronika Erdmann, Krzysztof Zawierucha	*M. alpigenum* Ehrenberg, 1853	i ii iii iv
DE.001	Germany, Tübingen, Bebenhausen, Schönbuch Nature Reserve, mixed forest	48°33'42"N 09°03'48"E 377 m asl	moss on soil	10.2002	Ralph O. Schill	*M. inceptum* **sp. nov.**	i ii iii iv
JP.010	Japan, Yokohama, Hiyoshi	35°33′10.6″N 139°39′01″E 35 m asl	moss (*Bryum argenteum*) on concrete wall	03.04.2000	Atsushi C. Suzuki	*M. inceptum* **sp. nov.**	i ii iv
CH.002	Switzerland, Zürich, corner of Rämistrasse and Schmelzbergstrasse	47°22'38"N 08°32'56"E 470 m asl	moss on concrete wall	10.12.2015	Łukasz Michalczyk, Grzegorz Kwiatkowski	*M. inceptum* **sp. nov.**	i ii iv
BG.058	Bulgaria, Shanovo, Kazanlak Valley, near the north slope of Sarnena Mt.	42°33'27"N 25°37'51"E 300 m asl	moss (*Grimmia* sp.) on brick wall	25.08.2015	Dilian Georgiev, Maria Yankova	*M. inceptum* **sp. nov.**	i ii iii iv

one day after hatching, (ii) reared to the juvenile stage and mounted one day after moulting, and (iii) cultured to the first adult instar and mounted one day after moulting. A comparison of the morphology between the first three instars permitted observation of any ontogenetic variability in taxonomically important traits such as the CC, cuticle morphology and the presence/appearance of cuticular bars under claws I–III.

Morphometrics. The number of measured specimens follow recommendations by Stec *et al.* (2016). Specimens were measured according to Tumanov (2006) and 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* values are given in *italics*.

Morphometric species delineation. Despite molecular analyses indicating clear genetic differences between the two species addressed in this study, the morphometric ranges (both absolute and relative values) overlapped (see below for details). Therefore, in order to test for statistical differences between the species, we used Principle Component Analysis (PCA) followed by a series of Student *t*-tests with α-level adjusted with the Benjamini-Hochberg correction. The PCA allows a reduction of the original dataset components while retaining the maximum possible variation of data. The analysis was performed in R 3.4.2. (R Core Team 2015) by prcomp function, using only the *pt* values as ratios to reduce allometric effects. The dataset had *ca.* 18% of missing measurements, which were replaced by median values of each variable in a given population to avoid losing statistical power. Afterwards, the variables were scaled to unit variance and zero mean to minimise the effect of different scales of measured traits. The results were visualised using package ggfortify (version 0.4.1, Tang *et al.* 2016).

Genotyping. First, we tested whether the five isolines of the *M. alpigenum* type locality (IT.057) and populations from Germany (DE.001), Japan (JP.010), Switzerland (CH.002), and Bulgaria (BG.058) represent a single or multiple species. This we achieved by sequencing two variable barcodes, the nuclear Internal Transcribed Spacer 2 (ITS-2), and the mitochondrial Cytochrome Oxidase C subunit I (COI), for eight individuals per population. We established that all Italian isolines represented a single species, and that all the remaining populations represented another species. With this knowledge, we further sequenced two conservative nuclear markers, small ribosomal subunit (18S rRNA), large ribosomal subunit (28 rRNA), for four individuals from each species (specifically, from populations IT.057 and DE.001).

DNA was extracted from individual tardigrades following the protocol of Chelex® 100 resin (Bio-Rad), extraction method by Casquet *et al.* (2012) with modifications by Stec *et al.* (2015). Primer sequences and sources as well as references for PCR programs are listed in Table 2. All sequences were handled in *BioEdit* ver. 7.2.5 (Hall 1999). COI sequences were translated into amino acids to test for potential pseudogenes. Additionally the uncorrected p-distances were calculated utilising MEGA 7 (Kumar *et al.* 2016) for comparisons between species and populations. All sequences were aligned using the default settings of *MAFFT* version 7 (Katoh *et al*. 2002; Katoh & Toh 2008). The obtained alignments were edited and checked manually in *BioEdit* and then trimmed to 585 bp (ITS-2) and 509 bp (COI).

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	SSU01_F	forward	AACCTGGTTGATCCTGCCAGT	Sands *et al.* (2008)	Zeller (2010)
	SSU82_R	reverse	TGATCCTTCTGCAGGTTCACCTAC		
28S rRNA	28SF0001	forward	ACCCVCYNAATTTAAGCATAT	Mironov *et al.* (2012)	Mironov *et al.* (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC		
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.* (2019)	Morek *et al.* (2019)
	COI_Mil.tar_Rr	reverse	CCTCCCCCTGCAGGATC		

Phylogenetic analysis. In order to visualise evolutionary relationships between the four *Milnesium* populations analysed in this study, phylogenetic trees using all available *Milnesium* ITS-2 and COI sequences were constructed. Thus, in addition to new sequences, the data set comprised the ITS-2 sequences for the following

species: *M. berladnicorum* Ciobanu, Zawierucha, Moglan & Kaczmarek, 2014; (KT951662 from Morek *et al.* 2016a); *M. dornensis* Ciobanu, Roszkowska & Kaczmarek, 2015; (MG923557 from Morek *et al.* 2019); *M. variefidum* Morek, Gąsiorek, Stec, Blagden & Michalczyk, 2016a; (KT951666–7 from Morek *et al*. 2016a); "*M. tardigradum*"; (GQ403681–2 from Schill, unpublished); *M. tardigradum sensu stricto* Doyère, 1840; (MG923551–5 from Morek *et al.* 2019). Additional COI sequences for the following species were also included: *M. berladnicorum* (KT951659 from Morek *et al.* 2016a); *M. dornensis* (MG923566 from Morek *et al.* 2019); *M. variefidum* (KT951663 from Morek *et al.* 2016a); *M. tardigradum s.s.* (MG923558–65 from Morek *et al.* 2019); *M.* cf. *alpigenum* (KU51342 from Kosztyła *et al.* 2016); "*M. tardigradum*" (EU244603–4 from Schill, unpublished); "*M. tardigradum*" (FJ435810 from Guil & Giribet 2012); "*M. tardigradum*" (JX683822–5 from Vicente *et al.* 2013); *M.* sp. (EF632553 from Sands *et al*., unpublished); *M.* sp. (KX306950 from Fox *et al*., unpublished); *M.* spp. (KJ857001–2, KP013598, KP013601 and KP013613 from Velasco-Castrillón *et al*. 2015). For the outgroup, sequences of *Diploechiniscus oihonnae* Richters, 1903 (MG063724 from Gąsiorek *et al.* 2017a and MG923556 from Morek *et al.* 2019) were used. Concatenation was run in SequenceMatrix (Vaidya *et al*. 2010) and the final ITS-2+COI alignment was 1094 bp long.

Using PartitionFinder version 2.1.1 (Lanfear *et al*. 2016), under the Akaike Information Criterion (AIC), the best substitution model was chosen for posterior phylogenetic analysis. As COI is a protein-coding gene, the alignment was divided into three data blocks representing three separated codon positions. As best-fit partitioning scheme, PartitionFinder suggested to retain three predefined partitions for the COI data set and four predefined partitions for the concatenated data set separately. As RAxML (Stamatakis 2014) allows for only a single model of rate heterogeneity (from the GTR family) in partitioned analyses, each data set was analysed twice: first to test all possible models implemented in the program (for Bayesian Inference, BI), and then for models from the GTR family (for Maximum Likelihood analysis, ML). The best fit-models for four partitions for BI were: TRN+I+G for the first codon position, K81UF+G for the second and the third codon position, and GTR+G for the ITS-2 partition. For ML, the best model was GTR+G.

BI marginal posterior probabilities were calculated using MrBayes v3.2 (Ronquist & Huelsenbeck 2003). Random starting trees were used and the analysis was run for ten million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of <0.01 was used as a guide to confirm that the two independent analyses had converged. The program Tracer v1.3 (Rambaut *et al.* 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct "burn-in" for the analysis, which was the first 10% of generations. The consensus tree was obtained after summarising the resulting topologies and discarding the "burn-in". In the BI consensus tree, clades recovered with posterior probability (PP) between 0.95 and 1.00 were considered well supported, those with PP between 0.90 and 0.94 were considered moderately supported and those with lower PP were considered unsupported. The consensus tree was viewed and visualised by FigTree v.1.4.3, available from http://tree.bio.ed.ac.uk/software/figtree. ML topologies were constructed using RAxML v8.0.19 (Stamatakis 2014). The strength of support for internal nodes of ML construction was measured using 1000 rapid bootstrap replicates. Bootstrap (BS) support values ≥70% on the final tree were regarded as significant statistical support.

Data deposition. Raw morphometric data are deposited in the Tardigrada Register (Michalczyk & Kaczmarek 2013) under http://tardigrada.net/register/0056.htm (*M. alpigenum*), http://tardigrada.net/register/0057.htm (*M. inceptum* **sp. nov.**) as well as in Supplementary Materials SM.1–5. The sequences of all haplotypes were uploaded to GenBank (accession numbers are listed under species redescription/description and in the Appendix 1).

Results

Taxonomic accounts

Phylum Tardigrada Doyère, 1840

Class Eutardigrada Richters, 1926

Order Apochela Schuster *et al*., 1980

Family Milnesiidae Ramazzotti, 1962

Genus *Milnesium* Doyère, 1840

***Milnesium alpigenum* Ehrenberg, 1853**
Fig. 1, Table 3

M. tardigradum Marcus (1928)

Material examined: The neotype series consisting of the neotype and 37 neoparatypes (see Table 1 and "Type repositories" below for details).

Integrative redescription. Females (morphometrics in Table 3): Body slightly yellowish, rather slender as for a *Milnesium* (Fig. 1A). Eyes present in live specimens, quickly dissolving after fixation in Hoyer's medium. Cuticle smooth in SEM and with minute pseudopores visible on the caudo-dorsal part only under high quality PCM (Fig. 1C). Weakly outlined pseudoplates on the caudo-dorsal cuticle visible in some specimens only under SEM (Fig. 1B and D). Six peribuccal papillae present, with the ventral being the smallest. Six triangular peribuccal lamellae of unequal size; the two lateral being slightly smaller than the dorsal and ventral lamellae, *i.e.* with the 4+2 configuration (identifiable only in SEM; Fig. 1F). Two lateral papillae present. Buccal tube funnel-shaped (Fig. 1E). Claws slender, primary branches with tiny accessory points, more visible on claws IV. All secondary branches with three points, *i.e.* with the [3-3]-[3-3] CC (Fig. 1G–H). Spurs on secondary branches long and slender, especially on internal and anterior claws. Cuticular bars under claws I–III present.

Males: No males were found in the sample or culture, confirming that the neotype population is parthenogenetic (at least facultatively).

Juveniles: Morphologically identical to adults, except for the lack of pseudopores.

Hatchlings: Morphologically identical to adults, except for the lack of pseudopores and the absence of cuticular bars under claws I–III in the majority of examined specimens (7/8 specimens = 88%).

Ontogenetic variability: No developmental variability in the CC. Pseudopores visible only in adults. Cuticular bars under claws I–III mostly absent in hatchlings but always present in juveniles and adults.

Eggs: Oval, yellow, smooth and laid in exuviae. In the culture, up to 12 eggs were recorded in a single clutch.

DNA markers: All sequences were of a very good quality and every marker was represented by a single haplotype: 18S rRNA (1054 bp, MG996146); 28S rRNA (809 bp, MH000384); ITS-2 (530 bp, MH000382); and COI (560 bp, MH000380). Sequences are provided in Appendix 1.

Neotype locality: 45°58'13"N, 07°57'07"E; 1370 m asl: Italy, Monte Rosa massif, lower chair-lift station of Macugnaga; moss on roof.

Etymology: Ehrenberg (1853) did not explain the choice of the species name; however, it seems reasonable to assume that Christian Ehrenberg named the species after the Alps, the mountain chain in which the type locality, the Monte Rosa massif, is located.

Type repositories: The neotype series consist of the neotype (slide IT.057.17) and 37 "neoparatypes" (IT.057.01–16; 18–38). The neotype and 15 neoparatypes (IT.057.01–12; 45–47) are preserved at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland), further 14 neoparatypes (IT.057.13–16; 18–26) are deposited in Department of Animal Taxonomy and Ecology, Adam Mickiewicz University in Poznań, Umultowska 89, 61-614 Poznań, Poland, 10 neoparatypes (IT.057.27–38) are stored in the Marine Biology & Ecology Research Centre, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, United Kingdom, one paratype (IT.057.47) is deposited in Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom, and the remaining 6 neoparatypes (IT.057.39-44) are deposited in the collection of Binda & Pilato, Museum of the Department of Biological, Geological and Environmental Sciences, Section of Animal Biology "Marcello La Greca", University of Catania, Italy.

Phenotypic differential diagnosis: *Milnesium alpigenum* has the [3-3]-[3-3] CC and "smooth" cuticle (*i.e.* cuticle smooth in SEM and with minute pseudopores visible only under high quality PCM, but with no sculpturing, such as reticulation, on cuticle surface). This places it in the largest group of *Milnesium* species that share these characteristics (20 species; Morek *et al.* 2016a; Pilato & Lisi 2016; Young *et al*. 2016; Pilato *et al.* 2016; Pilato *et al.* 2017; Schlabach *et al.* 2018). Nevertheless, *M. alpigenum* differs specifically from:

TABLE 3. Measurements (in µm) and the *pt* values of selected morphological structures of 30 specimens (neotype and neoparatypes) of *Milnesium alpigenum* Ehrenberg, 1853 from type locality, Monte Rosa, Italy, 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		Neotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	30	383 – 983	*1164 – 1509*	614	*1292*	166	*84*	751	*1322*
Peribuccal papillae length	23	5.8 – 11.7	*15.1 – 22.0*	8.7	*17.8*	1.7	*1.5*	10.2	*18.0*
Lateral papillae length	28	5.6 – 11.7	*14.1 – 20.4*	8.6	*18.0*	2.1	*1.5*	10.4	*18.3*
Buccal tube									
Length	30	32.3 – 67.5	–	47.1	–	10.5	–	56.8	–
Stylet support insertion point	30	21.8 – 43.3	*61.1 – 70.3*	30.4	*64.9*	6.2	*2.1*	34.7	*61.1*
Anterior width	29	11.5 – 28.6	*29.6 – 44.8*	17.9	*38.1*	4.6	*3.5*	22.1	*38.9*
Standard width	30	8.6 – 23.6	*21.0 – 38.4*	14.8	*31.4*	4.0	*3.7*	19.7	*34.7*
Posterior width	30	9.7 – 22.3	*23.7 – 39.4*	15.6	*33.0*	4.0	*3.8*	20.9	*36.8*
Standard width/length ratio	30	21% – 38%	–	31%	–	4%	–	35%	–
Posterior/anterior width ratio	29	75% – 97%	–	86%	–	6%	–	95%	–
Claw 1 lengths									
External primary branch	30	13.7 – 27.0	*36.7 – 47.9*	19.1	*40.8*	3.9	*2.9*	21.6	*38.0*
External base + secondary branch	23	10.1 – 19.7	*26.8 – 34.4*	13.9	*29.6*	2.9	*2.1*	?	*?*
External spur	17	4.0 – 9.1	*11.3 – 17.8*	6.4	*13.3*	1.4	*1.5*	?	*?*
External branches length ratio	23	66% – 86%	–	73%	–	5%	–	?	–
Internal primary branch	29	13.2 – 26.6	*36.0 – 44.8*	18.5	*39.2*	3.6	*2.4*	21.0	*37.0*
Internal base + secondary branch	18	9.9 – 20.8	*25.1 – 32.7*	13.4	*29.2*	3.0	*2.1*	15.8	*27.8*
Internal spur	23	3.7 – 10.1	*11.2 – 16.9*	6.8	*14.2*	1.7	*1.2*	8.4	*14.8*
Internal branches length ratio	17	66% – 84%	–	74%	–	5%	–	75%	–
Claw 2 lengths									
External primary branch	29	15.3 – 29.9	*40.0 – 50.2*	21.2	*44.8*	4.3	*2.9*	24.4	*43.0*
External base + secondary branch	25	10.4 – 23.0	*27.8 – 34.2*	15.0	*31.5*	3.6	*1.9*	18.6	*32.7*
External spur	20	4.5 – 10.7	*12.7 – 17.5*	7.6	*15.3*	1.8	*1.4*	8.5	*15.0*

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TABLE 3. (Continued)

CHARACTER	N	RANGE		MEAN		SD		Neotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
External branches length ratio	24	62% – 80%	–	71%	–	4%	–	76%	–
Internal primary branch	27	15.2 – 29.0	*39.2 – 49.8*	21.0	*44.2*	4.1	*3.0*	25.0	*44.0*
Internal base + secondary branch	14	10.4 – 21.1	*28.1 – 34.4*	13.9	*31.8*	3.5	*1.5*	18.0	*31.7*
Internal spur	24	4.4 – 10.5	*12.9 – 18.4*	8.1	*16.3*	1.9	*1.4*	9.7	*17.1*
Internal branches length ratio	12	65% – 88%	–	71%	–	6%	–	72%	–
Claw 3 lengths									
External primary branch	28	15.9 – 32.4	*41.1 – 51.4*	22.0	*45.8*	4.5	*2.7*	24.4	*43.0*
External base + secondary branch	21	11.1 – 23.0	*29.9 – 35.7*	16.1	*32.7*	3.5	*1.8*	18.4	*32.4*
External spur	20	4.4 – 12.0	*12.4 – 17.8*	7.5	*15.2*	1.8	*1.4*	8.2	*14.4*
External branches length ratio	20	65% – 82%	–	72%	–	4%	–	75%	–
Internal primary branch	26	15.3 – 29.1	*39.7 – 51.7*	21.5	*45.6*	4.6	*2.7*	24.8	*43.7*
Internal base + secondary branch	13	10.5 – 18.8	*29.8 – 36.0*	13.6	*32.5*	3.0	*1.7*	18.7	*32.9*
Internal spur	21	4.5 – 9.8	*13.1 – 18.7*	7.6	*16.3*	1.9	*1.7*	9.5	*16.7*
Internal branches length ratio	12	62% – 80%	–	71%	–	5%	–	75%	–
Claw 4 lengths									
Anterior primary branch	30	19.6 – 37.9	*50.9 – 63.2*	26.6	*56.9*	5.4	*3.2*	30.7	*54.0*
Anterior base + secondary branch	29	11.2 – 25.5	*32.2 – 39.1*	16.3	*34.9*	3.8	*1.9*	18.4	*32.4*
Anterior spur	28	4.3 – 9.6	*12.5 – 17.0*	7.2	*15.0*	1.6	*1.1*	8.7	*15.3*
Anterior branches length ratio	29	57% – 72%	–	61%	–	4%	–	60%	–
Posterior primary branch	29	18.2 – 35.0	*46.9 – 60.8*	25.1	*53.8*	5.1	*3.8*	28.4	*50.0*
Posterior base + secondary branch	25	10.9 – 25.7	*30.7 – 39.8*	16.4	*35.1*	3.8	*2.3*	18.1	*31.9*
Posterior spur	27	4.2 – 10.8	*12.8 – 19.9*	8.0	*16.5*	2.1	*1.9*	8.9	*15.7*
Posterior branches length ratio	24	58% – 73%	–	65%	–	4%	–	64%	–

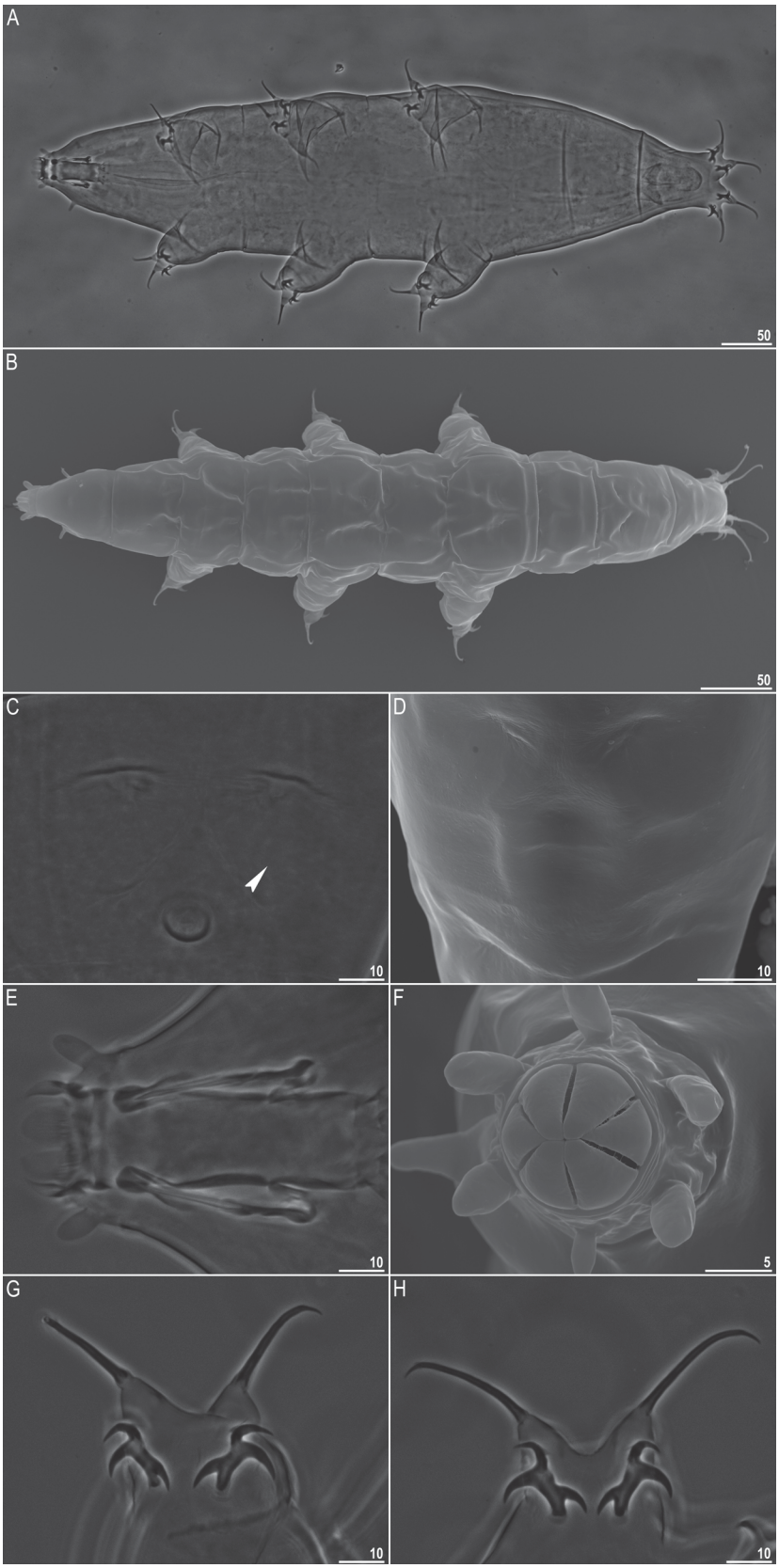

FIGURE 1. *Milnesium alpigenum* Ehrenberg, 1853. **A—**habitus, ventral view (neotype, PCM). **B—**habitus, dorsal view (SEM). **C—**dorsal cuticle, the arrow indicates area with visible pseudopores (neotype, PCM). **D**—dorsal cuticle with the barely visible outline of a pseudoplate (neoparatype, SEM). **E**—buccal apparatus (neotype, PCM). **F**—six peribuccal lamellae with the 4+2 configuration (neoparatype, SEM). **G**—claws III with the cuticular bar below (neoparatype, PCM). **H**—claws IV (neotype, PCM). All scale bars in μm.

- *M. antarcticum* Tumanov, 2006, only recorded from the Antarctic (Smykla *et al.* 2012), by the maximal length of the buccal tube (≤68 µm in *M. alpigenum vs* >68 µm in *M. antarcticum*), a smaller buccal tube standard width (8.6–23.6 µm in *M. alpigenum vs* 25.9–31.8 µm in *M. antarcticum*), and by a statistically lower *pt* of the stylet support insertion point (*61.1–70.3*, on average *64.9* in *M. alpigenum vs 70.0–73.7*, on average *71.5* in *M. antarcticum*; t_{38}=16.708, p<0.001).
- *M. argentinum* Roszkowska, Ostrowska & Kaczmarek, 2015, reported from Argentina, by the appearance of cuticle (faint pseudopores visible only with a high quality PCM on the caudal part of the dorsal cuticle in *M. alpigenum vs* well-visible pseudopores in *M. argentinum* on the entire dorsum with a standard PCM), and by the lower *pt* of the primary branches IV (*46.9–63.2* in *M. alpigenum vs 28.4–36.4* in *M. argentinum*).
- *M. asiaticum* Tumanov, 2006, recorded from Kirghizstan (type locality), China (Beasley & Miller 2007), Estonia (Zawierucha *et al.* 2014) and the Svalbard archipelago (Kaczmarek *et al.* 2012), by a statistically lower *pt* of primary branches III (*39.7–51.7*, on average *45.8* in *M. alpigenum vs 51.5–58.3*, on average *55.3* in *M. asiaticum*; t_{36}=15.385, p<0.001) and by a lower *pt* of primary branches IV (*46.9–63.2* in *M. alpigenum vs 63.9–76.0* in *M. asiaticum*).
- *M. barbadosense* Meyer & Hinton, 2012, only reported from the type locality in Barbados, by a lower *pt* of the stylet support insertion point (*61.1–70.3* in *M. alpigenum vs 71.6–82.1* in *M. barbadosense*) and by a higher *pt* of the primary branches IV (*46.9–63.2* in *M. alpigenum vs 28.4–42.2* in *M. barbadosense*).
- *M. beatae* Roszkowska, Ostrowska & Kaczmarek, 2015, only reported from the type locality in Argentina, by the appearance of cuticle (faint pseudopores visible only with a high quality PCM on the caudal part of the dorsal cuticle in *M. alpigenum vs* well-visible pseudopores in *M. beatae* on the entire dorsum with a standard PCM), by a more slender buccal tube (standard width/length ratio 21–38% in *M. alpigenum vs* standard width/length ratio 58–66% in *M. beatae*).
- *M. bohleberi* Bartels, Nelson, Kaczmarek & Michalczyk, 2014, recorded from North Carolina and Tennessee, USA, by a more slender buccal tube (standard width/length ratio 21–38% in *M. alpigenum vs* standard width/length ratio 54–64% in *M. bohleberi*).
- *M. brachyungue* Binda & Pilato, 1990, recorded from the type locality in Chile and south Argentina (Roszkowska *et al.* 2016), by a higher *pt* of primary branches of all claws (*36.0–63.2* in *M. alpigenum vs 22.9–33.1* in *M. brachyungue*).
- *M. burgessi* Schlabach, Donaldson, Hobelman, Miller & Lowman, 2018, reported from Kansas, USA, by a higher *pt* of the buccal tube standard width (*21.0–38.4* in *M. alpigenum vs 52.9–68.5* in *M. burgessi*) and by a lower *pt* of primary branches IV (*46.9–63.2* in *M. alpigenum vs 66.6–96.2.* in *M. burgessi*).
- *M. dornensis* Ciobanu, Roszkowska & Kaczmarek, 2015, recorded from Romania (type locality), Poland (Kaczmarek *et al*. 2018) and Tunisia (Gąsiorek *et al.* 2017b), by the appearance of cuticle (faint pseudopores visible only with a high quality PCM on the caudal part of the dorsal cuticle in *M. alpigenum vs* well-visible pseudopores in *M. dornensis* on the entire dorsum with a standard PCM) and by a statistically lower *pt* of the buccal tube standard width (*21.0–38.4*, on average *31.4* in *M. alpigenum vs 37.8–51.6*, on average *44.1* in *M. dornensis*; t_{43}=10.473, p<0.001).
- *M. eurystomum* Maucci, 1991, recorded from Greenland (type locality), Chile and Argentina (Maucci 1996), and Mongolia (Kaczmarek & Michalczyk 2006), by a more slender buccal tube (standard width/length ratio 21–38% in *M. alpigenum vs* standard width/length ratio 62–65% in *M. eurystomum*).
- *M. longiungue* Tumanov, 2006, reported from the type locality in the Himalayas (India) and China (Beasley & Miller 2007), by the presence of accessory points on primary branches, a lower *pt* of primary branches III (*39.7–51.7* in *M. alpigenum vs 57.1–73.5* in *M. longiungue*), and by a lower *pt* of primary branches IV (*46.9–63.2* in *M. alpigenum vs 81.8–92.4* in *M. longiungue*).
- *M. minutum* Pilato & Lisi, 2016, only reported from the type locality in Sicily, by a lower *pt* of the buccal tube standard width (*21.0–38.4* in *M. alpigenum vs 38.6–42.4* in *M. minutum*).
- *M. sandrae* Pilato & Lisi, 2016, only reported from the type locality in Hawaii, by a higher *pt* of the stylet support insertion point (*61.1–70.3* in *M. alpigenum vs 58.0–60.5* in *M. sandrae*) and by a lower *pt* of the buccal tube standard width (*21.0–38.4* in *M. alpigenum vs 44.9–48.0* in *M. sandrae*).
- *M. shilohae* Meyer, 2015, only reported from the type locality in Hawaii, by a lower *pt* of the stylet support insertion point (*61.1–70.3* in *M. alpigenum vs 75.5–77.5* in *M. shilohae*), a lower *pt* of the buccal tube standard width (*21.0–38.4* in *M. alpigenum vs 47.1–55.9* in *M. shilohae*), and by a higher *pt* of external spurs I–III (*11.3–17.8* in *M. alpigenum vs 1.9–7.5* in *M. shilohae*).

- *M. swansoni* Young, Chappell, Miller & Lowman, 2016, only reported from the type locality in USA, by a higher number of peribuccal lamellae (six in *M. alpigenum vs* four in *M. swansoni*), a lower *pt* of the buccal tube standard width (*21.0–38.4* in *M. alpigenum vs 39.2–42.2* in *M. swansoni*), a lower *pt* of the posterior buccal tube width (*23.7–39.4* in *M. alpigenum vs 39.9–42.2* in *M. swansoni*), and by a lower *pt* of primary branches I (*36.0–47.9* in *M. alpigenum vs 48.4–53.7* in *M. swansoni*). It should be noted that the number of peribuccal lamellae in *M. swansoni* was identified only with the use of PCM, thus until SEM observations are made, the number of lamellae should be treated as a working hypothesis.
- *M. tumanovi* Pilato, Sabella & Lisi, 2016, only reported from the type locality in Crimea, by a higher *pt* of the stylet support insertion point (*61.1–70.3* in *M. alpigenum* specimens 383–983 µm long *vs* ca. *52.3* in *M. tumanovi* in a specimen 774 µm long) and by a lower *pt* of the buccal tube standard width (*21.0–38.4* in *M. alpigenum* specimens 383–983 µm long *vs ca. 55.1* in *M. tumanovi* in a specimen 774 µm long).
- *M. validum* Pilato, Sabella, D'Urso & Lisi, 2017 only reported from the type locality in the Antarctic, according to the measurements presented in the description of *M. validum* all *pt* ranges overlap, but a comparison of specimens of a similar body length (414–509 µm in *M. alpigenum* and 424–482 µm in *M. validum*) shows that *M. alpigenum* has a shorter buccal tube (33.0–43.0 µm in *M. alpigenum* vs 44.1–55.6 µm in *M. validum*), moreover the two species differ in the shape of secondary branches (slender in *M. alpigenum vs* robust in *M. validum*, compare Fig. 1G–H here and Fig. 6B–D in Pilato *et al.* 2017), and in the shape of spurs (of typical width in *M. alpigenum vs* very thin in *M. validum*).
- *M. inceptum* **sp. nov.** (described below), recorded from Germany, Japan, Switzerland and Bulgaria—please see the section "Delineation of *M. alpigenum* and *M. inceptum* **sp. nov.**" below for a detailed differential diagnosis between these two pseudocryptic species.
- *M. zsalakoae* Meyer & Hinton, 2010, recorded from Arizona and New Mexico (USA), by the presence of accessory points on primary branches and by a lower *pt* of primary branches of all claws (*36.0–63.2* in *M. alpigenum vs 64.4–102.9* in *M. zsalakoae*).

Genotypic differential diagnosis: All sequences obtained for *M. alpigenum* were unique and distinct from the sequences deposited in GenBank. The ranges of the uncorrected p-distances between neotype *M. alpigenum* and sequences of other congeners are as follows:

- **18S rRNA:** 1.1%–3.6% (2.6% on average), with the most similar being *M. inceptum* **sp. nov.** from Europe (MH000383, present study) and the least similar being an undetermined species from Marion Island in the sub-Antarctic (EU266922, Sands *et al.* 2008).
- **28S rRNA:** 4.5%–8.0% (6.1% on average), with the most similar being an undetermined species from the USA (JX888585–7, Adams *et al*, unpublished) and the least similar being *M. tardigradum s.s.* from Poland (KC138809, Zawierucha, unpublished).
- **ITS-2:** 20.4%–23.2% (20.2% on average), with the most similar being *M. tardigradum s.s.* from Germany (JF951049, Michalczyk *et al.* 2012a) and the least similar being *M. tardigradum s.s.* from France (MG923555, Morek *et al.* 2019).
- **COI:** 14.8%–25.8% (17.4% on average), with the most similar being *M. variefidum* from the UK (KT951663, Morek *et al.* 2016a) and an undetermined species from the USA (KX306950, Fox *et al*., unpublished), whereas the least similar being an undetermined species from the Antarctic (KP013598, Velasco-Castrillón *et al*. 2015).

***Milnesium inceptum* sp. nov.**
Figure 2, Tables 4–5

M. tardigradum: Suzuki 2003, Schill *at al*. 2004, Suzuki 2006, Pfannkuchen *et al.* 2007, Schill 2007, Schill & Steinbruck 2007, Hengherr *et al.* 2008a, Hengherr *et al.* 2008b, Jönsson *et al.* 2008, Schill & Fritz 2008, Suzuki 2008, Takahashi *et al*. 2008, Förster *et al.* 2009, Hengherr *et al.* 2009a, Hengherr *et al.* 2009b, Neumann *et al.* 2009, Hengherr *et al.* 2010, Mali *et al.* 2010, Reuner *et al.* 2010a, Reuner *et al.* 2010b, Schökraie *et al.* 2010, Shcherbakov *et al.* 2010, Grohme *et al.* 2011, Förster *et al.* 2011, Schökraie *et al.* 2011, Wełnicz, *et al.* 2011, Beisser, *et al.* 2012, Förster *et al.* 2012, Schökraie *et al.* 2012, Grohme *et al.* 2013, Wang *et al.* 2014, Jönsson *et al.* 2016.

M. cf. *alpigenum* strain Mil.alp_DE.001: Kosztyła *et al*. (2016), Morek *et al*. (2016ab), Stec *et al*. (2016).

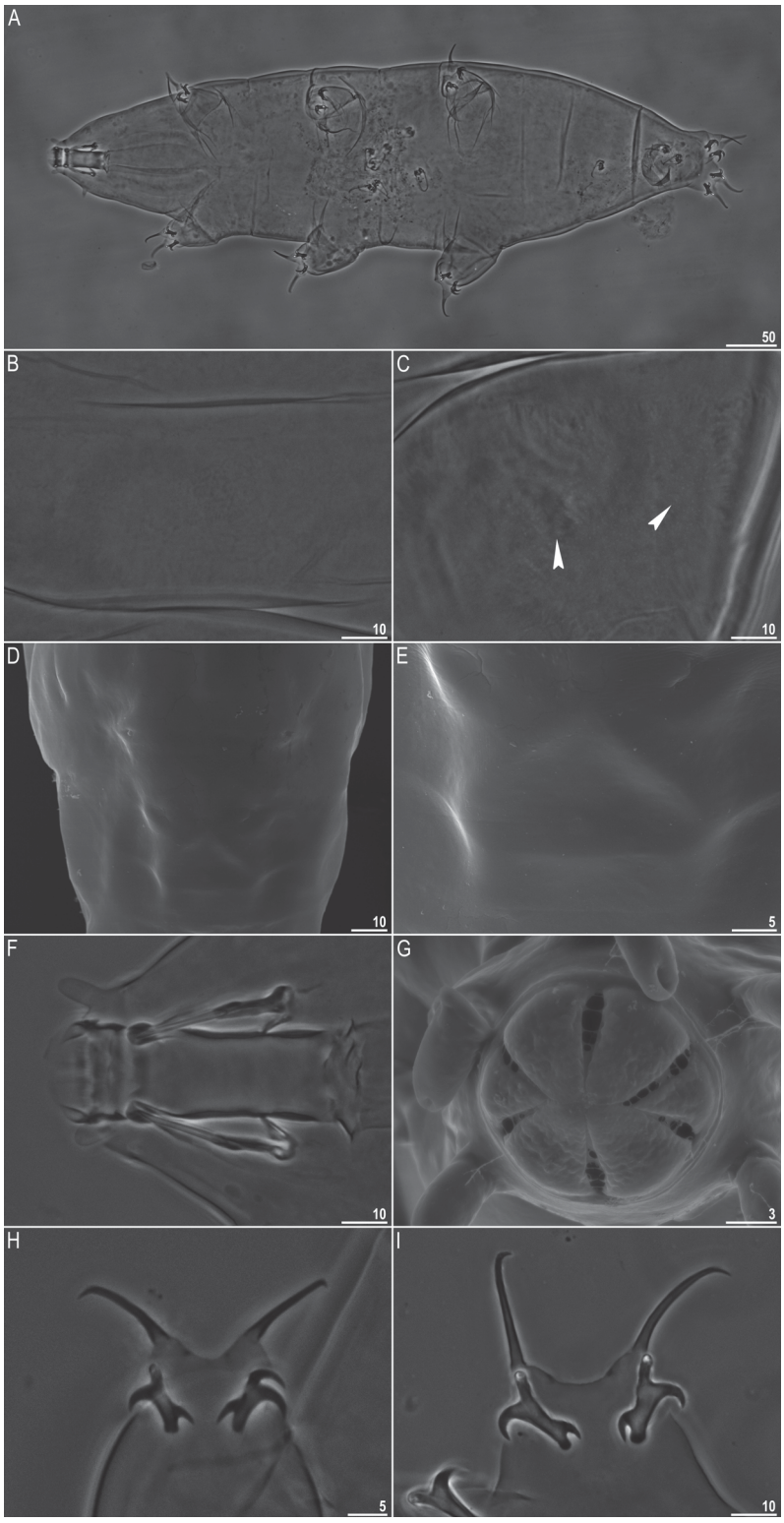

FIGURE 2. *Milnesium inceptum* **sp. nov. A**—habitus, ventral view (holotype, PCM). **B—**dorsal cuticle (holotype, German population, PCM). **C—**dorsal cuticle with faint pseudopores (specimen from Bulgaria, the white arrowhead indicates the area where the pseudopores are more densely arranged, PCM). **D**—dorsal cuticle with the barely visible outline of a pseudoplate (paratype, SEM). **E**—pseudoplate surface (paratype, SEM). **F**—buccal apparatus (holotype, PCM). **G**—six peribuccal lamellae with the 4+2 configuration. **H**—claws I with the cuticular bar below (paratype, PCM). **I**—claws IV (paratype, PCM). All scale bars in μm.

TABLE 4. Measurements (in µm) and the *pt* values of selected morphological structures of 30 specimens (holotype and paratypes) of *Milnesium inceptum* **sp. nov.** from the laboratory culture derived from the type locality, from Tübingen, Germany, 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		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	30	326 – 848	*1136 – 1588*	583	*1381*	164	*122*	743	*1561*
Peribuccal papillae length	13	4.5 – 11.1	*17.2 – 21.6*	8.6	*19.1*	2.0	*1.4*	10.3	*21.6*
Lateral papillae length	27	3.7 – 10.6	*13.4 – 19.8*	6.8	*15.5*	1.9	*1.6*	8.0	*16.8*
Buccal tube									
Length	30	25.8 – 53.5	–	41.8	–	9.7	–	47.6	–
Stylet support insertion point	30	17.1 – 34.3	*61.4 – 69.4*	27.1	*65.0*	5.9	*1.8*	30.9	*64.9*
Anterior width	30	8.4 – 20.9	*30.8 – 42.9*	15.4	*36.5*	4.4	*3.1*	20.4	*42.9*
Standard width	30	7.1 – 19.6	*23.1 – 37.8*	13.8	*32.5*	4.2	*3.7*	18.0	*37.8*
Posterior width	30	7.4 – 20.1	*25.2 – 39.9*	14.1	*33.0*	4.3	*3.7*	19.0	*39.9*
Standard width/length ratio	30	23% – 38%	–	32%	–	4%	–	38%	–
Posterior/anterior width ratio	30	81% – 99%	–	91%	–	4%	–	93%	–
Claw 1 lengths									
External primary branch	27	11.0 – 22.4	*35.1 – 45.6*	17.7	*40.8*	3.7	*2.9*	20.9	*43.9*
External base + secondary branch	28	8.3 – 17.1	*26.8 – 35.2*	12.6	*30.6*	2.9	*2.4*	15.6	*32.8*
External spur	16	2.1 – 6.6	*7.6 – 14.8*	4.6	*11.0*	1.4	*1.9*	5.0	*10.5*
External branches length ratio	25	67% – 79%	–	74%	–	4%	–	75%	–
Internal primary branch	28	10.0 – 21.4	*33.3 – 43.8*	16.6	*39.6*	3.7	*2.6*	20.7	*43.5*
Internal base + secondary branch	21	8.2 – 17.4	*24.4 – 32.7*	11.9	*29.7*	3.0	*2.6*	14.7	*30.9*
Internal spur	19	3.4 – 8.4	*11.5 – 15.7*	5.8	*13.6*	1.6	*1.4*	6.7	*14.1*
Internal branches length ratio	19	68% – 86%	–	74%	–	4%	–	71%	–
Claw 2 lengths									
External primary branch	29	10.8 – 25.0	*37.5 – 50.0*	18.3	*43.3*	4.2	*3.1*	21.6	*45.4*
External base + secondary branch	21	8.8 – 17.2	*27.4 – 36.7*	13.2	*31.6*	3.1	*2.4*	?	*?*
External spur	15	2.9 – 7.1	*9.2 – 15.2*	4.8	*11.8*	1.2	*1.5*	?	*?*

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TABLE 4. (Continued)

CHARACTER	N	RANGE		MEAN		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
External branches length ratio	20	67% – 81%	–	73%	–	4%	–	?	–
Internal primary branch	28	10.6 – 22.9	*38.8 – 46.3*	17.4	*42.4*	4.0	*2.3*	20.0	*42.0*
Internal base + secondary branch	16	8.3 – 17.7	*27.3 – 33.6*	12.3	*30.7*	3.3	*2.1*	15.3	*32.1*
Internal spur	16	3.6 – 9.1	*12.1 – 18.0*	6.3	*14.8*	1.8	*1.7*	6.2	*13.0*
Internal branches length ratio	14	68% – 77%	–	71%	–	2%	–	77%	–
Claw 3 lengths									
External primary branch	30	11.7 – 24.0	*38.8 – 51.2*	18.4	*44.2*	4.1	*2.8*	21.7	*45.6*
External base + secondary branch	26	8.5 – 18.2	*27.6 – 36.3*	13.5	*32.0*	3.2	*2.2*	15.7	*33.0*
External spur	15	3.3 – 7.9	*8.9 – 14.8*	5.0	*12.0*	1.5	*1.9*	?	*?*
External branches length ratio	26	67% – 80%	–	72%	–	3%	–	72%	–
Internal primary branch	28	10.6 – 22.2	*37.3 – 47.7*	17.4	*42.5*	4.0	*2.6*	20.0	*42.0*
Internal base + secondary branch	11	8.3 – 14.9	*25.9 – 33.8*	10.6	*30.4*	2.4	*2.3*	?	*?*
Internal spur	14	3.7 – 9.0	*13.1 – 17.8*	5.9	*15.1*	1.7	*1.5*	6.5	*13.7*
Internal branches length ratio	11	62% – 78%	–	70%	–	5%	–	?	–
Claw 4 lengths									
Anterior primary branch	29	13.3 – 27.9	*45.9 – 59.5*	22.2	*52.7*	4.9	*3.3*	24.7	*51.9*
Anterior base + secondary branch	29	8.8 – 19.9	*32.8 – 40.2*	15.2	*35.7*	3.7	*2.0*	17.6	*37.0*
Anterior spur	16	2.6 – 8.5	*7.4 – 18.1*	5.1	*12.0*	1.7	*2.7*	?	*?*
Anterior branches length ratio	28	61% – 76%	–	68%	–	4%	–	71%	–
Posterior primary branch	27	12.1 – 28.9	*44.1 – 58.4*	20.9	*49.8*	5.0	*3.2*	?	*?*
Posterior base + secondary branch	26	8.5 – 20.1	*29.7 – 40.3*	14.3	*34.4*	3.6	*2.6*	17.4	*36.6*
Posterior spur	25	3.0 – 9.8	*11.6 – 19.2*	6.7	*15.9*	2.0	*1.8*	7.0	*14.7*
Posterior branches length ratio	23	61% – 81%	–	68%	–	4%	–	?	–

Material examined: Type series consisting of 96 specimens (population DE.001) and additional 93 specimens (15 from JP.010 population, 9 from population CH.002, and 69 from BG.058 population). See Table 1 and "Type repositories" below for details.

Integrative description. Females: Body yellowish. Eyes present in live specimens, dissolved after fixation in Hoyer's medium in 50% of specimens (remained visible in 1/30 = 3% specimens of the German type series, 15/15 = 100% of the Japanese series, 9/9 specimens = 100% of the Swiss series, and in 14/23 specimens = 61% of the Bulgarian series). Cuticle with very small pseudopores (0.46 ±0.06μm, detectable only under a high quality PCM) in the German and the Swiss population and with slightly larger (but still small, 0.62 ±0.06 μm) pseudopores in the Bulgarian and the Japanese population (detectable under a standard PCM). In the German and the Swiss population, the cuticle on the entire body appears smooth under PCM (Fig. 2B), but under SEM a weak outline of a single dorsal pseudoplate is visible in some specimens in the caudal part of the body (Fig. 2D–E). In the Bulgarian and Japanese populations, no pseudoplates were detected either under PCM or in SEM. Six peribuccal papillae present, with the ventral being the smallest. Six triangular peribuccal lamellae of unequal size, with the two lateral being noticeable smaller than the two dorsal and the two ventral, *i.e.* with the 4+2 configuration (identifiable only in SEM; Fig. 2G). Two lateral papillae present. Buccal tube funnel-shaped (Fig. 2F). Primary branches with typically developed and clearly visible accessory points. All secondary branches with three points, *i.e.* with the [3-3]-[3-3] CC (Fig. 2H and I). Spurs on secondary branches of moderate length. Cuticular bars under claws I–III present in the majority of examined specimens (23/29 specimens = 79% in the German type population, 15/15 specimens = 100% in the Japanese population, 7/9 specimens = 78% in the Swiss population, and in 11/16 specimens = 69% in the Bulgarian population; Fig. 2H).

Males: No males were found in German, Swiss, or Bulgarian populations and culturing of isolated virgin females confirmed that the type population is parthenogenetic. However, males were found to appear spontaneously in an otherwise parthenogenetic culture of the Japanese strain (Suzuki 2008). This suggests that the species is facultatively parthenogenetic with males appearing only occasionally.

Juveniles: Morphologically identical to adults, except for the lack of the cuticular pseudopores.

Hatchlings: Morphologically identical to adults, except for the lack of cuticular bars under claws I–III in the majority of examined hatchlings (14/15 specimens = 93%), and the absence of cuticular pseudopores.

Ontogenetic variability: No developmental variability in the CC. Pseudopores visible only in adults. Cuticular bars under claws I–III mostly absent in hatchlings but usually present in juveniles and adults.

Eggs: Oval, yellow, smooth and laid in the exuviae, up to 18 in a single clutch were found in laboratory culture.

DNA markers: All sequences were of a very good quality. The 18S rRNA and 28S rRNA, sequenced only in the German type population, were 1070 bp (MH000383) and 817 bp (MH000385) long, respectively. In ITS-2, two haplotypes were found: H1 was shared by the German, the Japanese and the Swiss population (528 bp long, MH000386), whereas H2 was found in the Bulgarian population 528 bp, MH000387). The p-distance between the two ITS-2 haplotypes was 0.8%. The COI marker exhibited three haplotypes: H1 shared by the German and the Swiss population (658 bp, KU513422), H2 in the Japanese population (580 bp, MK628723), and H3 in the Bulgarian population (647 bp, MH000381). The p-distances between the COI haplotypes were as follows: 0.5% (H1 *vs* H2 and H1 *vs* H3), and 0.3% (H2 *vs* H3). Sequences with marked differences are provided in Appendix 1.

Morphology and genetic markers: The sample size of four populations does not allow us to formulate strong conclusions on the relationship between genetic markers and animal morphology. Nevertheless, it should be noted that populations with COI H1 (DE.001 and CH.002) exhibited statistically smaller pseudopores than populations with COI H2 (JP.010) and H3 (BG.058): 0.46 ±0.06 μm (DE.001) vs 0.62 ±0.06 μm (BG.058), t_{28}=7.450, p<0.001. No associations were observed between ITS-2 haplotypes and phenotypic taxonomic traits.

Type locality: 48°33'42"N, 09°03'48"E; 377 m asl: Germany, Tübingen, Bebenhausen; forest; moss on soil.

Etymology: The name of the new species originates from the Latin "*inceptor*", meaning "an initiator", or "a pioneer", as this species was among the very first tardigrade laboratory models. *Milnesium inceptum* **sp. nov.** has been used in a number of studies, including first studies on molecular mechanisms underlying cryptobiosis.

Type repositories: The type series consist of the holotype (slide DE.001.34) and 96 paratypes representing hatchlings, juveniles and adult females (slides DE.001.01–33). The holotype (DE.001.34) with 14 paratypes (DE.001.04–07; 32–33) are preserved at the Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland; 18 paratypes (DE.001.08–13) are deposited in Department of

Animal Taxonomy and Ecology, Adam Mickiewicz University, Poznań, Umultowska 89, 61-614 Poznań, Poland; 18 paratypes are deposited in the Department of Zoology, Institute of Biomaterials and Biomolecular Systems, Stuttgart University, Germany (DE.001.14–19), 18 paratypes (DE.001.20–25) are stored in Marine Biology & Ecology Research Centre, Plymouth University, Drakes Circus, Plymouth, PL4 8AA, United Kingdom, , one paratype (DE.001.34) is deposited in Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom, 18 paratypes (DE.001.26–31) are deposited in Department of Ecology and Environmental Conservation, Faculty of Biology, University of Plovdiv, Tzar Assen 24, BG-4000 Plovdiv, Bulgaria and the remaining 9 (DE.001.01–03) are deposited in the collection of Binda & Pilato, Museum of the Department of Biological, Geological and Environmental Sciences, Section of Animal Biology "Marcello La Greca", University of Catania, Italy.

TABLE 5. Measurements (in µm) and the *pt* values of selected morphological structures of 75 specimens of *Milnesium inceptum* **sp. nov.** from the type locality in Tübingen (Germany) and the additional localities from Hiyoshi (Japan), Zürich (Switzerland), and Kazanlak Valley (Bulgaria) 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	75	326–998	*1136–1841*	628.4	*1493*	181	*164*
Peribuccal papillae length	48	4.4–13.1	*14.9–24.0*	8.2	*19.0*	2.1	*1.7*
Lateral papillae length	69	3.4–11.2	*11.8–21.6*	7.1	*16.5*	2.1	*2.1*
Buccal tube							
Length	75	25.8–56.2	–	41.6		9.1	
Stylet support insertion point	73	17.1–36.4	*59.0–71.6*	26.9	*65.5*	5.4	*2.4*
Anterior width	75	8.4–23.8	*28.5–45.2*	15.5	*36.8*	4.4	*4.0*
Standard width	73	7.1–21.2	*23.1–41.7*	13.5	*32.2*	4.2	*4.5*
Posterior width	75	7.4–22.1	*25.2–42.7*	13.8	*32.6*	4.1	*4.4*
Standard width/length ratio	73	23%–42%	–	32%		5%	
Posterior/anterior width ratio	75	74%–101%	–	87%		7%	
Claw 1 lengths							
External primary branch	65	11.0–26.1	*34.2–51.4*	18.5	*43.2*	4.2	*3.7*
External base + secondary branch	61	8.3–19.7	*26.8–38.0*	12.8	*32.0*	3.1	*2.8*
External spur	31	2.1–6.8	*7.6–14.8*	4.4	*11.3*	1.3	*1.8*
External branches length ratio	53	67%–81%	–	73%		4%	
Internal primary branch	70	10.0–24.8	*32.5–50.2*	17.5	*42.0*	4.2	*3.5*
Internal base + secondary branch	57	8.2–21.2	*24.4–38.8*	12.7	*31.5*	3.5	*3.1*
Internal spur	43	3.0–8.5	*10.8–16.8*	5.7	*13.8*	1.7	*1.5*
Internal branches length ratio	54	68%–88%	–	73%		5%	
Claw 2 lengths							
External primary branch	72	10.8–28.4	*37.5–56.8*	19.6	*46.7*	4.8	*4.5*
External base + secondary branch	59	8.8–22.8	*27.4–41.8*	13.7	*33.5*	3.4	*2.9*
External spur	36	2.9–8.2	*9.2–16.6*	5.2	*12.9*	1.6	*2.0*
External branches length ratio	57	64%–83%	–	70%		4%	
Internal primary branch	70	10.6–27.6	*38.8–55.0*	18.8	*45.5*	4.8	*4.3*
Internal base + secondary branch	52	8.3–21.0	*27.3–40.7*	13.3	*32.9*	3.7	*2.9*
Internal spur	42	3.2–20.1	*11.8–36.8*	6.8	*16.4*	2.8	*4.0*
Internal branches length ratio	49	59%–81%	–	70%		5%	

.....continued on the next page

TABLE 5. (Continued)

CHARACTER	N	RANGE		MEAN		SD	
		µm	*pt*	µm	*pt*	µm	*pt*
Claw 3 lengths							
External primary branch	69	11.7–27.7	*38.8–54.5*	19.6	*46.9*	4.7	*4.3*
External base + secondary branch	64	8.5–21.9	*27.6–42.4*	13.9	*33.9*	3.3	*3.0*
External spur	37	3.3–8.7	*8.9–16.8*	5.1	*12.4*	1.6	*1.8*
External branches length ratio	59	64%–90%	–	71%		6%	
Internal primary branch	70	10.6–27.2	*37.3–55.7*	18.7	*45.5*	4.6	*4.4*
Internal base + secondary branch	42	8.3–20.5	*25.9–39.5*	13.0	*33.2*	3.8	*2.9*
Internal spur	44	3.3–11.0	*11.7–21.4*	6.6	*16.2*	2.1	*2.4*
Internal branches length ratio	40	62%–86%	–	71%		6%	
Claw 4 lengths							
Anterior primary branch	67	13.3–34.1	*44.2–65.7*	23.1	*55.5*	5.4	*4.7*
Anterior base + secondary branch	63	8.8–24.2	*31.7–46.2*	15.5	*37.5*	4.0	*3.2*
Anterior spur	45	2.6–11.1	*7.4–22.8*	6.0	*14.1*	2.3	*3.9*
Anterior branches length ratio	59	61%–76%	–	67%		4%	
Posterior primary branch	64	12.1–32.7	*44.1–65.7*	22.5	*54.3*	5.5	*5.8*
Posterior base + secondary branch	59	8.5–22.8	*29.7–43.8*	15.2	*36.7*	4.0	*3.6*
Posterior spur	48	3.0–10.7	*11.6–20.6*	6.9	*16.4*	1.9	*2.4*
Posterior branches length ratio	55	60%–81%	–	66%		4%	

Phenotypic differential diagnosis. *Milnesium inceptum* **sp. nov.** has the [3-3]-[3-3] CC and "smooth" cuticle (*i.e.* cuticle smooth in SEM and with minute pseudopores, but with no sculpturing, such as reticulation, on cuticle surface), which places it in the largest group of *Milnesium* species that share these characteristics (19 species). Nevertheless, *M. inceptum* **sp. nov.** differs from:

- *M. alpigenum* Ehrenberg, 1853 only reported from the type locality in Italy—please see the section "Delineation of *M. alpigenum* and *M. inceptum* **sp. nov.**" below for a detailed differential diagnosis between these two pseudocryptic species.
- *M. antarcticum* Tumanov, 2006, only reported from the Antarctic (Smykla *et al.* 2012), by the maximal length of the buccal tube ($\leq$57.0 µm in the new species *vs* >67.0 µm in *M. antarcticum*), by a lower buccal tube standard width (7.1–21.2 µm in the new species *vs* 25.9–31.8 µm in *M. antarcticum*), and by a statistically lower *pt* of the stylet support insertion point (*59.0–71.6*, on average *65.5* in the new species *vs 70.0–73.7*, on average *71.5* in *M. antarcticum*; t_{80}=20.590, p<0.001).
- *M. argentinum* Roszkowska, Ostrowska & Kaczmarek, 2015, recorded from Argentina, by the appearance of cuticle (faint pseudopores visible only with a high quality PCM on the caudal part of the dorsal cuticle in the new species *vs* well-visible pseudopores in *M. argentinum* on the entire dorsum with a standard PCM), the maximal length of the buccal tube (up to 57 µm in the new species *vs* up to 74 µm in *M. argentinum*), and by a lower *pt* of the primary branches IV (*44.1–65.7* in the new species *vs 28.4–36.4* in *M. argentinum*).
- *M. asiaticum* Tumanov, 2006, recorded from Kirghizstan (type locality), China (Beasley & Miller 2007), Estonia (Zawierucha *et al.* 2014), and the Svalbard archipelago (Kaczmarek *et al.* 2012), by a statistically lower *pt* of primary branches IV (*44.1–65.7*, on average *54.8* in the new species *vs 63.9–76.0*, on average *69.7* in *M. asiaticum*; t_{54}=26.040, p<0.001).
- *M. barbadosense* Meyer & Hinton, 2012, only reported from the type locality in Barbados, by a lower *pt* of the stylet support insertion point (*59.0–71.6* in the new species *vs 71.6–82.1* in *M. barbadosense*), and by a higher *pt* of the primary branches IV (*44.1–65.7* in the new species *vs 28.4–42.2* in *M. barbadosense*).
- *M. beatae* Roszkowska, Ostrowska & Kaczmarek, 2015, only reported from the type locality in Argentina, by the appearance of cuticle (faint pseudopores visible only with a high quality PCM on the caudal part of the

dorsal cuticle in the new species *vs* well-visible pseudopores in *M. argentinum* on the entire dorsum with a standard PCM), and by more elongated buccal tube (standard width/length ratio 23–42% in the new species *vs* standard width/length ratio 58–66% in *M. beatae*)

- *M. bohleberi* Bartels, Nelson, Kaczmarek & Michalczyk, 2014, recorded from North Carolina and Tennessee, USA, by the more slender buccal tube (standard width/length ratio 23–42% in the new species *vs* standard width/length ratio 54–64% in *M. bohleberi*).
- *M. brachyungue* Binda & Pilato, 1990, reported from the type locality in Chile and south Argentina (Roszkowska *et al.* 2016), by a higher *pt* of primary branches of claws I–III (*32.5–56.8* in the new species *vs 22.9–27.1* in *M. brachyungue*) and by the *pt* of primary branches IV (*44.1–65.7* in the new species *vs 33.1* in *M. brachyungue*).
- *M. burgessi* Schlabach, Donaldson, Hobelman, Miller & Lowman, 2018, recorded from Kansas, USA, by a higher *pt* of the buccal tube standard width (*23.1–41.7* in the new species *vs 52.9–68.5* in *M. burgessi*) and by the lower *pt* of primary branches IV (*44.1–65.7* in the new species *vs 66.6–96.2.* in *M. burgessi*).
- *M. dornensis* Ciobanu, Roszkowska & Kaczmarek, 2015, recorded from Romania (type locality), Poland (Kaczmarek *et al.* 2018) and Tunisia (Gąsiorek *et al.* 2017b), by the appearance of cuticle (faint pseudopores visible only with a high quality PCM on the caudal part of the dorsal cuticle in the new species *vs* well-visible pseudopores in *M. dornensis* on the entire dorsum with a standard PCM), and by a statistically lower *pt* of buccal tube standard width (*23.1–41.7*, on average *32.2* in the new species *vs 37.8–51.6,* on average *44.1* in *M. dornensis*; t_{22}=10.686, p<0.001).
- *M. eurystomum* Maucci, 1991, recorded from Greenland (type locality), Argentina and Chile (Maucci 1996), and Mongolia (Kaczmarek & Michalczyk 2006), by a more slender buccal tube (standard width/length ratio 23–42% in the new species *vs* standard width/length ratio 62–65% in *M. eurystomum*).
- *M. longiungue* Tumanov, 2006, reported from the Himalayas (India, type locality) and China (Beasley & Miller 2007), by the presence of accessory points on primary branches, a lower *pt* of primary branches III (*37.3–55.7* in the new species *vs 57.1–73.5* in *M. longiungue*), and by the lower *pt* of primary branches IV (*44.1–65.7* in the new species *vs 81.8–92.4* in *M. longiungue*).
- *M. minutum* Pilato & Lisi, 2016, only reported from the type locality in Sicily, by a statistically lower *pt* of the buccal tube standard width (*23.1–41.*7, on average *32.2* in the new species *vs 38.6–42.4*, on average *41.1* in *M. minutum*; t_3=7.990, p=0.002).
- *M. sandrae* Pilato & Lisi, 2016, only reported from the type locality in Hawaii, by a higher *pt* of the stylet support insertion point (*59.0–71.6*, on average *65.5* in the new species *vs 58.0–60.5*, on average *58.9* in *M. sandrae;* t_{22}=8.506, p<0.001) and by a lower *pt* of the buccal tube standard width (*23.1–41.*7 in the new species *vs 44.9–48.0* in *M. sandrae*).
- *M. shilohae* Meyer, 2015, only reported from the type locality in Hawaii, by a lower *pt* of the stylet support insertion point (*59.0–71.6* in the new *species vs 75.5–77.5* in *M. shilohae*) and by a lower *pt* of the buccal tube standard width (*23.1–41.7* in the new species *vs 47.1–55.9* in *M. shilohae*).
- *M. swansoni* Young, Chappell, Miller & Lowman, 2016, only reported from the type locality in the USA, by a higher number of peribuccal lamellae (six in the new species *vs* four in *M. swansoni*; but note that the number of peribuccal lamellae in *M. swansoni* was determined only with a PCM) and by a lower *pt* of the buccal tube standard width (*23.1–41.*7, on average *32.2* in the new species *vs 39.2–42.2*, on average *40.3* in *M. swansoni*; t_{12}=10.325, p<0.001).
- *M. tumanovi* Pilato, Sabella & Lisi, 2016, only reported from the type locality in Crimea, by a higher *pt* of the stylet support insertion point (*59.0–71.6* in the new species specimens being 326–998 µm long *vs 52.3* in *M. tumanovi* in a specimen 774 µm long) and by a lower *pt* of the buccal tube standard width (*23.1–41.7* in the new species in specimens 326–998 µm long *vs 55.1* in *M. tumanovi* in a specimen 774 µm long).
- *M. validum* Pilato, Sabella, D'Urso & Lisi, 2017, only reported from the type locality in the Antarctic; according to measurements presented in the description of *M. validum* all *pt* ranges overlap, but a comparison between specimens of similar body length (393–513 µm in the new species and 424–482 µm in *M. validum*) shows that *M. inceptum* **sp. nov.** has a shorter buccal tube (27.1–39.0 µm in the new species *vs* 44.1–55.6 in *M. validum*), moreover the two species differ in the shape of the secondary branches (typical in the new species *vs* robust in *M. validum*, compare Fig. 2H–I here and Fig. 6B–D in Pilato *et al.* 2017), and in the shape of spurs (moderate length and of normal width in the new species *vs* long and very thin in *M. validum*).

- *M. zsalakoae* Meyer & Hinton, 2010, recorded from Arizona and New Mexico (USA), by the presence of accessory points on primary branches, by a lower *pt* of primary branches I–III (*32.5–56.8* in the new species *vs 64.4–88.6* in *M. zsalakoae*) and by a lower *pt* of primary branches IV (*44.1–65.7* in the new species *vs 94.8–102.9* in *M. zsalakoae*).

Genotypic differential diagnosis: Four sequences deposited in GenBank prior to this publication, labelled as "*M. tardigradum*", in fact represent *M. inceptum*: two ITS-2 (GQ403681–2) and two COI (EU244603–4) (all by Schill, unpublished). The GQ403683 and EU244604 sequences originated from Germany and represent the same laboratory strain that was utilised herein to describe the new species. The sequences GQ403682 and EU244603 originated from Japan and represent the Japanese strain, also used in the present study.

The ranges of uncorrected p-distances between the new species and sequences of other congeners are as follows:

- **18S rRNA:** 1.1%–3.9% (2.9% on average), with the most similar being *M. alpigenum*, (MG996146, present study) and the least similar being an undetermined species from the USA (GQ925696, Chen *et al.* unpublished) as well as an undetermined species from South Georgia in the sub-Antarctic (EU266922, Sands *et al.* 2008).
- **28S rRNA:** 0.4%–8.8% (6.0% on average), with the most similar being an undetermined species from the USA (AY210826, Mallatt *et al*. unpublished) and another undetermined species also from the USA (JX888540–1, Adams *et al.* unpublished) and the least similar being an undetermined species from Spain (FJ435779–80, Guil & Giribet 2012).
- **ITS-2:** 19.6%–22.8% (20.3% on average), with the most similar being *M. tardigradum s.s.* from Hungary and Poland (MG923553, Morek *et al.* 2019) and the least similar being *M. tardigradum s.s.* from France (MG923555, Morek *et al.* 2019).
- **COI:** 17.8%–25.8% (19.7% on average), with the most similar being *M. dornensis* from Romania **(**MG923566, Morek *et al.* 2019) and an undetermined species from the USA (KX306950, Fox *et al*., unpublished) whereas the least similar being two undetermined species from the Antarctic (KP013601 and KP013598, Velasco-Castrillón *et al*. 2015).

Delineation of *M. alpigenum* and *M. inceptum* sp. nov.

The two species are genetically distinct but morphologically very similar, although not identical. Therefore, they could be classified as pseudocryptic species, *i.e.* species that can be differentiated morphologically but only with a detailed analysis; in this case—with the use of statistical testing of morphometric traits measured in a number of specimens, since the classical identification based on qualitative traits and non-overlapping morphometric ranges is not sufficient to tell the species apart.

The PCA analysis indicated three components comprising 59.3% of the total variance in the *pt* ratios. The three factors were as follows: PC1: the *pt* of the primary and secondary branches of all claws (38.2%), PC2: the *pt* of external and posterior spurs (11.3%), and PC3: the *pt* of buccal tube widths and stylet support insertion point (9.8%). The relationships between the principal components are shown in Fig. 3. The two species did not differ in PC1 and PC3 but in contrast, they differed in PC2, thus comparisons of the first three principal components did not result in congruent conclusions. Specifically, when PC1 and PC3 were compared (Fig. 3B), ranges for the two species largely overlapped. In the PC1 *vs* PC2 comparison (Fig. 3A), the overlap between species was smaller. Finally, when PC2 and PC3 were compared, the ranges barely overlapped (Fig. 3C). Thus, we compared only the traits constituting PC2 (*i.e.* the *pt* values of claw spurs) with a series of *t*-tests and adjusted α-levels. Student's *t*-tests revealed significant differences in three of the eight compared traits (mean values, ±SD, and [ranges] for *M. alpigenum vs M. inceptum* **sp. nov.**):

- spur on external claw I: *13.3 ±1.5* [*11.3–17.8*] *vs 11.3 ±1.3* [*7.6–14.8*], t_{46}=4.002, p<0.001;
- spur on external claw II: *15.3 ±1.4* [*12.7–17.5*] *vs 12.9 ±2.0* [*9.2–16.6*], t_{54}=4.630, p<0.001;
- spur on external claw III: *15.2 ±1.4* [*12.4–17.8*] *vs 12.4 ±1.8* [*8.9–16.8*], t_{48}=5.758, p<0.001.

In other words, the analysis showed that *M. alpigenum* has statistically longer (relatively to the buccal tube length) external spurs than *M. inceptum* **sp. nov.** (compare also Fig. 1G–H and 2H–I).

In contrast to subtle morphometric differences, the two species exhibit considerable genetic distances in all four analysed markers. Specifically, they differ by 1.0% in 18S rRNA, 5.2% in 28S rRNA, 21.6% in ITS-2, and by 18.1% in COI. Most importantly, however, the two species are not immediately related to each other (see Fig. 4 for the positions of both species on the *Milnesium* phylogenetic tree), which is the strongest evidence that the two species represent separate phylogenetic lineages.

To conclude, differences both in phenotypic and genetic traits unequivocally show that *M. inceptum* **sp. nov.** is a *bona species*. Nevertheless, an extreme care must be taken when identifying these species using solely phenotypic data.

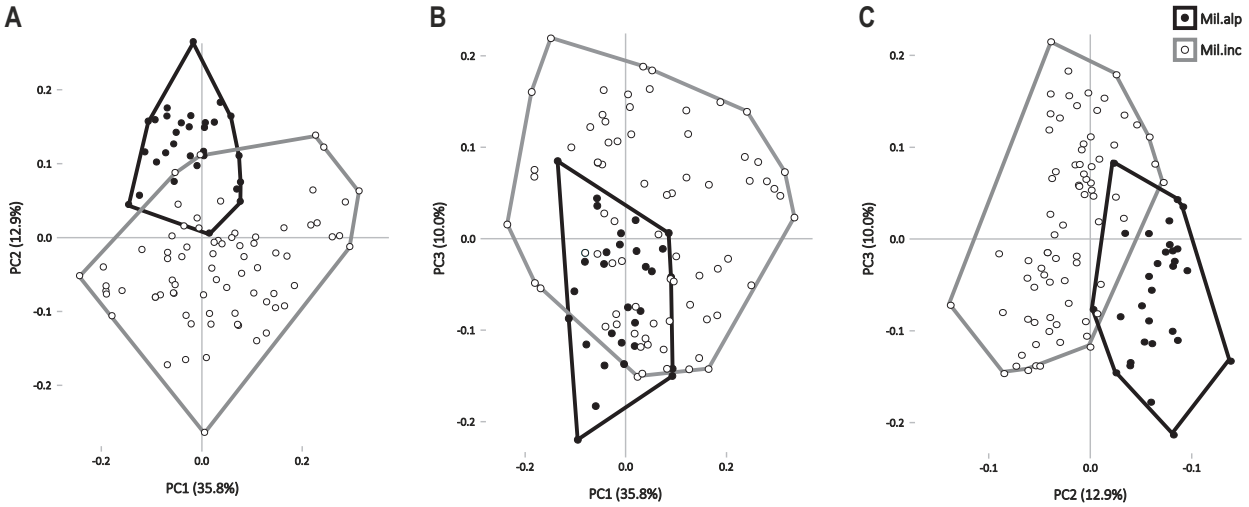

FIGURE 3. Graphs illustrating the relationships between the first three principal components revealed by the PCA for the single population of *M. alpigenum* Ehrenberg, 1853 and the three pooled populations of *M. inceptum* **sp. nov.**

Phylogenetic relationships

Phylogenetic trees obtained with BI and ML methods did not exhibit the same topologies. Importantly, however, majority of nodes were weakly supported in the ML tree, thus we considered it uninformative and we focused on the BI tree, which had good statistical support (Fig. 4). The first clade encompasses *M. tardigradum s.s.* and a sister clade composed of *M. berladnicorum, M. dornensis* and *M. variefidum*. This group is in a sister relationship to the clade comprising *M. inceptum* **sp. nov.** and an undescribed species ("*Milnesium hisatsinomorum*"; KX306950; Fox *et al*., unpublished). The abovementioned taxa are in polytomy with *M. alpigenum* and an unknown species (described as *"M.* cf. *tardigradum"*; JX683822–5; Vicente *et al*. 2013). The following three undescribed species: EF632553 (Sands *et al*., unpublished), KP013613 and KJ857002 (Velasco-Castrillón *et al*. 2015) formed a clade that, together with another undescribed species KJ857001 (Velasco-Castrillón *et al*. 2015), were in polytomy with the abovementioned species. Finally, an undescribed species represented by two sequences (KP013598 and KP013601; Velasco-Castrillón *et al*. 2015) was a sister group to all remaining *Milnesium* species. Thus, our analysis indicated that *M. inceptum* **sp. nov.** and *M. alpigenum* are not sister species, even though the relationships of *M. alpigenum* with other congeners were not fully resolved.

Discussion

Nearly a century since the synonymisation of *M. alpigenum* with *M. tardigradum* by Marcus (1928), *M. alpigenum* is now redescribed and reinstated utilising the tools of integrative taxonomy. Moreover, the redescription of *M. alpigenum* allowed us to verify the taxonomic status of the commonly used German "Tübingen" and Japanese

"Hiyoshi H-1" *Milnesium* laboratory strains. Both strains were originally identified as "*M. tardigradum*" (Suzuki 2003; Schill *et al.* 2004), but the redescription of *M. tardigradum* by Michalczyk *et al.* (2012a, b) showed that the original identifications were incorrect (see also Morek *et al*. 2019 for an updated delineation of *M. tardigradum*). In fact, Michalczyk *et al.* (2012a) hypothesised that the "Tübingen" strain might represent a new species. More recently, Morek *et al.* (2016a) tentatively classified the "Tübingen" strain as "*M.* cf. *alpigenum*" since the morphology of the strain fit the limited original description of *M. alpigenum*. However, the present study has shown unambiguously that both laboratory strains indeed represent a distinct taxon, *M. inceptum* **sp. nov.**

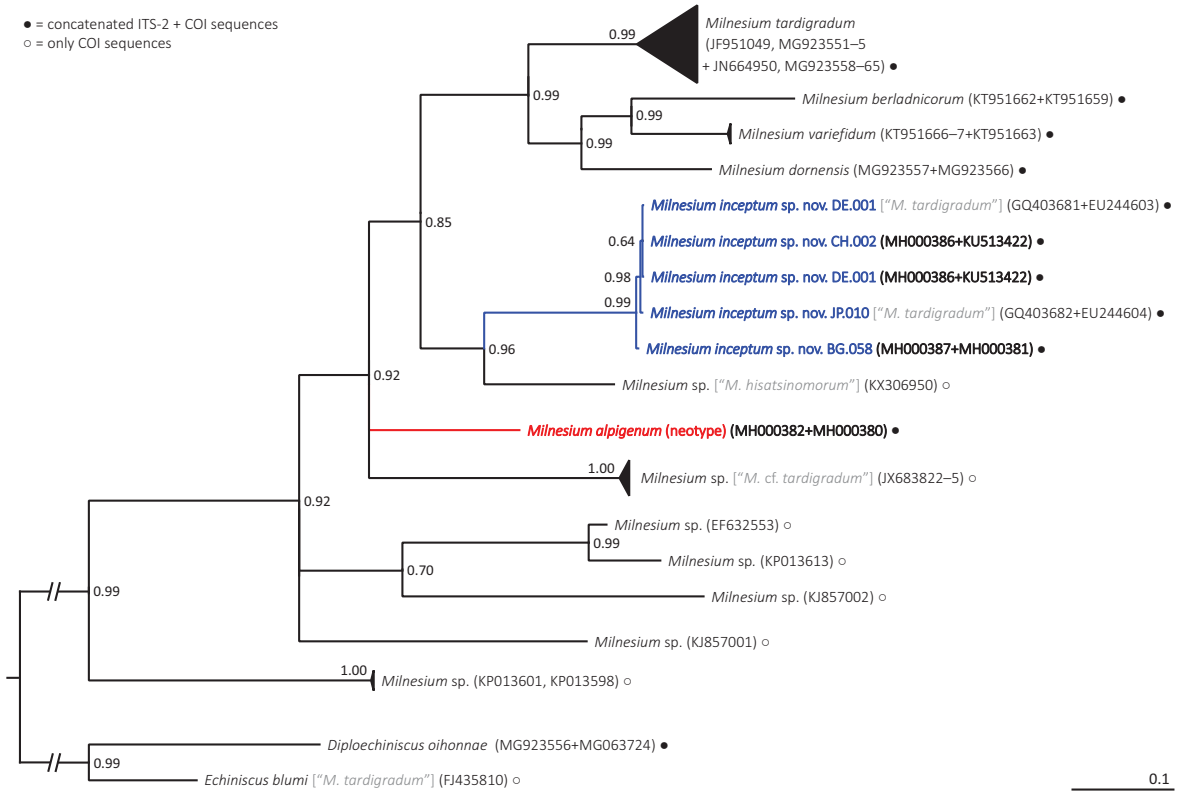

FIGURE 4. The positions of *M. alpigenum* Ehrenberg, 1853 and *M. inceptum* **sp. nov.** on the *Milnesium* phylogenetic tree based on the concatenated data set of ITS-2 and COI sequences. Branch support values are BI posterior probabilities. Species names in square brackets and in grey font are GenBank labels that are incorrect species identifications, uncertain identifications, or invalid names (correct identifications are provided in black font, before the incorrect labels). Filled circles represent both ITS-2 and COI sequences, empty circles indicate COI sequences only. Scale bar shows the number of substitutions per site.

Interestingly, *M. alpigenum* and *M. inceptum* **sp. nov.** are barely distinguishable using standard morphometric traits, even though they are genetically distant (not even immediate kin; see Fig. 4). In fact, the two species would have been unrecognised using classical taxonomic methods alone and only the use of molecular markers allowed a *post hoc* identification of statistical phenotypic differences. Still, the two species differ morphometrically, which means that they are not truly cryptic. As the identified differences are minor, and can only be revealed by the use of statistical tests, *M. alpigenum* and *M. inceptum* **sp. nov.** should be termed "pseudocryptic", meaning that they are species that exhibit minor morphometric differences, which are not detectable using classical diagnostic keys that rely on the presence or absence of qualitative (morphological) traits and non-overlapping ranges of quantitative (morphometric) traits. This example shows explicitly how important it is to base new species descriptions on a proper sample size and on both phenotypic and genetic traits.

In our opinion, species described with a few measured individuals and without associated molecular data pose a serious threat to the development of tardigrade taxonomy. At the time of description (Ehrenberg 1853), *M. alpigenum* was easy to differentiate from the only congener, *M. tardigradum*, because there were no other known *Milnesium* species with the [3-3]-[3-3] CC. However, now there are 22 *Milnesium* species with this CC, with most

of them only differentiated by morphometrics. At the time of Doyère and Ehrenberg, the knowledge and available analytical methods were, of course, limited compared to the present time. However, despite the undeniable increase in knowledge of tardigrade biology and progress in taxonomic methodology, the same process may occur again and again if new species are continually described with classical methods and low numbers of individuals. Even if a new species is obviously new (*e.g.* because it exhibits a unique qualitative trait) but is not integratively described, it may hinder the detection of similar species in the future. When describing a new species with a unique trait, it cannot be predicted as to whether this is the only such species in the world or whether it is the first representative of a complex of cryptic, pseudocryptic, or even only roughly similar taxa. Therefore, if the species is the first representative of a group of similar species, many of those species may go unnoticed for decades, until the nominal species is integratively redescribed. Furthermore, if individuals collected from distant locations that represent morphologically similar species are erroneously identified as the nominal species, the geographic range of the nominal species may be highly overestimated.

The history of confusion with the taxonomic identity of the "Tübingen" *Milnesium* laboratory strain also underlines the vital importance of integrative redescriptions. As mentioned above, the taxonomic identification of the strain was possible only after the redescriptions of *M. tardigradum* and *M. alpigenum*. Importantly, however, it needs to be underlined that if *M. alpigenum* was redescribed classically, the Tübingen *Milnesium* laboratory strain would not have been recognised as a separate species, since statistical differences in a fraction of morphometric traits—even if they were detected—would not have been accepted by the taxonomic community as sufficient evidence to erect a new species. Recently, another popular laboratory strain, originally identified as "*Hypsibius dujardini* (Doyère, 1840)", was shown to represent a new species, *Hypsibius exemplaris* Gąsiorek *et al.*, 2018. The reason for the misidentification of the *Hypsibius* strain was similar to *M. inceptum* **sp. nov.**, *i.e.* the original description of *H. dujardini* was insufficient to identify other morphologically similar species such as *H. exemplaris* (Gąsiorek *et al.* 2018). As there are numerous species requiring integrative redescriptions (many of them being nominotypical taxa for genera and higher taxonomic ranks), we would like to urge taxonomists to prioritise redescribing existing taxa over describing new species (*e.g.* see Meier & Dikow 2004). Only when species with older, limited descriptions are integratively redescribed and new taxa are described integratively, may we hope to reliably estimate tardigrade diversity, species geographic distributions, and identify evolutionary mechanisms underlying these phenomena.

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References

Bartels, P.J., Nelson, D.R., 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.
https://doi.org/10.11646/zootaxa.3826.2.5

Beasley, C.W. & Miller, W.R. (2007) Tardigrada of Xinjiang Uygur Autonomous Region, China. *Journal of Limnology*, 66, 49–55.
https://doi.org/10.4081/jlimnol.2007.s1.49

Beisser, D., Grohme, M.A., Kopka, J., Frohme, M., Schill, R.O., Hengherr, S., Dandekar, T., Klau, G.W., Dittrich, M. & Müller, T. (2012) Integrated pathway modules using time-course metabolic profiles and EST data from *Milnesium tardigradum*. *BMC Systems Biology*, 6 (1), 72.
https://doi.org/10.1186/1752-0509-6-72

Binda, M.G. & Pilato, G. (1990) Tardigradi di Terra del Fuoco e Magallanes. I. *Milnesium brachyungue*, nuova specie di

Tardigrado Milnesiidae. *Animalia*, 17, 105–110.
Bryce, D. (1892) On the Macrotrachelous Callidinae. *Journal of the Quekett Microscopical Club*, 2, 15–23.
Casquet, J., Thebaud, C. & Gillespie, R.G. (2012) Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. *Molecular Ecology Resources*, 12, 136–141. https://doi.org/10.1111/j.1755-0998.-2011.03073.x
Ciobanu, D.A., Zawierucha, K., Moglan, I. & Kaczmarek, Ł. (2014) *Milnesium berladnicorum* sp. n. (Eutardigrada, Apochela, Milnesiidae), a new species of water bear from Romania. *ZooKeys*, 429, 1–11. https://doi.org/10.3897/zookeys.429.7755
Ciobanu, D.A., 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. https://doi.org/10.11646/zootaxa.3941.4.4
Dastych, H. (1988) The Tardigrada of Poland. *Monografie Fauny Polski*, 16, 1–255.
Doyère, M. (1840) Mémoire sur les Tardigrades. *Annales des Sciences Naturalles, Zoologia, Paris, Series 2*, 14, 269–362.
Ehrenberg, C.G. (1853) Diagnoses novarum formarum. *Verhandlungen der Königlich Preussische Akademie der Wissenschaften zu Berlin*, 8, 526–533.
Förster, F., Liang, C., Shkumatov, A., Beisser, D., Engelmann, J.C., Schnölzer, M., Frohme, M., Müller, T., Schill, R.O. & Dandekar, T. (2009) Tardigrade workbench: comparing stress-related proteins, sequence-similar and functional protein clusters as well as RNA elements in tardigrades. *BMC Genomics*, 10, 469. https://doi.org/10.1186/1471-2164-10-469.
Förster, F., Beisser, D., Frohme, M, Schill, R.O. & Dandekar, T. (2011) Bioinformatics identifies tardigrade molecular adaptations including the DNA-j family and first steps towards dynamical modelling. *Journal of Zoological Systematics and Evolutionary Research*, 49 (1), 120–126. https://doi.org/10.1111/j.1439-0469.2010.00609.x
Förster, F., Beisser, D., Grohme, M.A., Liang, C., Mali, B., Siegl, A.M., Engelmann, J.C., Shkumatov, A.V., Schokraie. E., Müller, T., Schnölzer, M., Schill, R.O., Frohme, M. & Dandekar, T. (2012) Transcriptome Analysis in Tardigrade Species Reveals Specific Molecular pathways for stress Adaptations. *Bioinformatics and Biology Insights*, 6, 69–96. https://doi.org/10.4137/BBI.S9150
Gąsiorek, P., Stec, D., Morek, W. & Michalczyk, Ł. (2017a) An integrative redescription of *Echiniscus testudo* (Doyère, 1840), the nominal taxon for the class Heterotardigrada (Ecdysozoa: Panarthropoda: Tardigrada). *Zoologischer Anzeiger*, 270, 107–122. https://doi.org/10.1016/j.jcz.2017.09.006
Gąsiorek, P., Morek, W., Stec, D., Marnissi, J. & Michalczyk, Ł. (2017b) The tardigrade fauna of Tunisia, with an integrative description of *Bryodelphax maculatus* sp. nov. (Heterotardigrada: Echiniscidae). *African Zoology*, 52 (2), 77–89. https://doi.org/10.1080/15627020.2017.1297688
Gąsiorek, P., Stec, D., Morek, W. & Michalczyk, Ł. (2018) An integrative redescription of *Hypsibius dujardini* (Doyère, 1840), the nominal taxon for Hypsibioidea (Tardigrada: Eutardigrada). *Zootaxa*, 4415 (1), 45–75. https://doi.org/10.11646/zootaxa.4415.1.2
Grohme, M.A., Mali, B., Schill, R.O. & Frohme, M. (2011) cDNA representational difference analysis for identifying transcripts regulated under anhydrobiosis in the tardigrade *Milnesium tardigradum. Journal of Zoological Systematics and Evolutionary Research*, 49, (1), 127–132. https://doi.org/10.1111/j.1439-0469.2010.00610.x
Grohme, M.A., Mali, B., Wełnicz, W., Michel, S., Schill, R.O. & Frohme, M. (2013) The Aquaporin Channel Repertoire of the Tardigrade *Milnesium tardigradum*. *Bioinformatics and Biology Insights*, 7, 153–165. https://doi.org/10.4137/BBI.S11497
Guil, N. & Giribet, G. (2012) A comprehensive molecular phylogeny of tardigrades adding genes and taxa to a poorly resolved phylum-level phylogeny. *Cladistics*, 28, 21–49. https://doi.org/0.1111/j.1096-0031.2011.00364.
Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.
Hengherr, S., Brümmer, F. & Schill, R.O. (2008a) Anhydrobiosis in tardigrades and its effects on longevity traits. *Journal of Zoology*, 275, 216–220. https://doi.org/10.1111/j.1469-7998.2008.00427.x
Hengherr, S., Heyer, A.G., Köhler, H.R. & Schill, R.O. (2008b) Trehalose and anhydrobiosis in tardigrades—evidence for divergence in responses to dehydration. *The FEBS Journal*, 275 (2), 281–288. https://doi.org/10.1111/j.1742-4658.2007.06198.x
Hengherr, S., Worland, M.R., Reuner, A., Brümmer, F. & Schill, R.O. (2009a) Freeze tolerance, supercooling points and ice formation: comparative studies on the subzero temperature survival of limno-terrestrial tardigrades. *The Journal of Experimental Biology*, 212, 802–807. https://doi.org/10.1242/jeb.025973
Hengherr, S., Worland, M.R., Reuner, A., Brümmer, F. & Schill, R.O. (2009b) High-temperature tolerance in anhydrobiotic tardigrades is limited by glass transition. *Physiological and Biochemical Zoology*, 82 (6), 749–755. https://doi.org/10.1086/605954

Hengherr, S., Reuner, A., Brümmer, F. & Schill, R.O. (2010) Ice crystallization and freeze tolerance in embryonic stages of the tardigrade *Milnesium tardigradum. Comparative Biochemistry and Physiology, Part A*, 156, 151–155. https://doi.org/10.1016/j.cbpa.2010.01.015

Jönsson, K.I., Rabbow, E., Schill, R.O., Harms-Ringdah, M. & Rettberg, P. (2008) Tardigrades survive exposure to space in low Earth orbit. *Current Biology*, 18 (17), R729–R731. https://doi.org/10.1016/j.cub.2008.06.048

Jönsson, K.I., Schill, R.O., Rabbow, E., Rettberg, P. & Harms-Ringdahl, M. (2016) The fate of the TARDIS offspring: no intergenerational effects of space exposure. *Zoological Journal of the Linnean Society*, 178 (4), 924–930. https://doi.org/10.1111/zoj.12499

Kaczmarek, Ł. & Michalczyk, Ł. (2006) The Tardigrada Fauna of Mongolia (Central Asia) with a Description of *Isohypsibius altai* sp. nov. (Eutardigrada: Hypsibiidae). *Zoological Studies*, 45(1), 11–23.

Kaczmarek, Ł., Zawierucha, K., Smykla, J. & Michalczyk, Ł. (2012) Tardigrada of the Revdalen (Spitsbergen) with the descriptions of two new species: *Bryodelphax parvuspolaris* (Heterotardigrada) and *Isohypsibius coulsoni* (Eutardigrada). *Polar Biology*, 35, 1013–1026. https://doi.org/10.1007/s00300-011-1149-0

Kaczmarek, Ł., Kosicki, J.Z. & Roszkowska, M. (2018) Tardigrada of Bory Tucholskie National Park, Zaborski Landscape Park, and their surroundings (Pomerania Province, Poland). *Turkish Journal of Zoology*, 42, 6–17. https://doi.org/10.3906/zoo-1705-44

Katoh, K., Misawa, K., Kuma, K. & Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Resources*, 30, 3059–3066. https://doi.org/10.1093/nar/gkf436

Katoh, K. & Toh, H. (2008) Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinformatics*, 9, 286–298. https://doi.org/10.1093/bib/bbn013

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, Z.M., Kaczmarek, Ł. & Michalczyk, Ł. (2016) Experimental taxonomy confirms the environmental stability of morphometric traits in a taxonomically challenging group of microinvertebrates. *Zoological Journal of the Linnean Society*, 178, 765–775. https://doi.org/10.1111/zoj.12409

Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870–1874. https://doi.org/10.1093/molbev/msw054

Lanfear, R., Hua, X. & Warren, D.L. (2016) Estimating the Effective Sample Size of tree topologies from Bayesian phylogenetic analyses. *Genome Biology and Evolution*, 8, 2319–2332. https://doi.org/10.1093/gbe/evw171

Mali, B., Grohme, M.A., Förster, F., Dandekar, T., Schnölzer, M., Reuter, D., Wełnicz, W., Schill, R.O. & Frohme, M. (2010) Transcriptome survey of the anhydrobiotic tardigrade *Milnesium tardigradum* in comprison with *Hypsibius dujardini* and *Richtersius coronifer*. *BMC Genomics*, 11, 168. https://doi.org/10.1186/1471-2164-11-168

Marcus, E. (1928) IV: Bartierchen (Tardigrada). *Die Tierwelt Deutschlands und der angrenzenden Meeresteile nach ihren Merkmalen und nach ihrer Lebensweise*, 12, 1–226.

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. *Zoological Journal of the Linnean Society*, 116, 185–204. https://doi.org/10.1111/j.1096-3642.1996.tb02343.x

Meier, R. & Dikow, T. (2004) Significance of Specimen Databases from Taxonomic Revisions for Estimating and Mapping the Global Species Diversity of Invertebrates and Repatriating Reliable Specimen Data. *Conservation Biology*, 18, 378–488. https://doi.org/10.1111/j.1523-1739.2004.00233.x

Meyer, H.A. (2015) Water bears (Phylum Tardigrada) of Oceania, with the description of a new species of *Milnesium*. *New Zealand Journal of Zoology*, 42, 173–186. https://doi.org/10.1080/03014223.2015.1062402

Meyer, H.A. & Hinton, J.G. (2010) *Milnesium zsalakoae* and *M. jacobi*, two new species of Tardigrada (Eutardigrada: Apochela: Milnesiidae) from the southwestern United States. *Proceedings of the Biological Society of Washington*, 123, 113–120. https://doi.org/10.2988/09-29.1

Meyer, H.A. & Hinton, J.G. (2012) Terrestrial Tardigrada of the Island of Barbados in the West Indies, with the Description of *Milnesium barbadosense* sp. n. (Eutardigrada: Apochela: Milnesiidae). *Caribbean Journal of Science*, 46, 194–202. https://doi.org/10.18475/cjos.v46i2.a8

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.

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.
Michalczyk, Ł. & Kaczmarek, Ł. (2013) The Tardigrada Register: a comprehensive online data repository for tardigrade taxonomy. *Journal of Limnology*, 72 (S1), 175–181.
https://doi.org/10.4081/jlimnol.2013.s1.e22
Mironov, S.V., Dabert, J. & Dabert, M. (2012) A new feather mite species of the genus *Proctophyllodes* Robin, 1877 (Astigmata: Proctophyllodidae) from the Long-tailed Tit *Aegithalos caudatus* (Passeriformes: Aegithalidae): morphological description with DNA barcode data. *Zootaxa*, 3253, 54–61.
Morek, W., Gąsiorek, P., Stec, D., Blagden, B. & Michalczyk, Ł. (2016a). Experimental taxonomy exposes ontogenetic variability and elucidates the taxonomic value of claw configuration in *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela). *Contributions to Zoology*, 85 (2), 173–200.
Morek, W., Stec, D., Gąsiorek, P., Schill, R.O., Kaczmarek, Ł. & Michalczyk, Ł. (2016b). An experimental test of eutardigrade preparation methods for light microscopy. *Zoological Journal of the Linnean Society*, 178, 785–793.
https://doi.org/10.1111/zoj.12457
Morek, W., Stec, D., Gąsiorek, P., Surmacz, B. & Michalczyk, Ł. (2019) *Milnesium tardigradum*: the first integrative study of inter-population variability in a tardigrade species. *Journal of Zoological Systematics and Evolutionary Research*, 57, 1–23.
https://doi.org/10.1111/jzs.12233
Nederström, P. (1919). Die bis jetzt asu Finnland bekannten Tardigraden. *Acta Societatis pro Fauna et Flora Fennica*, 46 (8), 1–25.
Nelson, D.R., Guidetti, R. & Rebecchi, L. (2015) Phylum Tardigrada. In: Thorp and Covich's freshwater invertebrates, vol 1, Chap 17. *Academic, Cambridge*, pp. 347–380.
https://doi.org/10.1016/B978-0-12-385026-3.00017-6
Neumann, S., Reuner, A., Brümmer, F. & Schill, R.O. (2009) DNA damage in storage cells of anhydrobiotic tardigrades. *Comparative Biochemistry and Physiology, Part A*, 153, 425–429.
https://doi.org/10.1016/j.cbpa.2009.04.611.
Pfannkuchen, M., Brümmer, F. & Schill, R.O. (2007) 3-Dimensional reconstruction of fluorescent structures in tardigrades. *Proceedings of the Tenth International Symposium on Tardigrada Journal of Limnology*, 66 (Suppl. 1), 158–163.
https://doi.org/10.4081/jlimnol.2007.s1.158
Pilato, G. (1981) Analisi di nuovi caratteri nello studio degli Eutardigradi. *Animalia,* 8, 51–57.
Pilato, G. & Binda, M.G. (1991) *Milnesium tetralamellatum*, New Species of Milnesiidae from Africa (Eutardigrada). *Tropical Zoology*, 4, 103–106.
https://doi.org/10.1080/03946975.1991.10539480
Pilato, G. & Lisi, O. (2016) *Milnesium minutum* and *Milnesium sandrae*, two new species of Milnesiidae (Tardigrada, Eutardigrada, Apochela). *ZooKeys*, 580, 1–12.
https://doi.org/10.3897/zookeys.580.6603
Pilato, G., Sabella, G. & Lisi, O. (2016) Two new species of *Milnesium* (Tardigrada: Milnesiidae). *Zootaxa*, 4132 (4), 575–587.
https://doi.org/10.11646/zootaxa.4132.4.9
Pilato, G., Sabella, G., D'Urso, V. & Lisi O. (2017) Two new species of Eutardigrada from Victoria Land, Antarctica. *Zootaxa*, 4317 (3), 541–558.
https://doi.org/10.11646/zootaxa.4317.3.6
R Core Team (2015) R: A language and environment for statistical computing. *R Foundation for Statistical Computing*. Austria, Vienna.
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.
Ramazzotti, G. & Maucci, W. (1983) Il Phylum Tardigrada. Terza edizione riveduta e aggiornata. *Memorie dell' Istituto Italiano di Idrobiologia*, 41, 1–1012.
Rambaut, A., Suchard, M.A., Xie, D. & Drummond, A.J. (2014) Tracer v1.6. Available from: http://beast.bio.ed.ac.uk/Tracer (Accessed 15 Apr. 2019)
Reuner, A., Hengherr, S., Brümmer, F. & Schill, R.O. (2010a) Comparative studies on storage cells in tardigrades during starvation and anhydrobiosis. *Current Zoology*, 56 (2), 259–263.
Reuner, A., Hengherr, S., Mali, B., Förster, F., Arndt, D., Reinhardt, R., Dandekar, T., Frohme, M., Brümmer, F. & Schill, R.O. (2010b) Stress response in tardigrades: differential gene expression of molecular chaperones. *Cell Stress and Chaperones*, 15, 423–430.
https://doi.org/10.1007/s12192-009-0158-1
Richters, F. (1903) Nordische Tardigraden. *Zoologischer Anzeiger*, 27, 168–172.
Richters, F. (1926) Tardigrada. *Handbuch der Zoologie*, III, 58–61.
Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19 (12), 1572–1574.
https://doi.org/10.1093/bioinformatics/btg180
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. *Zoological Studies*, 54, 1–17.
https://doi.org/10.1186/s40555-014-0082-7

Roszkowska, M., Stec, D., Ciobanu, D.A. & Kaczmarek Ł. (2016) Tardigrades from Nahuel Huapi National Park (Argentina, South America) with descriptions of two new Macrobiotidae species. *Zootaxa*, 4105 (3), 243–260.
https://doi.org/10.11646/zootaxa.4105.3.2

Sands, CJ., McInnes, S.J., Marley, M.J., Goodall-Copestake, WP., Convey, P. & Linse, K. (2008) Phylum Tardigrada: an 'individual' approach. *Cladistics*, 24, 1–11.
https://doi.org/0.1111/j.1096-0031.2008.00219

Schill, R.O., Steinbrück, G. & Köhler H.R. (2004) Stress gene (hsp70) sequences and quantitative expression in *Milnesium tardigradum* (Tardigrada) during active and cryptobiotic stages. *The Journal of Experimental Biolo*gy, 207, 1607–1613.
https://doi.org/10.1242/jeb.00935

Schill, R.O. (2007) Comparison of different protocols for DNA preparation and PCR amplification of mitochondrial genes of tardigrades. *Proceedings of the Tenth International Symposium on Tardigrada, Journal of Limnology*, 66 (Suppl. 1), 164–170.
https://doi.org/10.4081/jlimnol.2007.s1.164

Schill, R.O. & Steinbruck, G. (2007) Identification and differentiation of Heterotardigrada and Eutardigrada species by riboprinting. *Journal of Zoological Systematics and Evolutionary Research*, 45 (3), 184–190.
https://doi.org/10.1111/j.1439-0469.2007.00409.x

Schill, R.O. & Fritz, G.B. (2008) Desiccation tolerance in embryonic stages of the tardigrade. *Journal of Zoology*, 276, 103–107.
https://doi.org/10.1111/j.1469-7998.2008.00474.x

Schlabach, S., Donaldson, E., Hobelman, K., Miller, W.R. & Lowman, M.D. (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.
https://doi.org/10.1660/062.121.0204

Schökraie, E., Hotz-Wagenblatt, A., Warnken, U., Mali, B., Frohme, M., Forster, F., Dandekar, T., Hengherr, S., Schill, R.O. & Schnölzer M. (2010) Proteomic analysis of tardigrades: towards a better understanding of molecular mechanisms by anhydrobiotic organisms. *PLoS ONE*, 5 (3), e9502.
https://doi.org/10.1371/journal.pone.0009502

Schökraie, E., Hotz-Wagenblatt, A., Warnken, U., Frohme, M., Dandekar, T., Schill, R.O. & Schnölzer, M. (2011) Investigating heat shock proteins of tardigrades in active versus anhydrobiotic state using shotgun proteomics. *Journal of Zoological Systematics and Evolutionary Research*, 49 (1), 111–119.
https://doi.org/10.1111/j.1439-0469.2010.00608.x

Schökraie, E., Warnken, U., Hotz-Wagenblatt, A., Grohme, M.A., Hengherr, S., Forster, F., Schill, R.O., Frohme, M., Dandekar, T. & Schnölzer, M. (2012) Comparative proteome analysis of *Milnesium tardigradum* in early embryonic state versus adults in active and anhydrobiotic state. *PLoS ONE*, 7 (9), e45682.
https://doi.org/10.1371/journal.pone.0045682

Schuster, R.O., Nelson, D.R., Grigarick, A.A. & Christenberry, D. (1980) Systematic criteria of the Eutardigrada. *Transactions of the American Microscopical Society*, 99, 284–303.
https://doi.org/10.2307/3226004

Shcherbakov, D., Schill, R.O., Brümmer, F. & Blum, M. (2010) Movement behaviour and video tracking of *Milnesium tardigradum* Doyère, 1840 (Eutardigrada, Apochela). *Contributions to Zoology*, 79 (1), 33–38.

Smykla, J., Iakovenko, N., Devetter, M. & Kaczmarek, Ł. (2012) Diversity and distribution of tardigrades in soils of Edmonson Point (Northern Victoria Land, continental Antarctica). *Czech Polar Reports*, 2 (2), 61–70.
https://doi.org/10.5817/CPR2012-2-6

Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30, 1312–1313.
https://doi.org/10.1093/bioinformatics/btu033

Stec, D., Gąsiorek, P., Morek, W., Kosztyła, P., Zawierucha, K., Michno, K., Kaczmarek, Ł., Prokop, Z.M. & Michalczyk, Ł. (2016) Estimating optimal sample size for tardigrade morphometry. *Zoological Journal of the Linnean Society*, 178, 776–784.
https://doi.org/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). *Systematics and Biodiversity*, 16 (4), 357–376.
https://doi.org/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 (5), 501–526.
https://doi.org/10.11646/zootaxa.4052.5.1

Suzuki, A.C. (2003) Life history of *Milnesium tardigradum* Doyère (Tardigrada) under a rearing environment. *Zoological Science*, 20, 49–57.
https://doi.org/10.2108/zsj.20.49

Suzuki, A.C. (2006) Ovarian structure in *Milnesium tardigradum* (Tardigrada, Milnesiidae) during early vitellogenesis. *Hydrobiologia*, 558, 61–66.
https://doi.org/10.1007/s10750-005-1416-6

Suzuki, A.C. (2008) Appearance of males in a thelytokous strain of *Milnesium* cf. *tardigradum* (Eutardigrada: Apochela). *Zoological Science*, 25, 849–853
https://doi.org/10.2108/zsj.25.849.
Tang, Y., Horikoshi, M. & Li, W. (2016) ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages. *The R Journal*, 8 (2), 474–485.
https://doi.org/10.32614/RJ-2016-060
Takahashi, H., Kamiya., A., Ishiguro, A., Suzuki, A.C., Saitou, N., Toyoda, A. & Aruga, J. (2008) Conservation and diversification of Msx protein in metazoan evolution. *Molecular Biology and Evolution*, 25, 69–82.
https://doi.org/10.1093/molbev/msm228
Tumanov, D.V. (2006) Five new species of the genus *Milnesium* (Tardigrada, Eutardigrada, Milnesiidae). *Zootaxa*, 1122, 1–23.
Vaidya, G., Lohman, J.D. & Meier, R. (2010) SequenceMatrix: concatenation software for the fast assembly of multigene datasets with character set and codon information. *Cladistics*, 27, 171–180.
https://doi.org/10.1111/j.1096-0031.2010.00329.x
Velasco-Castrillón, A., McInnes, S.J., Schultz, MB., Arróniz-Crespo, M., D'Haese, C.A., Gibson, J.A.E., Adams, B.J., Page, T.J., Austin, A.D., Cooper, S.B.J. & Stevens, M.I. (2015). Mitochondrial DNA analyses reveal widespread tardigrade diversity in Antarctica. *Invertebrate Systematics*, 29, 578–590.
https://doi.org/10.1071/IS14019
Vicente, F., Cesari, M., Serrano, A. & Bertolani, R. (2013) The impact of fire on terrestrial tardigrade biodiversity: a first case-study from Portugal. *Journal of Limnology*, 72, 152–159.
https://doi.org/10.4081/jlimnol.2013.s1.e19
Wang, C., Grohme, M.A., Mali, B., Schill, R.O. & Frohme, M. (2014) Towards decrypting cryptobiosis—analyzing anhydrobiosis in the tardigrade *Milnesium tardigradum* using transcriptome sequencing. *PLoS ONE*, 9 (3), e92663.
https://doi.org/10.1371/journal.pone.0092663
Wełnicz, W., Grohme, M.A., Kaczmarek, Ł., Schill, R.O. & Frohme, M. (2011) ITS-2 and 18S rRNA data from *Macrobiotus polonicus* and *Milnesium tardigradum* (Eutardigrada, Tardigrada). *Journal of Zoological Systematics and Evolutionary Research*, 49, 34–39.
https://doi.org/10.1111/j.1439-0469.2010.00595
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.
https://doi.org/10.11646/zootaxa.4072.5.3
Zawierucha, K., Dziamięcki, J., Jakubowska, N., Michalczyk, Ł. & Kaczmarek, Ł. (2014) New tardigrade records for the Baltic states with a description of *Minibiotus formosus* sp. n. (Eutardigrada, Macrobiotidae). *ZooKeys*, 408, 81–105.
https://doi.org/10.3897/zookeys.408.6612
Zeller, C. (2010) *Untersuchung der Phylogenie von Tardigraden anhand der Genabschnitte 18S rDNA und Cytochrom c Oxidase Untereinheit 1 (COX I).* MSc Thesis, Technische Hochschule Wildau, 105 pp.

APPENDIX 1. Neotype and type DNA sequences

***Milnesium alpigenum* Ehrenberg, 1853**

18S rRNA (1054 bp, MG996146):

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TAGATCGTATCATCCTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCAAAAAGCCGTCTGGCCTCGTGTCAGCGGCG
CAGTTATTAGATTAAAACCAATATAGGCTTTTCGGGTCTATTAAACTTGTGATGAATCTGAATAACCGAAGCAAAGCGCATGGT
CTCGTACCGGCGCTAGATCTTTCAAGTGTCTGATCTATCAGCTTGTCGTTAGGTTATGTTCCTAACGTGGCTTCGACGGGTAAC
GGGGTATCAGGGTCCGATACCGGAGAGGGAGCCTGAGAAATGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCA
CTCCCAGTTCGGGGAGGTAGTGACGAAAAATAACGATGCGGGAGCATAATGCTTCCCGTAATCGGAATGAGTACACTTTAAATC
CTTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGCTG
CGGTTAAAAAGCTCGTAGTTGGATCTGGGTGTTTCGATGAGTGGTGCATCTATTCGATGTCTACTACTCCATCGACACCACAAG
CCAACCATGTCCTTCTATACCCTTCACTGGGCGTAGATAATGGGCGGTTGGAACGTTTACTTTGAAAAAATTAGAGTGCTCAAA
GCAGGCGTATGGCCTTGAATAATGGTGCATGGAATAATGGAATAGGACCTCGGTTCTATTTGTTGGTTTTCAAGAGCTCGAGGT
AATGATAAAGAGGAACAGACGGGGGCATTCGTATTGCGACGTTAGAGGTGAAATTCTTGGATCGTCGCAAGACGAACTACTGCG
AAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACC
ATAAACGATGCCAACCAGCGATCTGTCGGTGTTTATTTAACGACTCGACAGGCAGCTTCCGGGAAACCAAAGTGCTTAGGTTCC
GGGGGAAGTATGGTTGCAAAGCTGAAACTAAAGGAATGACGAAGCC
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28S rRNA (809 bp, MH000384):

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TACTAAGCGGGGGAAAAGAAACCAACGGGGATTCTCCTAGTAACTGCGAGTGAACGGAGAAAAGCCCAGCGCTGAATCCTGTAG
CTGGTAACGGTTATGGGAGCTGTAGCGTGAAGAAGGTGTACAACCATTGCAGTCAATACACGTAAGTCTCCCTGAGTGGAGCTC
CATCCCAAGGAGGGTGCAAGGCCCGTATCGTGTTTGACGCGTGATGGTATAGCATCTTCAGAGAGTCGGGTTGTTTGGGATTGC
AACCTAAAGCCGGTGGTAAACTCCATCGAAGGCTAAATATGACCACGAGTCCGATAGCGAACAAGTACCGTGAGGGAAAATTGA
AAAGCACTTTGAAGAGAGAGCGAAATAGTGCGTGAAACCGCTTAGAGGCAAGCAGATGGGGCCTCGAAGGTAGAGCAGCGAATT
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CAGCTTGCATTTCTGCTAGACTACTGTCGGCGTAGAGATCGTAAGACTCTTGTCGATGTAGGGTGTATAGTGGAATGTGAGTGC
ACTTTCGCTGTTTGTACGCCACCGCTGTTAAGTGTGCATCCGCTGTGGTCTTGCGTGAGGCCTTGAGTGGCTTGCTGCTCAAGT
CACCTACGCTTGGCTATTATGCAGCGCGTTTGCCTATTAACTGGACAAGTCATTCCTATGCCAGCATCGCTTCGGTGGTGTGAT
GTCGAACACTGGCGTGTTTATTGCTGCTTCGTGGCAGTTGACGTGCTTGCACGGCTTCAGCTGCTGGTGGTATACTGCGTCGGC
TCTACAGGCATAGTGTAGATTTGGTGGCGAGTAGATGGCTGCCCATCTAACCC

ITS-2 (530 bp, MH000382):
CTTTATGAACGTTGTTTCTTCGAACGCAAATTGCGGCTATGGGTTGACCGTAGCCACGTCTGGTTGAGGGTCAAACGAAAAAAA
AATGATAGCTACGTGTTTGCTATCGATTGTCTGTCATCCTATACTGGCCATCTCAGAGCCAGGCGAAGGCTGACAGATGAAGTA
TCAACCCTTTGACGAGCGTATTCCTGGTCTGTAGCGGATCGGAAGCCTACGGGCGCGCACATATGCGTATATGTATGTACGGGC
TGTGTGATGGCAGTAGGTTGGAGTCGCTGATAGGCTCTGTATCGCTTAGCTGGTTAATGCATGCGGCAGTTGTGCATATATAGA
GCCGGCTACGAGCAGAGTTTTGACCAATCGAACGACAGCCCACTAAAGTACATACTCGCATACACAATGCGCTTGGCTTCGAGT
ACAGATATCAGTACGCTGAATGGTCATAGGTGAAGCCATGTGTTTGTCGCTTGCGACATGCTCATACACATATACACTCATTAC
GTTGACCTCAGCTCAGGCGAGATTAC

COI (560 bp, MH000380):
GCCTTTTTGTAGGTTCAGCATTGAGTATATTAATTCGACTTGAATTATCTCAACCTAATTCTATACTTATAAGAGAAGATATTT
ATAATGCTTTTATTACAAGTCATGCTTTAGTAATAATTTTTTTTTTTGTTATACCAGTGTTAATTGGAGGGTTTGGTAATTGAT
TGGTTCCTTTAATAATTAGTTCTCCGGATATAGCTTTTCCTCGTATTAATAATGTGAGATTTTGAATGTTAATTGCTTCTTTTT
TGTTATTAATTTTAAGAATATTTTCTGGAGCTGGTGTAGGAGCAGGTTGAACTTTGTATCCTCCTCTTACTAATATTTATGGTC
ATAGAAATTCTTCAGTTGATTTTGCGATCTTATCATTGCATATTGCTGGTGCTTCTTCTGTGTTTAGAGCAATAAATTTTTTAA
CTACAATTTTTAATATACATTATTTTGGTCTTCGAATAGATAAATTACCTTTGTTTGTATGATCAATTTTTATTACGGCAATCT
TGTTAGTTTTGGCTTTGCCTGTTTTGGCTGGTGCTATTACAATATTAATTTTGATC

***Milnesium inceptum* sp. nov.**

18S rRNA (1070 bp, MH000383):
ATTAATCAGGTATGGGGTMCTAGATCGTATCATCCTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCAATGAAGCTG
TCTGGCCTTGTGTCAGCAGCGCAGTTATTAGATTAAAACCAATATAGGCTTTCGGGTCTATTAACTTGTGATGAATCTGAATAA
CCGAAGCAAAGCGCATGGTCTCGTACCGGCGCTAGATCTTTCAAGTGTCTGATCTATCAGCTTGTCGTTAGGTTATGTTCCTAA
CGTGGCTTCGACGGGTAACGGGGTATCAGGGTCCGATACCGGAGAGGGAGCCTGAGAAATGGCTACCACATCCAAGGAAGGCAG
CAGGCGCGCAAATTACCCACTCCCAGTTCGGGGAGGTAGTGACGAAAAATAACGATGCGGGAGCATAATGCTTCCCGTAATCGG
AATGAGTACACTTTAAATCCTTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATA
GCGTATATYAAAGTTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGGGTGTTTCGATGAGTGGTGCATCTATTCGATGTCTACT
ACTCCATCGACACCACAAGCCAACCATGTCCTTCTATACCCTTCACTGGGCGTAGATAATGGGCGGTTGGAACGTTTACTTTGA
AAAAATTAGAGTGCTCAAAGCAGGCGTATGGCCTTGAATAATGGTGCATGGAATAATGGAATAGGACCTCGGTTCTATTTGTTG
GTTTTCAAGAGCTCGAGGTAATGATAAAGAGGAACAGACGGGGGCATTCGTATTGCGACGTTAGAGGTGAAATTCTTGGATCGT
CGCAAGACGAACTACTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGAGGTTCGAAGGCGATCAGA
TACCGCCCTAGTTCTAACCATAAACGATGCCAACCAGCGATCTGTCGGTGTTTATTTAACGACTCGACAGGCAGCTTCCGGGAA
ACCAAAGTGCTTAGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAACTAAAGGAATTGACGAG

28S rRNA (817 bp, MH000385):
TACTAAGCGGAGGAAAAGAAACCAACGGGGATTCTCCTAGTAACTGCGAGTGAACGGAGAAAAGCCCAGCGCTGAATCCTGTAG
CTGGTAACGGTTACGGGAGCTGTAGCGTGAAGAAGGTGTACAACCATTGCAGTCAATACACGTAAGTCTCCCTGAGTGGAGCTC
CATCCCAAGGAGGGTGCAAGGCCCGTATCGTGTTTGACGCGTGATGGTATAGCATCTTCAGAGAGTCGGGTTGTTTGGGATTGC
AACCTAAAGCCGGTGGTAAACTCCATCGAAGGCTAAATATGACCACGAGTCCGATAGCGAACAAGTACCGTGAGGGAAAATTGA
AAAGCACTTTGAAGAGAGAGCGAAATAGTGCGTGAAACCGCTTAGAGGCAAGCAGATGGGGCCTCGAAGGTAGAGCAGCGAATT
CAGCTTGCATCTCTGCTGGACTACTGTTGGCGTAGAGATCGTAAGACTCTTGTCAATGTAGGCTGTCATAGTGGAATGTGAGTG
CACTTTCGCTGTTTGTACGCCACCGCTGATAAATGTTTCTGCATCCGCTGTGGCCTTGTGTGAGGCCTTGAGTGGCTTGCTACT
CAAGTAACCTACACTTGGCTATATACAGCGCGTTTGCCTTTTATCTGGTCGAGTCACATCCTATGCCGGCATTGCTTTACGGCG
ATGCAGTGTAGATACTAGCGTGTTTATTGCTACTTCGCATTGCGGTTGACGTGCTTGCACGGCTGCTGCGGCTGGTGGTATACT
GCGTTGGATCTACTGGTATAGTGTAGATTTGGTGGCGAGTAGACGGCTGCCCATCTAACCC

ITS-2/H1, Germany (DE.001), Japan (JP.010) and Switzerland (CH.002) (528 bp, MH000386):
CTTTATGAACGTTGTTTCTTCGAACGCAAATTGCGGCTATGGGTTGACCGTAGCCACGTCTGGTTGAGGGTCAAACGAAAAAAT
CACTGATAGCTACGTGTTTGCT**G**TCGATTGTCTGTCAGTCTATACTGGCCATTTTAGTGCCAGGTCAAGGCTGACAGATGAAGT
TTGGACCCTATGGCTAGCGTACTTTTGGTCTGTGACGGATCGG**G**AGC**T**GGCGCGTACATGCTCATATAGAGTTATACTGGGACT
GTGTGATCACAGTAGGTTGGAGTCGCTGA**T**GGGCTCTACCGTTTAGCTGACAATGCATGCGGCAGTTGTGCATAACTAGTCAGC
GACGCGTAGACGCTTGGCCAACCGAACGACAGTCCACTCACGGTATACTCTGGCATGTTCAAGCGTACGGCTACCGAGTGCAGC
TTCCAATACGCAGTGATATAGTCATAGGTTGATAAAGCGTGTGTCGTACGCTTCATGTGCGCGACGCATACACACTCATTACGT
TGACCTCAGCTCAGGCGAGATTAC

ITS-2/H2, Bulgaria (BG.058) (528 bp, MH000387):

CTTTATGAACGTTGTTTCTTCGAACGCAAATTGCGGCTATGGGTTGACCGTAGCCACGTCTGGTTGAGGGTCAAACGAAAAAAT
CACTGATAGCTACGTGTTTGCT**A**TCGATTGTCTGTCAGTCTATACTGGCCATTTTAGTGCCAGGTCAAGGCTGACAGATGAAGT
TTGGACCCTATGGCTAGCGTACTTTTGGTCTGTGACGGATCGG**A**AGC**C**GGCGCGTACATGCTCATATAGAGTTATACTGGGACT
GTGTGATCACAGTAGGTTGGAGTCGCTGA**C**GGGCTCTACCGTTTAGCTGACAATGCATGCGGCAGTTGTGCATAACTAGTCAGC
GACGCGTAGACGCTTGGCCAACCGAACGACAGTCCACTCACGGTATACTCTGGCATGTTCAAGCGTACGGCTACCGAGTGCAGC
TTCCAATACGCAGTGATATAGTCATAGGTTGATAAAGCGTGTGTCGTACGCTTCATGTGCGCGACGCATACACACTCATTACGT
TGACCTCAGCTCAGGCGAGATTAC

COI/H1, Germany (DE.001) and Switzerland (CH.002) (658 bp, KU513422):

TATATTGTATTTTATTTTTGGTATTTGATGTGCTTTTGTAGGTTCAGGTTTAAGTGTGTTAATTCGTCTTGAATTATCTCAGCC
TAACACAATATTAATAAGTGAAGATATTTATAATGCTTTTATTACAAGTCATGCTTTAGTAATGATTTTTTTTTTTGTTATACC
TGTTTTAATTGGAGGTTTTGGAAATTGA**T**TAGTTCCTCTTATAATTAGATCACCAGATATAGCTTTTCCTCGTATTAA**C**AATGT
AAGATTTTGAATATTAGTTGCTTCTTTTGGTTTGTTGCTTTTTAGAATATTTAGGGGTACAGGAGTAGGAGCTGGTTGAACACT
TTATCCTCCGTTAACTAGGTATAATGG**C**CATAGCAGTCATGCTGTCGATTATGCAATTTTGTCTTTACATATTGCAGGAGCATC
GTCAATTTTTAGTGCACTGAATTTTTTAACGACGATTATTAATATACACTATTTTGGAGTACGAATAGATAAATTACCGTTGTT
TGTGTGATCGATTTTTATTACTGCTCTATTGTTAGTTTTGGCTTTACCAGTACTTGCTGGAGCAATTACAATATTAATTTCTGA
TCGTAATTT**T**ACTACTACATTTTTTGATCCGGCAGGGGGAGGAGATCCTGTTTTATTTCAACATTTATTT

COI/H2, Japan (JP.010) (580 bp, MK628723):

TTTTGTAGGTTCAGGTTTAAGTGTGTTAATTCGTCTTGAATTATCTCAGCCTAACACAATATTAATAAGTGAAGATATTTATAA
TGCTTTTATTACAAGTCATGCTTTAGTAATGATTTTTTTTTTTGTTATACCTGTTTTAATTGGAGGTTTTGGAAATT**G**ATTAGT
TCCTCTTATAATTAGATCACCAGATATAGCTTTTCCTCGTATTAA**T**AATGTAAGATTTTGAATATTAGTTGCTTCTTTTGGTTT
GTTGCTTTTTAGAATATTTAGGGGTACAGGAGTAGGAGCTGGTTGAACACTTTATCCTCCGTTAACTAGGTATAATGG**T**CATAG
CAGTCATGCTGTCGATTATGCAATTTTGTCTTTACATATTGCAGGAGCATCGTCAATTTTTAGTGCACTGAATTTTTTAACGAC
GATTATTAATATACACTATTTTGGAGTACGAATAGATAAATTACCGTTGTTTGTGTGATCGATTTTTATTACTGCTCTATTGTT
AGTTTTGGCTTTACCAGTACTTGCTGGAGCAATTACAATATTAATTTCTGATCGTAATTT**C**ACTACTACATTTTTT

COI/H3, Bulgaria (BG.058) (647 bp, MH000381):

TTTTGTAGGTTCAGGTTTAAGTGTGTTAATTCGTCTTGAATTATCTCAGCCTAACACAATATTAATAAGTGAAGATATTTATAA
TGCTTTTATTACAAGTCATGCTTTAGTAATGATTTTTTTTTTTGTTATACCTGTTTTAATTGGAGGTTTTGGAAATTGA**C**TAGT
TCCTCTTATAATTAGATCACCAGATATAGCTTTTCCTCGTATTAA**T**AATGTAAGATTTTGAATATTAGTTGCTTCTTTTGGTTT
GTTGCTTTTTAGAATATTTAGGGGTACAGGAGTAGGAGCTGGTTGAACACTTTATCCTCCGTTAACTAGGTATAATGG**C**CATAG
CAGTCATGCTGTCGATTATGCAATTTTGTCTTTACATATTGCAGGAGCATCGTCAATTTTTAGTGCACTGAATTTTTTAACGAC
GATTATTAATATACACTATTTTGGAGTACGAATAGATAAATTACCGTTGTTTGTGTGATCGATTTTTATTACTGCTCTATTGTT
AGTTTTGGCTTTACCAGTACTTGCTGGAGCAATTACAATATTAATTTCTGATCGTAATTT**C**ACTACTACATTTTTTGATCCGGC
AGGGGGAGGAGATCCTGTTTTATTTCAACATTTATTTTGGTTTTTTGGGCATCCAGAAG

Supplementary Materials

Supplementary Materials **SM.01**—Morphometric measurements of the neotype (Italy, IT.057) population of *Milnesium alpigenum* Ehrenberg, 1853.

Supplementary Materials **SM.02**—Morphometric measurements of type population (Germany, DE.001) of *Milnesium inceptum* **sp. nov.**

Supplementary Materials **SM.03**—Morphometric measurements of the Japan (JP.010) population of *Milnesium inceptum* **sp. nov.**

Supplementary Materials **SM.04**—Morphometric measurements of the Swiss (CH.002) population of *Milnesium inceptum* **sp. nov.**

Supplementary Materials **SM.05**—Morphometric measurements of the Bulgarian (BG.058) population of *Milnesium inceptum* **sp. nov.**