

‘Everything is not everywhere’: time-calibrated phylogeography of the genus *Milnesium* (Tardigrada)

Witold Morek¹, Bartłomiej Surmacz¹, Alejandro López¹, and Lukasz Michalczyk¹

¹Jagiellonian University in Krakow

January 20, 2021

Abstract

There is ample evidence that macroscopic animals form geographic clusters termed as zoogeographic realms (zones), whereas distributions of species of microscopic animals are still poorly understood. The common view has been that micrometazoans, thanks to their putatively excellent dispersal abilities, are subject to the ‘Everything is Everywhere but environment selects’ hypothesis (EiE). One of such groups, <1 mm in length, are limnoterrestrial water bears (Tardigrada), which can additionally enter cryptobiosis that should further enhance their potential for long distance dispersion (e.g. by wind). However, an increasing number of studies, including the most recent phylogeny of a eutardigrade genus *Milnesium*, seem to question the general applicability of the EiE hypothesis to tardigrade species. Nevertheless, all the *Milnesium* phylogenies published to date were based on a limited number of populations, which are likely to falsely suggest limited geographic ranges. Thus, in order to comprehensively test the EiE hypothesis, here, we considerably enlarged the *Milnesium* dataset both taxonomically and geographically, and we analysed it in tandem with climate type and reproductive mode. Additionally, we time-calibrated our phylogeny to align it with major geological events. Our results show that, although cases of long distance dispersal are present, they seem to be rare and mostly ancient. Overall, *Milnesium* species are restricted to single zoogeographic realms, which suggests that these tardigrades have limited dispersal abilities. Finally, our results also suggest that the breakdown of Gondwana may influenced the evolutionary history of *Milnesium*. In conclusion, phylogenetic relationships within the genus seem to be determined mainly by paleogeography.

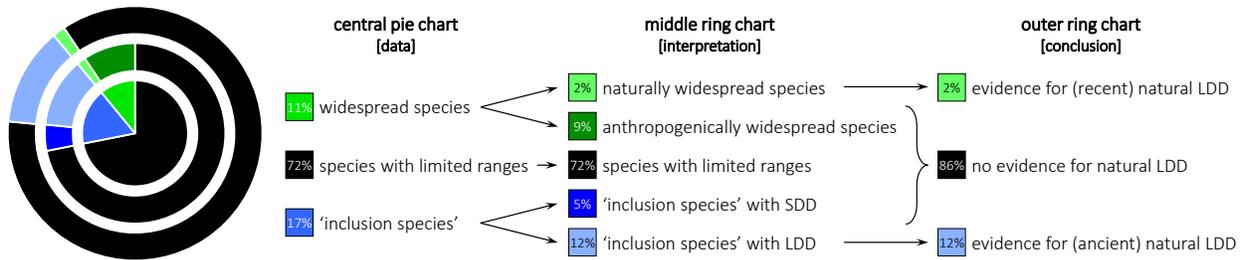


Figure 1: GRAPHICAL ABSTRACT.

Keywords: Apochela, EiE, long-distance dispersal (LDD), molecular clock, trait evolution, reproductive mode

Running title: *Milnesium* phylogeography

Introduction

The distribution of animals across the World has been studied since the nineteenth century, and one of the first results of global biogeographic analyses was the division of the Earth into zoogeographic realms (Wallace, 1876). However, such studies were conducted primarily on large, easily observable organisms, mainly vertebrates (Sclater, 1858). In contrast, for microscopic forms of life, the ‘Everything is Everywhere but environment selects’ hypothesis (EiE) was postulated (Baas-Becking, 1934; Fenchel & Finlay, 2004; Foissner, 2006): because of their small size, <1 mm, such organisms were believed to have unlimited long distance dispersal (LDD) abilities, and therefore they should not show any biogeographical patterns, dwelling wherever the environment is suitable. This hypothesis was argued to be especially applicable to groups with dormant stages, which can be easily dispersed over long distances (Fontaneto, Barraclough, Chen, Ricci, & Herniou, 2008; Incagnone, Marrone, Barone, Robba, & Naselli-Flores, 2015). Earlier studies, which were based on morphological characters, seemed to confirm the EiE hypothesis across different taxonomic groups (e.g. Fenchel, Esteban, & Finlay, 1997; Martiny et al., 2006; Heino et al., 2010). However, with the development of molecular tools, many species of microscopic animals that were previously thought to be cosmopolitan, were demonstrated to actually be species complexes (Fontaneto, Kaya, Herniou, & Barraclough, 2009; Cesari, Bertolani, Rebecchi, & Guidetti, 2009), with respective species that often exhibit limited geographic ranges. Therefore, recently, evidence against the EiE hypothesis in microscopic animals started to accumulate (e.g. Fontaneto et al. 2008; Baltanás & Danielopol, 2013; Garraffoni & Balsamo, 2017; Worsaae, Kerbl, Vang, & Gonzalez, 2019; Gąsiorek, Vončina, Zajac, & Michalczyk, in review), undermining the hypothesis or at least its universal application to all micrometazoan species.

One of the groups of microscopic animals (up to ca. 1 mm) that have the ability to withstand harsh environmental conditions are limnoterrestrial tardigrades (e.g. Hengherr & Schill, 2018). These ubiquitous invertebrates inhabit a wide variety of environments across the planet (e.g. Nelson, Bartels, & Guil, 2018). Many tardigrade species have been considered cosmopolitan, but the knowledge on the distribution of particular species is usually extremely fragmentary (Gąsiorek et al., 2019b). In recent years, the discussion on whether tardigrades do support or undermine the EiE hypothesis has been debated (e.g. Pilato & Binda, 2001; Guil, 2011), but only a limited number of geographically and/or taxonomically restricted studies on this topic have been conducted so far (e.g. Jørgensen, Møbjerg, & Kristensen, 2007; Guil Sanchez-Moreno, & Machordom, 2009; Cesari, McInnes, Bertolani, Rebecchi, & Guidetti, 2016; Morek, Stec, Gąsiorek, Surmacz, & Michalczyk, 2019a; Gąsiorek et al. 2019b; Gąsiorek, Vončina, Degma, & Michalczyk, 2020; Morek & Michalczyk 2020). Nevertheless, these initial studies already indicate that many species may not be as widely distributed as previously assumed. Importantly, in recent years, many species complexes and pseudocryptic species have been detected (e.g. Bertolani, Rebecchi, Giovannini, & Cesari, 2011; Stec, Morek, Gąsiorek, & Michalczyk, 2018; Morek et al., 2019a), further questioning the putative cosmopolitan distribution of tardigrade species. Moreover, the impact of geological events on the distribution of these animals has been hypothesised to be important (McInnes & Pugh, 1998; Guidetti, McInnes, Cesari, Rebecchi, & Rota-Stabelli, 2017), which could not be the case if tardigrade species were generally cosmopolitan. In parallel, anthropogenic dispersal has to be taken into consideration, as it is likely to obscure the natural distribution of tardigrade species (Gąsiorek Vončina, & Michalczyk, 2019a; Morek et al. 2019b) most likely by artificially broadening geographic ranges of at least some species, which may provide false evidence in favour of the EiE hypothesis.

One of the tardigrade genera with a long history of biogeographic records is *Milnesium* Doyère, 1840. Importantly, the genus was erroneously considered monotypic for many decades and, as a consequence, the type species, *Milnesium tardigradum* Doyère, 1840, was reported from numerous localities throughout the globe. Therefore, *M. tardigradum* was regarded cosmopolitan for over 170 years, but this view has been challenged by its integrative redescription by Michalczyk, Welnicz, Frohme, & Kaczmarek (2012ab) and further questioned by Morek et al. (2019b). Moreover, the most recent phylogenetic analysis of the genus *Milnesium* (Morek

& Michalczyk, 2020), despite the limited number of analysed populations (34) and species (25), suggested that the dispersal capability of *Milnesium* species is limited and that the geographic origin of species is a better predictor of a phylogenetic position of any given *Milnesium* species than the morphological characters traditionally used in the apochelan taxonomy.

Importantly, however, with small sample size, the detection of the same species in multiple localities is not likely, especially in species-rich genera in which species are not common/abundant. Thus, even if species are widespread and their geographic distributions conform to the EiE hypothesis, analyses based on small sample size may produce artefactual patterns. In other words, the correlation of geography with phylogeny reported by Morek & Michalczyk (2020) could be an artefact, resulting from undersampling, falsely suggesting low dispersal abilities of *Milnesium* species and prematurely rejecting the EiE hypothesis to explain the geographic distribution of species within this genus. Therefore, to verify the biogeographic conclusions stemming from Morek & Michalczyk (2020), we considerably enlarged the phylogenetic *Milnesium* dataset (from 34 in Morek & Michalczyk, 2020 to 127 populations herein), originating from nine zoogeographic realms (compared to six in Morek & Michalczyk, 2020). Moreover, in order to understand what geological or historical events may have shaped the diversification and distribution of *Milnesium*, we dated our phylogeny using molecular clock analysis for the first time in Eutardigrada Richters, 1926.

Materials and Methods

Sampling and specimens

We analysed a total of 127 *Milnesium* populations, including 83 newly sequenced. They originate from five continents and nine zoogeographic realms (according to Holt et al., 2013); from the most sampled: Afrotropical, Palaearctic, Oriental, Neotropical, Australian, Madagascan, Panamanian, Nearctic and Oceanian realm (approximate localities are depicted in Fig. 1, for detailed information see Table S1).

DNA sequencing

DNA was extracted individually from 1–12 specimens (in most cases 4) per population following the Chelex[®] 100 resin (Bio-Rad) extraction method by Casquet, Thebaud, & Gillespie (2012), with modifications by Stec, Smolak, Kaczmarek, & Michalczyk (2015). After extraction, exoskeletons, if found in the vial, were mounted on permanent slide as voucher specimens (hologenophores; Pleijel et al., 2008; 77 populations in total). Otherwise, vouchers were isogenophores/progenophores (offspring of sequenced mothers; 10 populations) or paragenophores (individuals from the same population; 40 populations). Four molecular markers, three nuclear and one mitochondrial, were Sanger-sequenced: the small ribosomal subunit (18S rRNA), the large ribosomal subunit (28S rRNA), Internal Transcribed Spacer 2 (ITS-2), and cytochrome oxidase c subunit 1 (COI). The PCR protocol followed Stec, Kristensen, & Michalczyk (2020); primers and PCR protocols with relevant references are listed in Table 1. All chromatograms were manually checked in BioEdit ver. 7.2.5 (Hall, 1999). COI sequences were translated into amino acids to test for potential pseudogenes using MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). All sequences are deposited in GenBank (accession numbers are listed in Table S2).

Phylogenetic analysis

We analysed only populations with at least three out of four molecular markers sequenced; thus some of the literature data and some sequences available at GenBank were not included. The available *Milnesium* sequences, together with those from outgroup taxa (see Table S1–S2), were aligned independently for each marker, using MAFFT version 7 (Kato, Misawa, Kuma, & Miyata, 2002; Kato & Toh, 2008). For 18S rRNA and 28S rRNA, the G-INS-I strategy was applied to consider the secondary structure of RNA, whereas for ITS-2 and COI, the default setting was used. The obtained alignments were checked manually in BioEdit and then trimmed to 1159 (18S rRNA), 887 (28S rRNA), 841 (ITS-2) and 569 bp (COI). Next, the four obtained alignments were concatenated in SequenceMatrix (Vaidya, Lohman, & Meier, 2011). We used the Bayesian Information Criterion in PartitionFinder version 2.1.1 (Lanfear, Hua, & Warren, 2016) to find the most suitable substitution model for posterior phylogenetic analysis. As COI is a protein coding

fragment, the alignment was previously divided into three data blocks corresponding to the three codon positions. The best fit-model was GTR+I+G for all the partitions, with the exception of the first and third codon positions in COI, for which SYM+I+G and K81UF+G had a better fit respectively.

Phylogenetic inference was carried out using two different approaches. The first one utilized the same exact methodology as Morek & Michalczyk (2020) in MrBayes v3.2 (Ronquist & Huelsenbeck 2003). Additional Bayesian Inference analyses were carried out in BEAST 1.10.4 (Drummond, Suchard, Xie, & Rambaut, 2012) using different clock (strict, lognormal relaxed, and exponential relaxed) and tree (coalescent with constant population size versus Yule) models. Different nucleotide substitution models and clock rates were set for each partition. In order to calibrate the molecular clock, and in the absence of any other reliable calibration, we used two calibration points: (i) we constrained the divergence between Hetero- and Eutardigrada using a normal distribution according to the estimates by Guidetti et al. (2017), and (ii) we set a lognormal distribution for the origin of *Milnesium* using the estimated age of the only know fossil assigned to this genus, *Milnesium swolenskyi* Bertolani & Grimaldi, 2000 as lower bound.

The analyses ran for 100 million generations in the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010), sampling every 10,000 steps. The best combination of clock and tree priors, was relaxed exponential clock model with Yule process as tree prior, which were selected according to the Bayes factors calculated in TRACER 1.6 (available from <http://beast.bio.ed.ac.uk>). The consensus tree for this best combination was built using TREEANNOTATOR (distributed with BEAST). Additionally, mutation rates for the four utilised markers, estimated in TRACER, are given in Table 2.

In all the trees, 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 poorly supported or unsupported. The consensus tree was visualised in FigTree v.1.4.3 (available from <http://tree.bio.ed.ac.uk/software/figtree>).

Species delineation

To estimate the number of species present in the dataset, we used a molecular species delimitation method, the Poisson tree process (PTP) (Zhang, Kapli, Pavlidis, & Stamatakis, 2013), conducted on the bPTP webserver (<http://species.h-its.org/ptp>). We applied the same methodology as in Morek & Michalczyk (2020), constructing the tree based on concatenated COI+ITS-2 sequences in MrBayes. The results were then compared with morphological data and genetic distances, to verify the putative species identified by bPTP. In parallel to genetic delineation, we attempted to identify species using the latest diagnostic key to the genus (Morek, Gąsiorek, Stec, Blagden, & Michalczyk, 2016). Species that could not be identified are designated as “sp. nov.” followed by population code (international country code and sample number). If there were two species from the same population, an additional number/letter was added to discriminate between them. In order to aid navigation through phylogeny, all species identified and delineated in the present study are given running numbers, from #1 to #64.

Phylogeography and the ‘Everything is Everywhere’ hypothesis

In order to test the EiE hypothesis, samples from distant geographic locations that exhibit similar climate conditions have to be collected and analysed using phylogenetic methods. Specifically, the EiE hypothesis can be tested in two non-exclusive ways: (i) by the comparison of geographic localities of populations representing individual species; and (ii) by the analysis of phylogenetic relationships of species collected in different zoogeographic realms, but in localities characterised by similar climate conditions. In the first case, multiple populations representing the same species are required, whereas in the second analysis species may be represented by single populations. Correspondingly, the EiE hypothesis predicts that: (i) populations of the same species will be found in geographically distant and isolated localities, provided that the localities are characterised by similar climate conditions; and (ii) closely related species will likely differ in geographic localities in which they were collected (i.e. clades will be geographically diversified). If, however, geographic distributions of *Milnesium* species are not subject to the EiE hypothesis, then: (i) populations representing the same species will be found exclusively in a single zoogeographic realm; and (ii) groups of closely related

species will share the same zoogeographic zone but not necessarily the same climate zone.

Given that our samples came from nine different zoogeographic realms, with many localities similar to each other in terms of climate conditions, we were able to test the EiE hypothesis by the two ways described above. Specifically, we mapped the zoogeographic origin at the level of the zoogeographic realm as well as climate type, of analysed populations onto the molecular phylogeny. Moreover, we utilised RASP 4 (Yu, Blair, & He, 2020) to statistically determine the area of origin, as well as the climate type for the main clades. The analysis was carried out on the consensus tree obtained with the BEAST (see Results) with the Bayesian Binary MCMC using the default settings. Zoographic realms were specified according to Holt et al. (2013), whereas main climate categories follow Peel, Finlayson, & McMahon (2007). The RASP analysis provided information on the geographic origin of the major clades, which allowed for an identification of ‘inclusion species’, i.e. species that were found in a different zoogeographic realm than the majority of species in a given clade (such species suggest ancient LDD). In parallel, a comparison of the geographic origin of multiple populations representing a single species allowed for an identification of recent LDD if such populations were found in more than one zoogeographic realm.

As the mode of reproduction was hypothesised to influence the dispersal abilities (Maynard Smith, 1978), this information (dioecy or parthenogenesis) was also mapped on the phylogenetic tree. The mode was tested for with PCM and individual culturing whenever possible (Rebecchi & Nelson, 1998); mode of reproduction was classified as unknown if culturing was not possible and if a population comprised only females but there were below ten adult specimens available, as in such a case the probability of not detecting males was too high to identify parthenogenesis. Although this does not allow for the distinction between facultative and obligatory parthenogenesis was, even facultative parthenogenesis should increase dispersal potential, thus it should be taken into consideration when investigating biogeography.

Results

Molecular phylogeny

The phylogenies obtained with BEAST and MrBayes have very similar topologies, with six distinct clades (named A to F for convenience; BEAST topology is shown in Figs 2–5, whereas MrBayes topology is available in Supplementary Materials SM.03). As multiple basal nodes in the MrBayes tree are poorly supported/unsupported (PP=0.56–0.78; see SM.03), only the topology obtained with BEAST is showed and discussed in detail (Figs 2–5).

The BEAST topology is, therefore, as follows: clade A and B are in a sister relationship, with high support (PP=0.94 for clade A, PP=1.00 for clade B, and PP=1.00 for clade A+B), with the exact composition and relationships being depicted in Fig. 3. Clade A groups 40 populations representing 17 species, whereas the sister clade B includes 23 populations representing 10 species. The (A+B) clade is sister to the clade that groups clades C and D. Clade C+D is highly supported (PP=0.95 for clade C, PP=1.00 for clade D, and PP=0.96 for clade C+D; Fig. 4). Clade C consists of 31 populations representing 19 species and clade D include 8 populations representing 7 species. The node indicating the split between (A+B) and (C+D) is moderately supported, with PP=0.84. Finally, clade E (PP=1.00; 21 populations representing 11 species; Fig. 5) is related to a small clade F (PP=1.00; three populations, classified as two species Fig. 5) but with low support.

The phylogeny largely corresponds to the relationships presented by Morek & Michalczyk (2020) (Fig. 2), but with some noticeable exceptions. Specifically, our clades A, B and D correspond to the previously identified clades A, B and D but are enriched with multiple species and populations (indicated by solid arrows in Fig. 2). Clades C and E herein correspond to clade E and C in the previous contribution, but their positions are different (dashed and dotted arrows in Fig. 2). Specifically, whereas in the previous study their clade E was the most basal, it is denoted here as clade C and is a part of the ‘crown group’ in the present study. In contrast, clade denoted as clade C and sister to clades (A+B) in Morek & Michalczyk (2020) is now denoted as clade E and is placed in the basal polytomy. Similarly to clades A, B and D, clades C and E in the present analysis are also enriched with new populations/species. Finally, clade F obtained in this study is new and

is formed exclusively by new samples.

Molecular clock estimations

Age estimation of the nodes is burdened with relatively wide 95% Highest Posterior Density (95% HPD; partly transparent blue horizontal bars in Fig. 2), indicating considerable uncertainty in node dating (see also Table 3), which was expected taking into account the limited input tree priors with only two available calibration points. The split between Eutardigrada and Heterotardigrada corresponds to the range introduced as a prior, based on the date calculated by Guidetti et al. (2017). The split between Apochela and Parachela dates back to 432 million years ago (Mya) with 95% HPD ranging from 323 to 540 Mya, thus it most likely took place in the Palaeozoic but the exact geological period is not possible to ascertain with the current data. The most recent common ancestor of *Milnesium* observed in our tree is estimated to have lived 162 Mya, with 95% HPD ranging from 116 to 207 Mya, thus possibly correlating with the initial stages of the Gondwana breakdown (160–180 Mya, Jokat, Boebel, König, & Meyer, 2003; partly transparent vertical grey bar in Fig. 2). Due to the polytomy present in the basal part of the tree (Fig. 2), it is difficult to estimate the exact ages of particular clades; however the lack of support for these basal nodes suggests a relatively rapid diversification after the first split between the extant lineages in the genus, recovered in our analysis. The split between clades A–D and the remaining species took place ca. 138 Mya, with 95% HPD ranging from 98 to 178 Mya.

Species delimitation

The bPTP analyses identified 76 entities in both the Maximum Likelihood and BI approaches, with supports ranging from 0.65 to 1.00 (0.97 on average). However, after considering the lack of morphological differences (data not shown) and low genetic variance among some of the delimited entities, and considering that bPTP and other tree-based species delimitation methods usually oversplit species into multiple entities (Dellicour & Flot, 2018), we concluded that the following populations represent single species: the eleven *M. tardigradum* populations (indicated as two entities by the bPTP; following Morek et al., 2019a), the six populations of *M. eurystomum* Maucci, 1991 (recovered as three entities by the bPTP; following Morek, Blagden, Kristensen, Michalczyk, 2020a), the three populations of *M. quadrifidum* Nederström, 1919 (indicated as two entities by the bPTP) both populations of *M. variefidum* Morek et al., 2016 (recovered as two entities by the bPTP), *Milnesium* sp. TZ.075, ZA.204 and ZA.180+ZA.218 (indicated as three entities by the bPTP); *Milnesium* sp. ID.432+ID.940, ID.711+ID.950, PH.014 and VN.045 (recovered as four entities by the bPTP); and *Milnesium* sp. AR.437+AR.470 and GF.089+GF.093+GF.193 (indicated as two entities by the bPTP). Thus, we concluded that our dataset comprises 64 species recognised as distinct taxa when both molecular and morphological data were in agreement. Slightly more than a half of the species (around 56%) were represented by a single population (singletons), whereas 44% were formed by multiple populations.

The most abundant species were: *M. tardigradum* (represented by eleven populations), *M. eurystomum* (six populations); *M. inceptum* Morek et al., 2019b (five populations), *M. reductum* Tumanov, 2006 (five populations), and two undescribed species, one from the Oriental realm, *Milnesium* sp. #54 (six populations), and the second one from the South America, *Milnesium* sp. #55 (five populations). Whether the Oriental and South American populations represent single species or groups of species, should be a subject of further research. Among the 64 delimited species, as little as 15 include previously described taxa. The other 49 species are most likely new to science, which would sharply increase the number of recognised species in the genus *Milnesium*, currently amounting to 43 (as of 23.09.2020; Degma, Bertolani, & Guidetti, 2009–2020; Morek, Ciosek, & Michalczyk, 2020b).

Phylogeography and the ‘Everything is Everywhere’ hypothesis

Geography

Overall, the geographic origin of analysed populations correlated with molecular phylogeny (Figs 2–5). Specifically, clade A comprises in the vast majority Palaeartic populations (35/40; 87%) with a small fraction of Afrotropical (4/10; 10%) and a single Nearctic population. Clade B consists of mostly Palaeartic po-

populations (18/23; 78%), with two Afrotropic, two Australian and a single Oriental population. Clade C is composed of African populations, belonging to two realms: Afrotropic (28/31; 90%) and Madagascan (3/31; 10%). Clade D groups eight populations, with seven belonging to the Australian realm (87%) and a single Oriental population (13%). Clade E consists of populations representing multiple realms, but all except one (*Milnesium bohleberi* US.065/species #57) come from a tropical or a subtropical climate: Oriental (7/21; 33%), Neotropic (6/21; 29%), Panamanian (3/21; 14%), Afrotropic (3/21; 14%), Oceanian (1/21; 5%) and Nearctic (1/21; 5%). Finally, clade F consists of three Afrotropical populations. The correlation between the geographic origin and phylogeny is also reflected by the results of the RASP analysis (SM.04), which suggest that all clades except one (clade E) originated in a single realm. Specifically, clades A and B originated in the Palaeartic (99% support), clades C and F in the Afrotropic (99% support), and clade D in the Australian realm (97% support). Only clade E has a mixed origin, with 45% and 44% probability for the Oriental and Neotropic origin, respectively. Thus, even though the two most ‘basal’ lineages have been collected in the Oriental realm, the mixed origin of the remaining species gives the clade the pantropical character (Figs 2, 5).

There are a total of 11 species (17% of all species delineated in our dataset), collected exclusively in a different zoogeographic realm than the majority of species in a given clade (Figs 2–4 and Table 4). Such species are termed here as ‘inclusion species’ and they suggest plausible ancient LDD events: a preliminarily delineated species *Milnesium* sp. nov. US.071 (species #16), from the Nearctic, embedded in the chiefly Palaeartic clade A (Fig. 3); *Milnesium* sp. nov. MY.025 (putative species #19), from the Oriental realm, and *Milnesium* sp. nov. UG.006 (putative species #23), from the Afrotropic realm, both embedded in the mainly Palaeartic clade B (Fig. 3); *Milnesium matheusi* MG (species #33) and *Milnesium wrightae* MG (species #46), from the Madagascan realm, both embedded in the overwhelmingly Afrotropic clade C (Fig. 4); *Milnesium* sp. nov. ID.947 (putative species #53), from the Oriental realm, embedded in the chiefly Australian clade D (Fig. 4). Given that clade E (Fig. 5) consists mainly of a mixture of Oriental and Neotropic lineages, the identification of ‘inclusion species’ is not straightforward in contrast to the remaining major clades which all have a clearly defined geographic origin. Thus, depending whether the Oriental or the Neotropic is assumed as the realm chiefly characterising the clade, species of the other origin could be considered as ‘inclusions’. To overcome this problem, we decided to identify ‘inclusion species’ as lineages that differ in geographic origin from their closest ‘basal’ relatives. Thus, given that the most ‘basal’ lineage comprises an Oriental species (#62), the following species were classified as ‘inclusions’: *Milnesium* sp. nov. BR.007 (putative species #61) found in the Neotropic; *Milnesium bohleberi* US.065 (species #57) from the Nearctic, making it stand out not only geographically but also by climate type; putative new species #56 represented by two populations from the Afrotropic; putative new species #55 represented by five populations from South America; and a preliminarily delineated new species #54 represented by six populations from Far East Asia (see Table 4 and Fig. 5 for details).

Among the 27 species represented by more than one population in our data set, 20 (74%) species were found in a single zoogeographic realm and 7 (26%) species were recorded from more than one realm (Figs 3–5 and Table 5). Such species with wide geographic ranges, termed here as ‘widespread species’, signify recent LDD events: *Milnesium tardigradum* (species #1) and *Milnesium berladnicorum* (species #3), both present in the Palaeartic and the Afrotropic (both in clade A; Fig. 3); *Milnesium inceptum* (species #26), found in the Palaeartic, Afrotropic and the Australian realm (clade B; Fig. 3); a preliminarily delineated new species #40 recorded from the Madagascan and Afrotropic realms (clade C; Fig. 4); putative new species #54 found in the Oriental and the Oceanic realm (clade E; Fig. 5); putative new species #55 present in the Neotropic and the Panamanian realm (clade E; Fig. 5), and putative new species #58 found in the Neotropic and the Afrotropic (clade E; Fig. 5).

Climate

Milnesium populations were found in all five main climate categories, with the largest number of populations found in the temperate climate (49/127, 39% pops.; clades A–E). Except the smallest clade (F), which represents a single climate category, all other clades comprise species that were found in multiple (three to

four) climate types (Figs 3–5). Species representing both arctic and cool climate were present exclusively in clades A and B, those dwelling in dry climate were present in clades A and C–F, and the tropical climate was recovered in clades B–E. Specifically, clade A includes populations found in the following climate types: cool (21/40, 52%), dry (10/40, 25%) temperate (8/40, 20%), and arctic (1/40, 3%). Clade B comprises populations mainly from cool climate (10/23 44%), with an equal number of species collected in temperate and arctic climates (each 6/23, 26%), and a single tropical population. Clade C groups populations mainly from temperate climate (23/31, 74%) and four populations from dry and tropical climate (13% each). Clade D consist chiefly of populations found in dry climate (5/8, 63%), with an addition of temperate (2/8, 25%) and tropical type (1/8, 12%). In clade E, the majority of populations represent tropical climate (11/21, 52%) with additional populations dwelling in temperate (9/21, 43%) and single in dry climate. Finally, clade F consists of populations originating from dry climate. The RASP analysis (SM.05) showed that lineages within the main six clades are generally correlated with particular climate types, which is evidenced by high supports of the ‘ancestral’ states: 89% and 88% support for cool climate for clades A and B, temperate for clades C, D and E (with 98%, 94% and 84% support, respectively), and dry climate for clade F (97% support).

In the aspect of the EiE hypothesis, it is particularly interesting to compare climate types in which widespread species dwell with climate types dominating in their respective clades. On the other hand, ‘inclusion species’, assuming that they represent ancient LDD events, are less important in relation to the EiE hypothesis, because they had considerable amounts of time to adapt to different climate conditions. Thus, out of 28 non-singleton species (i.e. species represented by more than one population in our dataset), 13 were found in more than one climate type (46%). Except *M. tardigradum* (species #1), whose populations dwell in three different climate types (cool, temperate and dry), the remaining 12 species were found in two climate types (species #2, #3, #6, #8, #13, #18, #20, #26 #28, #40, #55 #58), representing six combinations of climate types: temperate+cool (spp. #2, #6, #8, #26), tropical+temperate (spp. #28, #40, #55), dry+cool (spp. #3, #13), dry+temperate (sp. #58), temperate+arctic (sp. #18), and cool+arctic (sp. #20). Thus, four possible climate type pairs were absent (tropical+dry, tropical+cool, tropical+arctic and dry+arctic). Importantly, however, the missing pairs concern mostly the climate types in which the lowest numbers of species were found, i.e. arctic (8) and tropical (18) climate, whereas all combinations with the most sampled type (temperate) were present.

Reproductive mode

The majority of the analysed populations were identified as most likely (at least facultatively) parthenogenetic (72; 57% populations), whereas dioecious populations added up to 13% (17 populations) and they were scattered along all the clades. Moreover, there were two cases of species with both dioecious and putatively parthenogenetic populations: *M. eurystomum* (GL.045, NO.398 and GL.043, GL.048, GL.052, GB.005, respectively) and an undescribed species from Africa and Tanzania (ZA.204 and TZ.075, ZA.180, ZA.218, respectively). In the remaining 38 populations (30%) where no males were found, the number of specimens were too low to reliably assess the reproduction mode.

Discussion

***Milnesium* phylogeography and the ‘Everything is Everywhere’ hypothesis**

Our phylogeographic analyses provide evidence for limited dispersal abilities of the great majority of *Milnesium* species, suggesting that in general the EiE hypothesis does not explain the geographic distribution of apochelan tardigrades. This conclusion is supported by (i) the homogeneous geographic character of almost all major clades along their evolutionary history (Fig. 2), (ii) the low percentage of ‘inclusion species’ (Figs 3–6 and Table 4), and (iii) the low percentage of species with geographic ranges including more than one zoogeographic realm, with at least some being likely examples of ecdemism caused by human activity (Figs 3–6 and Table 5; see also Gąsiorek et al. 2019a). The following paragraphs discuss these three lines of evidence in detail.

Macroevolutionary patterns

All clades, except clade E, are restricted mostly to single zoogeographic realms, with the percentage of populations recorded in their respective realms varying from 78% to 100% (89% on average) per clade (Fig. 2). Moreover, the vast majority of analysed species (89%; 57/64) are restricted to a single realm. This is also translated to unequivocal results of the RASP analysis, which indicated a single zoogeographic zone as the place of origin of all clades except clade E (97%–99% and 45% support, respectively). In other words, most species in five of the six major clades recovered in our analysis, i.e. clades A–D and F, seem to have evolved and remained restricted to a single zoogeographic realm during their evolutionary history. In contrast, clade E, comprising widely distributed lineages across tropics and subtropics (Fig. 5), seems to support the EiE hypothesis to some extent.

Clade E (Fig. 5) is the most biogeographically complex group, which is also mirrored in the RASP analysis, with nearly equally supported Oriental (45%) as well as a Neotropical (44%) origin of the ancestor of this clade. In fact, clade E should be regarded as pantropical, considering that it comprises populations from multiple zoogeographic realms distributed along low latitude and often also low altitude areas in: the Orient (7 populations), Neotropic (6 pops), Afrotropic (3 pops), the Panamanian realm (3 pops), and the Oceanic realm (1 pop). Importantly, however, among the nine species preliminarily delineated in clade E, only three (#54, #55, and #58) were found in more than one tropical or subtropical zoogeographic realm (but see two subsections down for more details suggesting that only species #58 can be considered as naturally pantropical). The remaining six species are restricted to single zoogeographic zones, which suggests that dispersal, although it is more frequent than in other clades, is still rare (i.e. one event every several My; see Fig. 5). The pantropical character of clade E may be explained by LDD mediated by trade winds which tend to channel both northern and southern subtropical air masses that could drag tardigrade propagules towards and along the equator and effectively disperse them in a pantropical manner (as was observed in some insects, e.g. Gatehouse, 1997 or plant seeds, e.g. Nie, Deng, Meng, Sun, & Wen, 2013; which may be ecologically similar to dormant stages of tardigrades or their eggs). Given that low altitude tropical regions (which constitute the majority of the tropics) are characterised by similar climate conditions and habitats (mainly tropical and temperate climate types), tardigrade species exhibiting tropical preferences are likely to proliferate and dwell on any continents and islands along the equator provided they had a chance to arrive to there. In contrast, longitudinal dispersal may be much more difficult, as the latitudinally arranged subtropical and tropical areas may constitute a significant barrier, sort of a firewall, for species with temperate climate preferences that could potentially be dispersed by birds migrating between the north and south hemisphere (Mogle, Kimball, Miller, & McKown, 2018; but note that direct evidence for avian dispersal is missing).

Considering that clades C–F comprise exclusively tropical and subtropical species (except for *M. bohleberi* US.065/species #57 in clade E), it is intriguing why only clade E is pantropical, whereas clades C–D and F are mostly restricted to single zoogeographic realms (Afrotropic and the Australian realm; see Fig. 4 vs Fig. 5). We hypothesise that this could be due to the large geographic distances between South Africa and Australia (ca. 7,800 km) for the southern westerlies to efficiently disperse tardigrades between the continents compared to much smaller distances between land masses within the rings of trade winds (e.g. a continuous land mass between the east coast of northern Africa and west parts of the Orient, or ca. 3,000 km between the west coast of Africa and east coast of South America). In other words, the further south from the equator, the less likely it is to disperse pantropically. Thus, South Africa and Australia may be more efficiently isolated than central Africa and Orient, which is reflected in the geographically conservative compositions of clades C and D. If this hypothesis is true, when the Nearctic is sampled to a greater extent than in the present study, we should expect to observe at least some Holarctic tardigrade species because there are nearly continuous land masses extending from Western Europe via Siberia to North America, and the distance between the western coast of Europe and east coast of North America are separated by ca. 3,000 km. If, however, the latitudinal dispersal is restricted to the tropics and subtropics, then we should observe distinct Palaearctic and Nearctic clades in the northern hemisphere.

Our hypothesis about the limited dispersal capability of *Milnesium* species is also supported by the temporal correlation of the diversification of the six main clades recovered in our study with the initial stages of

the Gondwana breakdown (but see also the subsection ‘Diversification of the six main lineages’ below). Obviously, both dates are burdened with a considerable estimation error, however, it cannot be ruled out that there is a causal relationship between the Gondwana split and ancient age of the clades that are characterised by geographic clustering. Under this scenario, the barriers created by oceans wedging between the new continents provided effective geographic isolation to drive divergence between the clades. Thus, *Milnesium* lineages generally rarely dispersed between the drifting continents, which resulted in the extant pattern in which the major clades are still strongly correlated with their geographic origin. Further studies, including more populations from so far unsampled parts of the world, should be carried out to test whether this correlation is maintained. Ideally, the areas should include former Gondwanan fragments, such as India, the southern parts of South America and the Antarctic, as they could provide crucial data for untangling the origin and early diversification of the genus.

‘Inclusion species’ – evidence for sporadic ancient dispersal?

Among the 64 delineated species, we identified only 11 (17%) that were collected in a different zoogeographic realm than the majority of species in their respective clades (we termed such species as ‘inclusion species’ as their geographic localities stand out from the localities of other species in their clades; Table 4). Given that all these species diverged from their closest relatives (as identified in our dataset) ca. 13–91 Mya (47 Mya, on average), we interpret them as examples of ancient dispersal. Although we do not know when the dispersal occurred, the deep divergences observed between ‘inclusion species’ and their most close relatives suggest that the colonisations of new zoogeographic realms occurred in the deep past rather than recently. Alternatively, ‘inclusion species’ may be artefacts resulting from undersampling. Specifically, it is possible that if more populations of these species are collected (currently, all except three are singletons), these new populations will represent the dominating zoogeographic realm in the clade. In such a case, ‘inclusion species’ would become examples of species with wide geographic ranges, indicating recent dispersal events. Thus, more intense sampling is required to test these alternative hypotheses. Nevertheless, current data support the first hypothesis about ancient dispersal and although the existence of ‘inclusion species’ constitutes evidence that LDD occurs in *Milnesium*, the low percentage of such incidents suggests that LDD is rare. Moreover, the classification of one of the ‘inclusion species’, the Oriental *Milnesium* sp. nov. ID.947 (species #53) is questionable, because it was collected in Sulawesi, i.e. in the proximity of the Australian realm in which all other species in clade D were found, therefore it is an example of a short-distance dispersal (SDD) rather than LDD, even though the new locality lies in a different realm. Another examples of a potential SDD could be the two Madagascan species (#33 and #46) embedded in the otherwise South African clade C.

Species with broad geographic ranges – evidence for recent LDD?

Of the 27 species represented by more than one population in our data set, seven (26%) species were found in more than one zoogeographic zone (Figs 3–5 and Table 5). Such species with wide geographic ranges signify recent LDD events, but the question is whether these LDD examples represent natural or human-mediated dispersal. In our opinion, four (57%) of them are likely a result of anthropogenic dispersal: *M. tardigradum* (species #1, clade A), *M. berladnicorum* (species #3, clade A), *M. inceptum* (species #26, clade B), and a preliminarily delineated new species #54 (clade E). Specifically, the presence of Palaearctic species (#1, #3, and #26) in the Republic of South Africa is not surprising, as hundreds of invertebrate species have been unintentionally introduced from Europe to South Africa by humans (e.g. see Janion-Scheepers et al. 2016). The anthropogenic origin of these populations is even more probable, as *M. tardigradum* and *M. berladnicorum* were found in a single sample (ZA.040) and all four African populations of these two species were collected on the roadsides in the Western Cape Province, where the number of invasive species is among the highest in the RSA (e.g. Janion-Scheepers & Griffiths 2020). Interestingly, *M. inceptum* seems to be an example of a cosmopolitan taxon, as it has been reported from five continents and an oceanic island (Europe and Far East Asia in Morek et al. 2019b, Australia in Morek & Michalczyk 2020, and South Africa and New Zealand in the present contribution). In all five cases, *M. inceptum* was found in urban or rural areas, thus possibly it is a synanthropic species whose dispersal is facilitated by human activity, and it would not be surprising if the species is found in other localities around the World that are associated with human settlements.

Finally, species #54 (clade E) seems to be a predominately Oriental taxon, with one of the six analysed populations recorded in the Oceanic realm. Importantly, however, the Oceanic locality (ID.711) comes from Indonesia, as further three populations do (ID.432, ID.940 and ID.950), although these are classified within the Oriental realm. Given that Indonesia is one of the most densely populated countries, with high rates of human travel between the numerous and closely arranged islands, the ID.711 population is likely to be an example of anthropogenic dispersal (from the Oriental to the Oceanic realm). Alternatively, given that the locality in the Oceanic realm is only 650 km away from the closest known locality in the Oriental realm, this could be also a case of natural SDD. Thus, we consider the four abovementioned species as most probably artificially dispersed and therefore not supporting the EiE hypothesis despite their wide geographic ranges.

The remaining three species with broad geographic ranges, i.e. preliminarily delineated new species #40 (clade C), #55 and #58 (both in clade E), could be examples of recent natural dispersal. Except sample ZA.360, they were collected in sparsely populated and/or places that are not popular among tourists (e.g. French Guiana or North Argentina), further decreasing potential of human mediated dispersion. However, populations representing species #40 and #55 were found in adjacent zoogeographic realms: the Afrotropic and the Madagascan realm (ca. 2000 km apart), and the Neotropic and the Panamanian realm (ca. 3500 km apart), respectively. Moreover, there is no geographic barrier in the latter case. Thus, even though the geographic ranges of species #40 and #55 span more than one zoogeographic realm, they probably should not be considered as examples of LDD, but rather as cases of SDD.

Importantly, determining whether recent dispersal events are natural or anthropogenic is notoriously difficult to test in tardigrades (Gąsiorek et al. 2019a), thus mechanisms behind recent dispersal events of all seven species discussed above (Table 5) should be treated as working hypotheses.

Milnesium biology and dispersal

Considering that microscopic size is a key requirement for passive LLD, two factors are likely to limit dispersal abilities of *Milnesium* species: body size and oviposition strategy. The great majority of apochelan species represent the largest known tardigrades, with some individuals exceeding 1 mm in length (e.g. Morek et al. 2020a). Although tardigrades shrink when they enter the cryptobiotic tun stage, milnesiids are still on average larger than other tardigrade genera, which may reduce their dispersal potential. Moreover, *Milnesium* deposit their eggs inside the shed cuticle, so it is the size of the exuvia, that are only slightly smaller than the adult female, instead of the egg itself that determine the dispersal potential. Thus, oviposition strategy may also have a prominent role in the reduction of the dispersal potential in *Milnesium*, in contrast to multiple parachelan genera that freely deposit their eggs (e.g. species of the superfamily Macrobioidea Thulin, 1928). Apart from the size of animals and their propagules, also cryptobiotic abilities and reproductive mode have been hypothesised to affect dispersal potential of microscopic animals (Hörandl, 2009; Fontaneto, 2011; Incagnone et al., 2015). Specifically, greater cryptobiotic survival (both in terms of time and suboptimal environmental conditions) and parthenogenesis are predicted to enhance dispersion. Although *Milnesium inceptum*, the only species of the genus in which cryptobiosis has been investigated (Schill, Steinbrück, & Köhler, 2004; Wang, Grohme, Mali, Schill, & Frohme, 2014) exhibits high cryptobiotic abilities (and large geographic distribution for that matter), this cannot be easily extrapolated onto other species in the genus. Although many milnesiids are found in xerothermic habitats, which suggests good cryptobiotic abilities, variation – as in any other phenotypic trait – should be expected. Therefore, until cryptobiotic capabilities in a number of *Milnesium* spp. under a controlled common garden design are assessed, the effect of this trait on dispersal cannot be evaluated. Furthermore, in the great majority of investigated populations represented by at least ten adults, no males have been found (72/89; 81%), which suggest (at least facultative) parthenogenesis. If this reproductive mode indeed prevails in the genus, the lack of evidence for a widespread LLD suggests that this trait is not a key factor that shapes geographic distributions of *Milnesium* species.

Everything is not Everywhere (Fig. 6)

To sum up, our analyses discussed above suggest that the great majority (72%, 46/64) of *Milnesium* species have restricted distributions, indicating that Everything is *not* Everywhere and that natural LDD events are

rare. Overall, only 28% (18/64) species indicate LDD, either ancient (17%, 11/64) or recent (11%; 7/64); see Tables 4 and 5 for details. Moreover, after discarding examples that are likely to be the results of anthropogenic dispersal (species #1, #3, #26, and #54, all LDD), and cases of dispersal between localities close to the borders of adjacent zoogeographic realms (i.e. the Afrotropic and the Madagascan realm in Africa, the Neotropic and Panamanian zones in South America, and the Australian and Oriental realms) and treat them as examples of SDD rather than LDD (species #33, #40, #46, #53, and #55), then we end up with only 14% (9/64) species exhibiting likely natural LDD: 12% (8/64) species with evidence for ancient LDD (species #16, #19, #23, #54, #55, #56, #57, and #61) and 2% (1/64) species with evidence for recent LDD (species #58). Thus, the great majority of these LDD cases are examples of ancient dispersal and half of them happened in the tropics and subtropics, meaning that LDD is generally rare and most likely to occur in the zones affected by trade winds. In other words, there seem to be evidence for natural (mainly ancient) LDD in only 14% (9/64) species (Fig. 6).

The fact that we found multiple climate types within the majority of the main clades (Figs 3–5), indicates that *Milnesium* species may quickly adapt to new climates or are capable of dwelling in a wide spectrum of environments. Either way, this indicates that geographic ranges of *Milnesium* species should be even wider than the E_iE predicts (i.e. not limited to a single climate type). Thus, since our data suggest that the great majority of species have limited geographic ranges, climate is not the main factor limiting species distribution. In other words, this underlines that *Milnesium* species exhibit limited dispersal abilities and their geographic ranges are determined mainly by paleogeography.

Similar patterns seem to emerge also in other tardigrade groups. For example, the first phylogeny of an echiniscid genus *Bryodelphax* Thulin, 1928 (Heterotardigrada) uncovered two clades: one restricted to the Western Palaearctic and the other to the Orient (Gąsiorek et al., 2020). Furthermore, Gąsiorek et al., (in review) who analysed 64 populations representing 25 species of another echiniscid, *Pseudechiniscus* Thulin, 1911, from 6 continents (8 realms) found that the great majority of species are limited to single zoogeographic zone and only two were found to have pantropical distributions. Finally, among the several known species of the *Echiniscus virginicus* Riggan, 1962 complex, only one has a wide distribution and, again, it is pantropical in character (Gąsiorek et al., 2019b). Thus, in line with our study, works on heterotardigrades suggest that, in general, tardigrades may have limited dispersal abilities and geographic ranges. Moreover, if a tardigrade species happens to exhibit a broad geographic distribution at all, it is usually a pantropical range, which indicates that trade winds might be the key factor needed for LDD of these animals. However, any categorical general conclusions would be premature at this stage, because the accurate delineation of species geographic ranges and detection of LDD requires thorough sampling, whereas detailed molecular faunistic datasets concerning different geographic regions are extremely limited for tardigrades. Thus, even though the present study offers the largest sample size in terms of the number of sequenced species collected in various zoogeographic realms of any tardigrade genus to date, enlarging the dataset both in terms of taxonomy and geography may alter some of the current key conclusions. For example, the proportion of ‘inclusion species’ and species with broad geographic ranges may increase and some of the ‘inclusion species’ may turn out to be species that exhibit wide geographic ranges and lose their current status. If this were indeed true, then it would mean that LDD is more frequent than inferred from this study.

Finally, our results should be compared to other microscopic animals with similar ecology, ideally coexisting with limnoterrestrial tardigrades in the same habitats, such as moss and lichen-dwelling rotifers and nematodes. Unfortunately, global biogeographic analyses are not yet available for nematodes, but several studies concerning rotifers have been conducted (e.g. Mills, Lunt, & Gómez, 2007 or Fontaneto et al., 2008). The most influential study, Fontaneto et al. (2008), based on worldwide sampling (although with a European bias) indicated that rotifer species generally do not conform to the ‘Everything is everywhere’ hypothesis, with the isolation by distance being present and significant. However, many species were found to be widespread and some were classified as cosmopolitan. In contrast to many widespread *Milnesium* species, wide ranges of rotifer species are not hypothesised to be human mediated (Fontaneto et al., 2008). Moreover, whereas widespread tardigrade species are more often found to have pantropical distributions (Gąsiorek et al., 2019b; Gąsiorek et al., in review; this study) rather than disjunct temperate ranges in the northern and

southern hemisphere (Gąsiorek et al., 2019a), the precedence of pantropical distributions has not been found in rotifers (see Fontaneto et al., 2008; Jaturapruerk, Fontaneto, Meksuwan, Pholpunthin, & Maiphae, 2018), as some temperate widespread species were found, e.g., in Europe, Africa and New Zealand. Importantly, we have not identified such disjunct temperate distributions in *Milnesium* that are likely to be natural (see subsection *Macroevolutionary patterns* above). It is premature to explain why bdelloid rotifers have apparently greater LDD potential than apochelan tardigrades, but most obvious could be body size (ca. 200 vs 700–1000 μm) and oviposition strategy (eggs laid freely into the environment *vs* eggs laid in clutches into exuviae).

Molecular clock dating

Molecular clock estimations of the first split within *Milnesium* lineages present in our dataset is dated for the Jurassic, 162 million years (My) ago. Therefore, the analysis confirms the previously hypothesised ancient origin of the genus (Bertolani & Grimaldi, 2000; Guil & Giribet, 2012; Morek & Michalczyk, 2020), which may be even older than the first split observed in our analysis. Moreover, this suggest that the remaining genera within Milnesiidae Ramazzotti, 1962 should be even older, possibly >200 My old (late Triassic/early Jurassic or older). This, however, requires the assumption that all three are valid genera, and are not nested within the currently recognised *Milnesium* lineages. The age of split between the Apochela and the Parachela is dated for 432 Mya (Silurian) but has a very wide 95% HPD, (540 to 323 Mya; early Cambrian to Carboniferous). This is the first ever estimation of this divergence event between the two eutardigrade orders. However, given the scarce available fossil data for *Milnesium* (Bertolani & Grimaldi, 2000) and tardigrades in general (Guidetti et al., 2017), the uncertainty of the exact node ages is expected and needs to be verified and narrowed down by a more robust phylogenetic analysis, encompassing more genetic data for the numerous parachelan lineages.

The molecular clock analysis also allowed us to estimate mutation rates for all four utilised molecular markers (Table 3). As expected, 18S rRNA is characterised by the lowest rate whereas the COI fragment mutates the fastest. Importantly, our estimation of the 18S rRNA mutation rate (2.37×10^{-4} substitutions per site per million years; SSM) is lower than the previously calculated joint 18S and 28S rRNA rate for the entire Ecdysozoa (1.56×10^{-3} SSM; Rota-Stabelli, Daley, & Pisani, 2013) by an order of magnitude. The estimation of the 28S rRNA mutation rate indicated 1.05×10^{-3} SSM, which is also lower than the joined 18S and 28S rRNA for Ecdysozoa. The much lower mutation rates obtained in our analysis indicate that tardigrades are most likely characterised by slower pace of evolution, which is mirrored in the lower mutation rate. However, given that this is the first time that substitution rates have been calculated exclusively for tardigrades, they should be used with extreme caution, especially when applied to other groups of tardigrades. This is so because the substitution rates have been estimated in the genus *Milnesium*, which is characterised by a considerable morphological stasis compared to the parachelans, and thus is it possible that this lineage also exhibits a slower pace of evolution than other tardigrades. Therefore, analyses concerning taxa belonging to all major tardigrade lineages and a larger number of calibration points should be taken into consideration to assess the values of these rates for the entire phylum, or independently for each of the classes and/or orders.

Diversification of the six main lineages

The phyletic relationships obtained in this contribution are overall in line with the first comprehensive phylogeny of the genus *Milnesium* (Morek & Michalczyk, 2020; see also Fig. 2 herein). Three out of the five main clades obtained in the previous paper were also recovered in the analysis presented herein (A, B and D), and only the two smallest clades (E and C in Morek & Michalczyk, 2020, consisting therein of one and two species, respectively) changed their composition and slightly changed their position in the currently presented tree. These changes are not surprising given the inclusion of a large portion of new data in the current analysis (Fig. 2) and the shallow divergence of the deep nodes.

The short branches and weakly supported nodes in the basal part of the tree may suggest ancient and rapid diversification within the genus. On the other hand, such a pattern could be an artefact resulting from fragmentary sampling. Although both taxonomic and geographic sampling in the present study are widest

to date, the considerable number of species that were sequenced for the first time (see also below for details) suggests that new phyletic lineages could be out there, but have not been detected yet. Apart from new species, DNA sequences for the remaining three apochelan genera are likely to shed some strong light on the evolution of the genus and entire family. Moreover, the relatively long branch from the Apochela-Parachela split to the first diversification observed in the ingroup (187–270 My long; see Fig. 2) suggests that there probably were ancient lineages that did not persist to the present time. If this is indeed the case, it is possible that the genus diversified also earlier or later than the diversification recovered in our analysis. However, assuming that our phylogenetic tree accurately reflects the evolutionary history of the genus, the question what may have caused the rapid divergence calls for a hypothesis.

There are two mutually non-exclusive possible explanations for such a scenario: the breakdown of Gondwana and the switch to carnivory of the common ancestor of extant *Milnesium* species. The first hypothesis stems from the coincidence of the divergence and Gondwana breakdown (see Fig. 2). Under this scenario, the force behind the diversification was geographic isolation. The apparent low dispersal abilities of apochelans, as demonstrated in the present study, seem to support this hypothesis. On the other hand, the sister order Parachela started to diversify 83 My earlier and, if continental drift was supposed to be responsible for the diversification of limnoterrestrial tardigrades in general, we should expect to see roughly the same temporal pattern in both apochelans and parachelans. Moreover, it needs to be stressed that the Gondwana breakdown was a long process that did not result in an immediate geographic isolation and our molecular clock analysis is burdened with considerable error, meaning that the time correlation between geology and phylogeny observed in the present study may be accidental.

Whereas the Gondwana split hypothesis concerns an external, abiotic factor, the carnivory hypothesis concerns a physioecological property of *Milnesium* itself. All known species in the genus are strictly carnivorous and are not able to reproduce on a herbivorous diet (Bryndová, Stec, Schill, Michalczyk, & Devetter, 2020). At the same time, all limnoterrestrial heterotardigrades and many eutardigrades are bacteri-, herbi- and/or fungivorous, only with macrobiotids being mostly omnivorous. Apart from tardigrades, there are no predators within this body size range dwelling in habitats occupied by apochelans that would hunt for the same prey (i.e. mainly nematodes, rotifers and non-apochelan tardigrades; Bryndová et al. 2020). In other words, the ecological niche of a microscopic predator in moss and lichen habitats is occupied exclusively by apochelans. Moreover, taking into consideration that global biodiversity grows over geological time (Crame, 2001), it is even more likely than the ecological niche of a microscopic predator was unoccupied at the time of the diversification of the genus *Milnesium* uncovered in our analysis (ca. 160 My ago or even earlier). Assuming that *Milnesium* was carnivorous at the time of the observed lineage diversification, which is the most parsimonious scenario, our analysis also suggests that the genus is older than other tardigrades that feed on micrometazoans, such as the omnivorous *Paramacrobotus* Guidetti, Schill, Bertolani, Dandekar, & Wolf, 2009 (162 My vs 138 My, respectively), further lending credence to this hypothesis. On the other hand, this hypothesis requires a bold assumption that carnivory evolved within a relatively short period of time and relatively late in the natural history of the apochelan lineage (if it appeared earlier, then the divergence should have occurred earlier too). Moreover, the other three apochelan genera (which are probably also carnivorous) must have diverged at a similar time as the ‘basal’ lineages of *Milnesium*. Given that no molecular data for *Bergtrollus* Dastych, 2011, *Limmenius* Horning, Schuster & Grigarick, 1978 and *Milnesioides* Claxton, 1999 are available, this hypothesis cannot be currently tested. Similarly, the Gondwana breakdown hypothesis remains a speculation and may require fossil evidence to be verified, meaning that it is likely to remain untested for a long time.

Milnesium species diversity

The addition of new populations to the dataset resulted in a substantial growth in the number of species analysed in this study. Specifically, compared with the most recent *Milnesium* phylogeny (Morek & Michalczyk, 2020), the number of populations increased from 34 to 127 (3.7 times), the number of delineated species raised from 25 to 64 (2.5 times), and the number of species represented by single population decreased (from 84% to 56%). Although species delineation in this study is preliminary and taxonomic identity of the analy-

sed populations requires further investigation, including a detailed morphological and ontogenetic analyses, the 49 identified putative unknown species constitute a greater number than the number of the currently described species in the genus *Milnesium* (i.e. 43; Morek et al., 2020b). This explicitly demonstrates that that species diversity in this genus is grossly underestimated, indicating that there are hundreds rather than tens of extant *Milnesium* species. This prediction, combined with the apparent limited geographic ranges of the majority of apochelan species, suggests that each survey of any given unexplored or poorly investigated region will almost certainly uncover multiple new species, provided the essential molecular tools are applied in tandem with detailed morphological and ontogenetic analyses to provide the high resolution of species delineation required in the taxonomy of the genus (e.g. see Morek et al., 2016; Surmacz, Morek, & Michalczyk, 2019).

Future directions

Future research on *Milnesium* should concentrate on the Nearctic realm to verify whether taxa from this region are mixed with the species currently known only from the Palaeartic, within clades A and B, or whether they constitute their own, separate clade(s). Given that the Nearctic and the Palaeartic have only limited connection through the Bering strait, but they share the same climate types, mixed clades would support the EiE hypothesis. If, however, Nearctic and Palaeartic species form separate clades, then this would further strengthen the evidence that *Milnesium* species generally exhibit limited natural dispersal abilities. Furthermore, in order to uncover the early evolution of the genus *Milnesium*, sampling ought to focus in Antarctica, Australia and New Zealand, as the areas in the Southern Hemisphere seem to encompass the most basal lineages and are likely to help us understand the effect of the Gondwana breakdown on the early diversification of *Milnesium*.

Crucially, the remaining milnesiid genera need to be included in a phylogenetic framework to date the diversification events within this group more reliably. Moreover, as the basal part of the tree is characterised by shallow branching, which does not allow for resolving the phylogenetic relationships between the major clades, new molecular markers or entire genomes should be analysed. Furthermore, the verification of the mutation rates for other groups of tardigrades could provide important enhancement to the molecular clock analysis, and help us better understand the evolution of this phylum. Last but not least, a greater taxonomic effort should be made to describe the apparently overwhelming number of putative new species in the genus *Milnesium*, without neglecting the need to integratively redescribe the number of classically delineated species.

Acknowledgments

The authors are very grateful to the following friends and colleagues for providing us with some of the samples used in this study: Piotr Gasiorek and Daniel Stec (Jagiellonian University, Poland), Reinhardt M. Kristensen (University of Copenhagen, Denmark), Diane Nelson (East Tennessee State University, USA), Paul Bartels (Warren Wilson College, USA), Maarten Christenhusz (Curtin University, Australia/Plant Gateway, UK), Maciej Barczyk (SBIK-F, Germany) and Yevgen Kiosya. The samples were collected under permits No: CN35-285316, OP 3570/2018 and within a project “Reconstruction of the phylogeny of the order Apochela (Tardigrada: Eutardigrada)” carried within iSimangaliso Wetland Park, RSA, a UNESCO World Heritage Site; all to WM. We are deeply grateful to Carol Simon (Stellenbosch University, RSA) for her invaluable help with the field sampling in South Africa. The study was supported by the Polish National Science Centre via the *Sonata Bis* programme (grant no. 2016/22/E/NZ8/00417 to LM) and via the *Preludium* grant (no. 2019/35/N/NZ8/04487 to WM, supervised by LM). WM is a recipient of the *Etiuda* stipend (2020/36/T/NZ8/00306, funded by the Polish National Science Centre). Open-access publication of this article was funded by the BioS Priority Research Area under the programme “Excellence Initiative – Research University” at the Jagiellonian University in Kraków, Poland.

Author contributions

WM : conceived the study, collected morphological data and part of molecular data, ran phylogenetic analyses, prepared figures, drafted the manuscript and provided part of the funding.

BS : collected remaining molecular data, ran initial phylogenetic analysis, prepared figures and drafted the manuscript.

ALL : ran phylogenetic analyses, prepared figures and drafted the manuscript.

LM : conceived the study, supervised the entire process, drafted the manuscript, prepared figures and provided part of the funding.

References

Baas-Becking, L. G. M. (1934). *Geobiologie of inleiding tot de milieukunde* . The Hague, the Netherlands: W.P. Van Stockum & Zoon.

Baltanás, A., & Danielopol, D. L. (2013). Body-size distribution and biogeographical patterns in non-marine ostracods (Crustacea: Ostracoda). *Biological Journal of the Linnean Society* , 109 , 409–423. doi: 10.1111/bij.12041

Bertolani, R., & Grimaldi, D. (2000). A New Eutardigrade (Tardigrada: Milnesiidae) in Amber from the Upper Cretaceous (Turonian) of New Jersey. *Studies on Fossils in Amber, with Particular Reference to Cretaceous of New Jersey* , 103–110.

Bertolani, R., Rebecchi, L., Giovannini, I., & Cesari, M. (2011). DNA barcoding and integrative taxonomy of *Macrobiotus hufelandi* C.A.S. Schultze 1834, the first tardigrade species to be described, and some related species. *Zootaxa* , 2997 , 19–36. doi: 10.11646/zootaxa.2997.1.2

Binda, M.G., & Pilato, G. (1972). Tardigradi muscicoli di Sicilia (IV Nota). *Bollettino delle sedute della Accademia Gioenia di Scienze Naturali in Catania* 4 , 11 , 47–60.

Bryndova, M., Stec, D., Schill, R. O., Michalczyk, L., & Devetter, M. (2020) Tardigrade dietary preferences and diet effects on tardigrade life history traits. *Zoological Journal of the Linnean Society* , 188 , 865–877. doi: 10.1093/zoolinnean/zlz146

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. doi: 10.1111/j.1755-0998.2011.03073.x

Cesari, M., Bertolani, R., Rebecchi, L., & Guidetti, R. (2009). DNA barcoding in Tardigrada: The first case study on *Macrobiotus macrocalix* Bertolani & Rebecchi 1993 (Eutardigrada, Macrobiotidae). *Molecular Ecology Resources* , 9 , 699–706. doi: 10.1111/j.1755-0998.2009.02538.x

Cesari, M., McInnes, S. J., Bertolani, R., Rebecchi, L., & Guidetti, R. (2016). Genetic diversity and biogeography of the south polar water bear *Acutuncus antarcticus* (Eutardigrada: Hypsibiidae) – Evidence that it is a truly pan-Antarctic species. *Invertebrate Systematics* , 30 , 635–649. doi: 10.1071/IS15045

Claxton, S.K. (1999). *Milnesioides exsertum* gen. n. sp. n., a new tardigrade from Australia (Tardigrada: Milnesiidae). *Zoologischer Anzeiger* , 238 , 183–190.

Crame, J.A. (2001). Taxonomic diversity gradients through geological time. *Diversity and Distributions* , 7 , 175–189. doi: 10.1111/j.1472-4642.2001.00106.x

Dastych, H. (2011). *Bergtrollus dzimbowski* gen. n., sp. n., a remarkable new tardigrade genus and species from the nival zone of the Lyngen Alps, Norway (Tardigrada: Milnesiidae). *Entomologische Mitteilungen aus dem Zoologischen Staatsinstitut und Zoologischen Museum Hamburg* , 15 , 335–359.

Degma, P., Bertolani, R., & Guidetti, R. *Actual checklist of Tardigrada species* . doi: 10.25431/11380.-1178608. Accessed 2020.09.23.

Dellicour, S., & Flot, J. F. (2018). The hitchhiker’s guide to single-locus species delimitation. *Molecular Ecology Resources* , 18 , 1234–1246. doi: 10.1111/1755-0998.12908.

- Doyère, M. (1840). Mémoire sur les Tardigrades. *Annales des Sciences Naturelles , Zoologia, Paris, Series 2 ,14* , 269–362.
- Drummond, A., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* , 22 , 1185–1192. doi: 10.1093/molbev/mss075
- Fenchel, T., Esteban, G. F., & Finlay, B. J. (1997). Local versus Global Diversity of Microorganisms: Cryptic Diversity of Ciliated Protozoa. *Oikos* , 80 , 220–225. doi: 10.2307/3546589
- Fenchel, T., & Finlay, B. J. (2004). The ubiquity of small species: patterns of local and global diversity. *BioScience* , 54 , 777–784. doi: 10.1641/0006-3568(2004)054[0777:TUOSSP]2.0.CO;2
- Foissner, W. (2006). Biogeography and Dispersal of Micro-organisms: A Review Emphasizing Protists. *Acta Protozoologica* , 45 , 111–136.
- Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* , 3, 294–299.
- Fontaneto, D., Barraclough, T. G., Chen, K., Ricci, C., & Herniou, E. A. (2008). Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. *Molecular Ecology* , 17 , 3136–3146. doi: 10.1111/j.1365-294X.2008.03806.x
- Fontaneto, D., Kaya, M., Herniou, E. A., & Barraclough, T. G. (2009). Extreme levels of hidden diversity in microscopic animals (Rotifera) revealed by DNA taxonomy. *Molecular Phylogenetics and Evolution* , 53 , 182–189. doi: 10.1016/j.ympev.2009.04.011
- García-Reina, A., López-López, A., Serrano, J., & Galián, J. (2014). Phylogeographic patterns of two tiger beetle species at both sides of the strait of Gibraltar (Coleoptera: Cicindelini). *Annales de la Société entomologique de France* , 50 , 399–406. doi: 10.1080/00379271.2014.984954
- Garraffoni, A. R. S., & Balsamo, M. (2017). Is the ubiquitous distribution real for marine gastrotrichs? Detection of areas of endemism using Parsimony Analysis of Endemicity (PAE). *Proceedings of the Biological Society of Washington* , 130 (1) , 198–211. doi: 10.2988/17-00011
- Gatehouse, A. G. (1997). Behavior and ecological genetics of wind-borne migration by insects. *Annual Review of Entomology* , 42 , 475–502. doi: 10.1146/annurev.ento.42.1.475
- Gasiorek, P., Stec, D., Zawierucha, K., Kristensen, R. M., & Michalczyk, L. (2018). Revision of *Testechiniscus* Kristensen, 1987 (Heterotardigrada: Echiniscidae) refutes the polar temperate distribution of the genus. *Zootaxa* , 4472 , 261–297. doi: 10.11646/zootaxa.4472.2.3
- Gasiorek, P., Vončina, K., & Michalczyk, L. (2019a). *Echiniscus testudo* (Doyère, 1840) in New Zealand: anthropogenic dispersal or evidence for the ‘Everything is Everywhere’ hypothesis? *New Zealand Journal of Zoology* , 46 , 174–181. doi: 10.1080/03014223.2018.1503607
- Gasiorek, P., Jackson, K. J., Meyer, H. A., Zajac, K., Nelson, D. R., Kristensen, R. M., & Michalczyk, L. (2019b). *Echiniscus virginicus* complex: the first case of pseudocryptic allopatry and pantropical distribution in tardigrades. *Biological Journal of the Linnean Society* , 128 , 789–805. doi: 10.1093/biolinnean/blz147
- Gasiorek, P., Vončina, K., Degma, P., & Michalczyk, L. (2020). Small is beautiful: the first phylogenetic analysis of *Bryodelphax* Thulin, 1928 (Heterotardigrada: Echiniscidae). *Zoosystematics and Evolution* , 96 , 217–236. doi: 10.3897/zse.96.50821
- Gasiorek, P., Vončina, K., Zajac, K., & Michalczyk, L. (In review). Phylogeography and morphological evolution of *Pseudechiniscus* (Heterotardigrada: Echiniscidae). *Scientific Reports* .
- Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology*

Resources , 13 , 851–861. doi: 10.1111/1755-0998.12138.

Guidetti, R., Schill, R.O., Bertolani, R., Dandekar, T. & Wolf, M. (2009). New molecular data for tardigrade phylogeny, with the erection of Paramacrobotus gen. nov. *Journal of Zoological Systematics and Evolutionary Research* , 47 , 315–321. doi: 10.1111/j.1439-0469.2009.00526.x

Guidetti, R., McInnes, S. J., Cesari, M., Rebecchi, L., & Rota-Stabelli, O. (2017). Evolutionary scenarios for the origin of an Antarctic tardigrade species based on molecular clock analyses and biogeographic data. *Contributions to Zoology* , 86 , 97–110.

Guil, N., Sanchez-Moreno, S., & Machordom, S. (2009). Local biodiversity patterns in micrometazoans: Are tardigrades everywhere? *Systematics and Biodiversity* , 7 , 259–268. doi :10.1017/S1477200009003016

Guil, N. (2011). *Molecular approach to micrometazoans. Are they here, there and everywhere?* Chapter 14 in *Biogeography of Microscopic Organisms: Is Everything Small Everywhere?*

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. doi: 10.1111/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.

Heino, J., Bini, L. M., Karjalainen, S. M., Mykra Soininen, J., Galli Vieira, L. C., & Diniz-Filho J. A. F. (2010). Geographical patterns of micro-organismal community structure: are diatoms ubiquitously distributed across boreal streams? *Oikos* , 119 , 129–137. doi: 10.1111/j.1600-0706.2009.17778.x

Hengherr, S., & Schill, R. O. (2018). *Environmental adaptations: cryobiosis* . Chapter 11: Tardigrade Ecology. R. O. Schill (ed.) *Water Bears: The Biology of Tardigrades*. Zoological Monographs 2 pp. 295–310

Holt, B. G., Lessard, J. P., Borregaard, M. K., Fritz, S. A., Araújo, M. B., ... & Rahbek, C. (2013). An Update of Wallace's Zoogeographic Regions of the World. *Science* , 339 , 74–78. doi: 10.1126/science.1228282

Horning, D. S., Schuster, R. O., & Grigarick, A. A. (1978). Tardigrada of New Zealand. *New Zealand Journal of Zoology* , 5 , 185–280.

Hörandl, E. (2009). *Geographical parthenogenesis: opportunities for asexuality* . In: Schön I et al. (eds). *Lost Sex: the Evolutionary Biology of Parthenogenesis*. Springer: Dordrecht. pp 161–186.

Incagnone, G., Marrone, F., Barone, R., Robba, L., & Naselli-Flores, L. (2015). How do freshwater organisms cross the “dry ocean”? A review on passive dispersal and colonization processes with a special focus on temporary ponds. *Hydrobiologia* , 750 , 103–123. doi: 10.1007/s10750-014-2110-3.

Janion-Scheepers, C., Measey, J., Braschler, B., Chown, S. L., Coetzee, L., ... Wilson, J. R. U. (2016). Soil biota in a megadiverse country: current knowledge and future research directions in South Africa. *Pedobiologia* , 59 , 129–175. doi: 10.1016/j.pedobi.2016.03.004

Janion-Scheepers, C., & Griffiths, C. L. (2020). *Alien Terrestrial Invertebrates in South Africa* . In: van Wilgen B., Measey J., Richardson D., Wilson J., Zengeya T. (eds) *Biological Invasions in South Africa. Invading Nature - Springer Series in Invasion Ecology*, vol 14. Springer, Cham. doi: 10.1007/978-3-030-32394-3_7

Jaturapruerk, R., Fontaneto, D., Meksuwan, P., Pholpunthin, P., & Maiphae, S. (2018). Planktonic and periphytic bdelloid rotifers from Thailand reveal a species assemblage with a combination of cosmopolitan and tropical species. *Systematics and Biodiversity* , 16 (2), 128–141. doi: 10.1080/14772000.2017.1353554

Jokat, W., Boebel, T., König, M., & Meyer, U. (2003). Timing and geometry of early Gondwana breakup. *Journal of Geophysical Research* , 108 , 2428. doi: 10.1029/2002JB001802

- Jørgensen, A., Møbjerg, N., & Kristensen, R. M. (2007). A molecular study of the tardigrade *Echiniscus testudo* (Echiniscidae) reveals low DNA sequence diversity over a large geographical area. *Journal of Limnology* , 66 , 77–83. doi: 10.4081/jlimnol.2007.s1.77
- 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 , 3059–66. doi: 10.1093/nar/gkf436
- Katoh, K., & Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinformatics* , 9 , 286–298. doi: 10.1093/bib/bbn013
- Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* , 35 , 1547–1549. doi: 10.1093/molbev/msy096
- 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. doi: 10.1093/gbe/evw171
- Martiny, J., Bohannan, B., Brown, J., Colwell, R. K., Fuhrman, J. A., ... Staley, J. T. (2006). Microbial biogeography: putting microorganisms on the map. *Nature Reviews Microbiology* , 4 , 102–112. doi: 10.1038/nrmicro1341
- Maucci, W. (1954). Tardigradi nuovi della fauna italiana. *Atti della Società Italiana di Scienze Naturali* , 93 , 576–585.
- Maucci, W. (1991). Tre nuove specie di Eutardigradi della Groenlandia Meridionale. *Bollettino del Museo Civico di Storia Naturale di Verona* , 15 , 279–289.
- Maynard Smith, J. (1978). The evolution of sex. Cambridge; New York: Cambridge University Press
- McInnes, S. J., & Pugh, P. J. A. (1998). Biogeography of limno-terrestrial Tardigrada, with particular reference to the Antarctic fauna. *Journal of Biogeography* , 25 , 31–36.
- Michalczyk, L., Wehnicz, W., Frohme, M., & Kaczmarek, L. (2012a). Redescriptions of three *Milnesium* Doyère, 1840 taxa (Tardigrada: Eutardigrada: Milnesiidae), including the nominal species for the genus. *Zootaxa* , 3154 , 1–20.
- Michalczyk, L., Wehnicz, W., Frohme, M., & Kaczmarek, L. (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.
- Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop (GCE), New Orleans, LA, 2010, pp. 1–8, doi: 10.1109/GCE.2010.5676129.
- Mills, S., Lunt, D.H. & Gómez, A. (2007) Global isolation by distance despite strong regional phylogeography in a small metazoan. *BMC Evolutionary Biology* , 7 , 225. doi: 10.1186/1471-2148-7-225
- 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. doi: 10.11646/zootaxa.3253.1.2
- Mogle, M. J., Kimball, S. A., Miller, W. R., & McKown, R. D. (2018). Evidence of avian-mediated long distance dispersal in American tardigrades. *PeerJ* , 6 , e5035. doi: 10.7717/peerj.5035
- Morek, W., Gasiorek, P., Stec, D., Blagden, B., & Michalczyk, L. (2016). Experimental taxonomy exposes ontogenetic variability and elucidates the taxonomic value of claw configuration in *Milnesium* Doyère, 1840 (Tardigrada: Eutardigrada: Apochela). *Contributions to Zoology* , 85 , 173–200. doi: 10.1163/18759866-08502003

- Morek, W., Stec, D., Gasiorek, P., Surmacz, B., & Michalczyk, L. (2019a). *Milnesium tardigradum* Doyère, 1840: The first integrative study of interpopulation variability in a tardigrade species. *Journal of Zoological Systematics and Evolutionary Research* , 57 , 1–23. doi: 10.1111/jzs.12233
- Morek, W., Suzuki, A. C., Schill, R. O., Georgiev, D., Yankova, M., Marley, N. J., & Michalczyk, L. (2019b). Redescription of *Milnesium alpigenum* Ehrenberg, 1853 (Tardigrada: Apochela) and a description of *Milnesium inceptum* sp. nov., a tardigrade laboratory model. *Zootaxa* , 4586 , 35–64. doi: 10.11646/zootaxa.4586.1.2
- Morek, W., & Michalczyk, L. (2020). First extensive phylogeny of the genus *Milnesium* Doyère, 1840 (Tardigrada) reveals no congruence between genetic markers and morphological traits. *Zoological Journal of the Linnean Society* , 188 , 681–693. doi: 10.1093/zoolinnean/zlz040
- Morek, W., Blagden, B., Kristensen, R.M., Michalczyk, L. (2020a). The analysis of inter- and intrapopulation variability of *Milnesium euryostomum* Maucci, 1991 reveals high genetic divergence and a novel type of ontogenetic variation in the order Apochela. *Systematics and Biodiversity* , 18 , 614–632. doi: 10.1080/14772000.2020.1771469
- Morek, W., Ciosek, J. A., & Michalczyk, L. (2020b). Description of *Milnesium pentapapillatum* sp. nov., with an amendment of the diagnosis of the order Apochela and abolition of the class Apotardigrada (Tardigrada). *Zoologischer Anzeiger* , 288 , 107–117. doi: 10.1016/j.jcz.2020.07.002
- Nelson, D. R., Bartels, P. J., & Guil, N. (2018). Chapter 7: Tardigrade Ecology. R.O. Schill (ed.) *Water Bears: The Biology of Tardigrades*. *Zoological Monographs* 2 pp. 163–210.
- Nederström, P. (1919). Die bis jetzt asu Finnland bekannten Tardigraden. *Acta Societatis pro Fauna et Flora Fennica* , 46 (8).
- Nie, Z. L, Deng, T., Meng, M., Sun, H., & Wen, J. (2013). Post-Boreotropical dispersals explain the pantropical disjunction in *Paederia* (Rubiaceae). *Annals of Botany* , 111 , 873–886. doi: 10.1093/aob/mct053
- Peel, M. C., Finlayson, B. L., & McMahon, T. A. (2007). Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences* , 11 , 1633–1644. doi: 10.5194/hess-11-1633-2007
- Pilato, G., & Binda, M. G. (2001). Biogeography and Limno-terrestrial Tardigrades: Are They Truly Incompatible Binomials? *Zoologischer Anzeiger* , 240 , 511–516. doi: 10.1078/0044-5231-00061
- Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., ... Thollesson, M. (2008). Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution* , 48 , 369–371. doi: 48:369-71.10.1016/j.ympev.2008.03.024
- 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 di Milano*, 101 , 275–287.
- Rebecchi, L., & Nelson, D. R. (1998). Evaluation of a secondary sex character in eutardigrades. *Invertebrate Biology* , 117 , 194–198.
- Richters, F. (1926). Tardigrada. *Handbuch der Zoologie* , III , 58–61.
- Riggin, G. T. (1962). Tardigrada of southwest Virginia: with the addition of a description of a new marine species from Florida. *Virginia Agricultural Experimental Station, Technical Bulletin* , 152 , 1–145.
- Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* , 19 , 1572–1574. doi: 10.1093/bioinformatics/btg180
- Rota-Stabelli, O., Daley, A.C., & Pisani, D. (2013). Molecular Timetrees Reveal a Cambrian Colonization of Land and a New Scenario for Ecdysozoan Evolution. *Current Biology* , 2 , 392–398. doi: 10.1016/j.cub.2013.01.026

- 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 Biology* , 207 , 1607–1613. doi: 10.1242/jeb.00935
- Schultze, C. A. S. (1834). *Macrobiotus Hufelandii* animal e crustaceorum classe novum, reviviscendi post diuturnam asphixiam et ariditatem potens, etc. 8, 1 tab. *C. Curths, Berlin, 6 pp., I Table.*
- Slater, P. L. (1858). On the general Geographical Distribution of the Members of the Class Aves. *Zoological Journal of the Linnean Society* , 2 , 130–136. doi: 10.1111/j.1096-3642.1858.tb02549.x
- Stec, D., Smolak, R., Kaczmarek, L., & Michalczyk, L. (2015). An integrative description of *Macrobiotus paulinae* sp. nov. (Tardigrada: Eutardigrada: Macrobiotidae: *hufelandi* group) from Kenya. *Zootaxa* , 4052 , 501–526. doi: 10.11646/zootaxa.4052.5.1
- Stec, D., Morek, W., Gasiorek, P., Kaczmarek, L. & Michalczyk, L. (2017). Determinants and taxonomic consequences of extreme egg shell variability in *Ramazzottius subanomalous* (Biserov, 1985) (Tardigrada). *Zootaxa* , 4208 , 176–188. doi: 10.11646/zootaxa.4208.2.5
- Stec, D., Morek, W., Gasiorek, P., & Michalczyk, L. (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 , 357–376. doi: 10.1080/14772000.2018.1424267
- Stec, D., Kristensen, R. M., & Michalczyk, L. (2020). An integrative description of *Minibiotus ioculator* sp. nov. from the Republic of South Africa with notes on *Minibiotus pentannulatus* Londoño et al., 2017 (Tardigrada: Macrobiotidae). *Zoologischer Anzeiger* , 286 , 117–134. doi: 10.1016/j.jcz.2020.03.007
- Stec, D., Vecchi, M., Calhim, S. & Michalczyk, L. (202?) New multilocus phylogeny reorganises the family Macrobiotidae (Eutardigrada) and unveils complex morphological evolution of the *Macrobiotus hufelandi* group. *Molecular Phylogenetics & Evolution* . doi: 10.1016/j.ympev.2020.106987
- Surmacz, B., Morek, W., & Michalczyk, L. (2019). What If Multiple Claw Configurations Are Present in A Sample? A Case Study with the Description of *Milnesium pseudotardigradum* sp. nov. (Tardigrada) with Unique Developmental Variability. *Zoological Studies* , 58 , 32. doi:10.6620/ZS.2019.58-32
- Thulin, G. (1911). Beitrage zur Kenntnis der Tardigradenfauna Schwedens. *Arkiv för zoologi* , 7 , 1–60.
- Thulin, G. (1928). Über die Phylogenie und das System der Tardigraden. *Hereditas* , 11 , 207–266. doi: 10.1111/j.1601-5223.1928.tb02488.x
- Tumanov, D. V. (2006). Five new species of the genus *Milnesium* (Tardigrada, Eutardigrada, Milnesiidae). *Zootaxa* , 1122 , 1–23.
- 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. doi: 10.1371/journal.pone.0092663
- Wallace, A. R. (1876). The geographical distribution of animals: with a study of the relations of living and extinct faunas as elucidating the past changes of the Earth’s surface. *Macmillan and Company: Troon, Scotland.*
- Worsaae, K., Kerbl, A., Vang, A., & Gonzalez, B. C. (2019). Broad north Atlantic distribution of a meiobenthic annelid – against all odds. *Scientific Reports* , 9 , 15497 doi: 10.1038/s41598-019-51765-x
- Vaidya, G., Lohman, J. D., & Meier, R. (2011). SequenceMatrix: concatenation software for the fast assembly of multigene datasets with character set and codon information. *Cladistics* , 27 , 171–180. doi: 10.1111/j.1096-0031.2010.00329.x

Yu, Y., Blair, C., & He, X. J. (2020). RASP 4: Ancestral State Reconstruction Tool for Multiple Genes and Characters. *Molecular Biology and Evolution*, 37(2), 604–606. doi: 10.1093/molbev/msz257

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.

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

Tables and Figure legends

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source
18S rRNA	18S_Tar_Ff1	forward	AGGCGAAACCGCGAATGGCTC	Stec <i>et al.</i> (2017)
	18S_Tar_Rr1	reverse	GCCGCAGGCTCCACTCCTGG	
28S rRNA	28S_Eutar_F	forward	ACCCGCTGAACTTAAGCATAT	Gasiorek <i>et al.</i> (2017) Mironov <i>et al.</i> (2017)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	
ITS-2	ITS2_Eutar_Ff	forward	GCATCGATGAAGAACGCAGC	Stec <i>et al.</i> (2018)
	ITS2_Eutar_Rr	reverse	TCCTCCGCTTATTGATATGC	
COI	COI.Mil.tar_Ff	forward	TATTTTATTTTTGGTATTTGATGTGC	Morek <i>et al.</i> (2017)
	COI.Mil.tar_Rr	reverse	CCTCCCCCTGCAGGATC	
	LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> 1991
	HCO2198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	
	LCO1490_JJ	forward	CHACWAAYCATAAAGATATYGG	Geller <i>et al.</i> 2013
HCO2198_JJ	reverse	AWACTTTCVGGRTGVCCAAARAATCA		

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

mutation rate	18S rRNA	28S rRNA	ITS-2	COI
mean	2.37×10^{-4}	1.05×10^{-3}	3.64×10^{-3}	5.69×10^{-3}
SD	3.00×10^{-5}	1.49×10^{-4}	4.65×10^{-4}	8.78×10^{-4}

Table 2 . Estimated mean mutation rates for the four used molecular markers calculated in the BEAST. The values indicate the number of substitutions per site per million years, SD = Standard Deviation.

Most recent common ancestor	Estimated age (95% HPD) [Mya]	Estimated age (95% HPD) [Mya]	Estimated age (95% HPD) [Mya]
	Minimal	Mean	Max
Eutardigrada & Heterotardigrada	545	547	548
Apochela & Parachela	323	432	540
all <i>Milnesium</i>	116	162	207
<i>Milnesium</i> clade (A+B) & (C+D)	98	138	178
<i>Milnesium</i> clade A & B	81	115	148
<i>Milnesium</i> clade C & D	90	128	165

Table 3 . Estimated ages for the main divergence events in the phylogenetic tree (Fig. 2). HPD = Highest Posterior Density; Mya = millions years ago.

#Species

(clade)

Inclusion species

Species origin

Clade origin

16 (A)

Milnesium sp. nov.

Nearctic (USA)

Palaeartic

19 (B)

Milnesium sp. nov.

Orient (Malaysia)

Palaeartic

23 (B)

Milnesium sp. nov.

Afrotropic (Uganda)

Palaeartic

33 (C)

Milnesium matheusi

Madagascan (Madagascar)

Afrotropic

46 (C)

Milnesium wrightae

Madagascan (Madagascar)

Afrotropic

53 (D)

Milnesium sp. nov.

Orient (Indonesia)

Australian

54 (E)

Milnesium sp. nov.

Orient/Oceanic (Indonesia, Philippines, Vietnam)

Orient/Neotropic

55 (E)

Milnesium sp. nov.

Neotropic/Panamanian (Argentina, French Guiana)

Orient/Neotropic

56 (E)

Milnesium sp. nov.

Afrotropic (RSA)

Orient/Neotropic

57 (E)

Milnesium bohleberi

Nearctic (USA)

Orient/Neotropic

61 (E)

Milnesium sp. nov.

Neotropic (Brazil)

Orient/Neotropic

Table 4. ‘Inclusion species’, i.e. species that were found in a different zoogeographic realm than the majority of species in a given clade (such species suggest ancient LDD).

#Species (clade)	Species	Afrotropic	Palaeartic	Madagascar	Neotropic	Australian	Orient	Panamanian	Oceania
1 (A)	<i>Milnesium tardigradum</i>								
3 (A)	<i>Milnesium berladnicorum</i>								
26 (B)	<i>Milnesium inceptum</i>								
40 (C)	<i>Milnesium</i> sp. nov.								
54 (E)	<i>Milnesium</i> sp. nov.								/
55 (E)	<i>Milnesium</i> sp. nov.				/			/	
58 (E)	<i>Milnesium</i> sp. nov.								

Table 5. Species recorded from more than one zoogeographic realm, i.e. evidence for recent LDD (-

the most probable realm of origin; – most probably natural dispersion; – most probably anthropogenic dispersion).

Figure 1. Approximate localities of the 127 *Milnesium* populations analysed in this study (see Supplementary Materials SM.01 for details). Yellow circles indicate the newly analysed populations (N=83), whereas red circles stand for data retrieved from the literature (N=44).

Figure 2. Simplified Bayesian Inference time calibrated phylogenetic tree based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST (left) and its relation to the most recent published phylogeny of *Milnesium* by Morek & Michalczyk (2020) (right). The left side tree: the upper values at nodes indicate the 95% Highest Posterior Density, which is graphically presented as the blue bar, whereas the lower values provide the Posterior Probability (PP) supports. Yellow circles superimposed on nodes indicate the two calibration points used as tree priors. The dashed branches indicate phylogenetic uncertainty. The main geological eras as well as the estimated time of the Gondwana breakup (vertical grey bar) are marked. The Mya stands for Million years ago. The six main clades within *Milnesium* (A–F) are collapsed and their sizes indicate the number of populations within each clade in both phylogenetic trees. The percentages within the collapsed clades indicate the support for the origin of the ancestor in given zoogeographic realm (see Results for more details). See Figs 3–5 for detailed relationships within each of the main clades uncovered in this study. The right side tree: values at nodes represent PP supports and the scale bar shows the number of substitutions per site. The arrows between both trees show the relationship between clades obtained in this contribution and in the phylogeny by Morek & Michalczyk (2020). The pie chart below indicates the number of species detected in the genus *Milnesium* to date divided into three categories (blue – species described under the integrative taxonomy framework; green – classically described species, i.e. of which phylogenetic position is unknown; yellow – undescribed putative new species detected in this study).

Figure 3. *Milnesium* phylogeny: clades A and B reconstructed using the time calibrated Bayesian Inference based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST (see Fig. 2 for the entire tree). Values at nodes represent Posterior Probability (PP) supports and the scale indicates the time before present in Millions of years. Black vertical bars right to the population codes encompass species delineated using both phylogeny and morphology, whereas thin grey vertical lines indicate putative species suggested by the bPTP analysis alone and that did not agree with the integrative species delineation; numbers within vertical black bars are running species numbers (see Results for more details on species delineation). Further right, zoogeographic origin, climate type and reproductive mode are indicated by numbered and differently coloured squares. Colour and number coding are explained in the legend in Fig. 5.

Figure 4. *Milnesium* phylogeny: clades C and D reconstructed using the time calibrated Bayesian Inference based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST (see Fig. 2 for the entire tree). Values at nodes represent Posterior Probability (PP) supports and the scale indicates the time before present in Millions of years. Black vertical bars right to the population codes encompass species delineated using both phylogeny and morphology, whereas thin grey vertical lines indicate putative species suggested by the bPTP analysis alone and that did not agree with the integrative species delineation; numbers within vertical black bars are running species numbers (see Results for more details on species delineation). Further right, zoogeographic origin, climate type and reproductive mode are indicated by numbered and differently coloured squares. Colour and number coding are explained in Fig. 5.

Figure 5. *Milnesium* phylogeny: clades E and F reconstructed using the time calibrated Bayesian Inference based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST (see Fig. 2 for the entire tree). Values at nodes represent Posterior Probability (PP) supports and the scale indicates the time before present in Millions of years. Black vertical bars right to the population codes encompass species delineated using both phylogeny and morphology, whereas thin grey vertical lines indicate putative species suggested by the bPTP analysis alone and that did not agree with the integrative species delineation; numbers within vertical black bars are running species numbers (see Results for more details on

species delineation). Further right, zoogeographic origin, climate type and reproductive mode are indicated by numbered and differently coloured squares. Colour and number coding are explained in the legend at the bottom of the tree.

Figure 6. The summary and interpretation of distribution patterns of *Milnesium* species recovered in this study. Species with limited ranges = species found only in a single zoogeographic realm; widespread species = species found in more than one zoogeographic realm (i.e. suggesting recent Long Distance Dispersal, LDD); ‘inclusion species’ = species that were found in a different zoogeographic realm than the majority of species in a given clade (i.e. suggesting ancient dispersal); SSD = Short Distance Dispersal (refers to species found in localities close to the borders of adjacent zoogeographic realms). Overall, there were 86% species showing no evidence for natural LDD and 14% species suggesting natural LDD (mostly ancient, only rarely recent).

Supplementary Materials

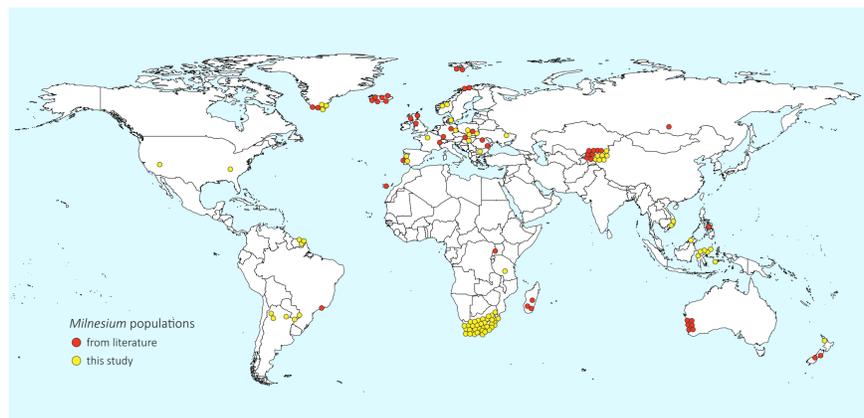
SM.01. Information about collection data of 127 *Milnesium* spp. populations utilised in the study.

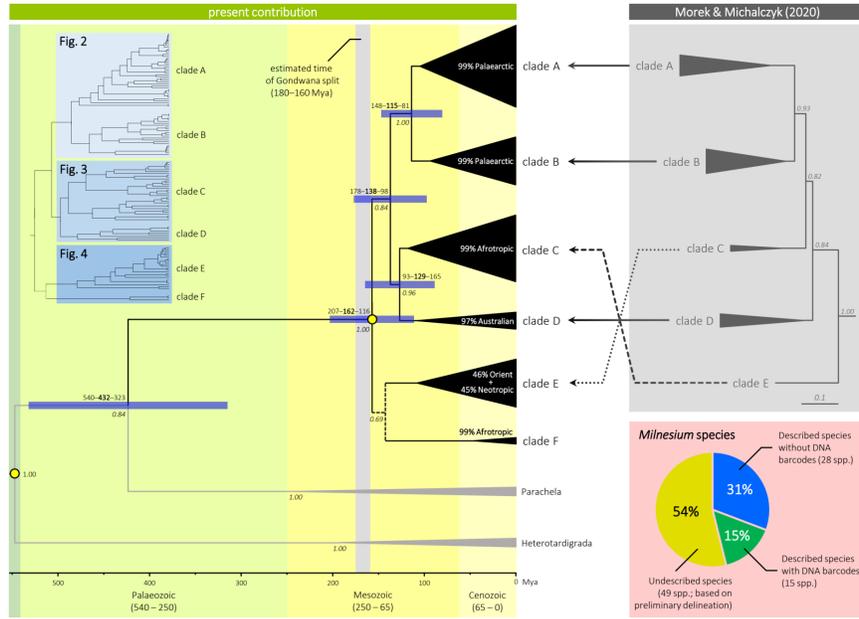
SM.02. GenBank accession numbers for all *Milnesium* spp. sequences used in the analyses. New sequences are bolded.

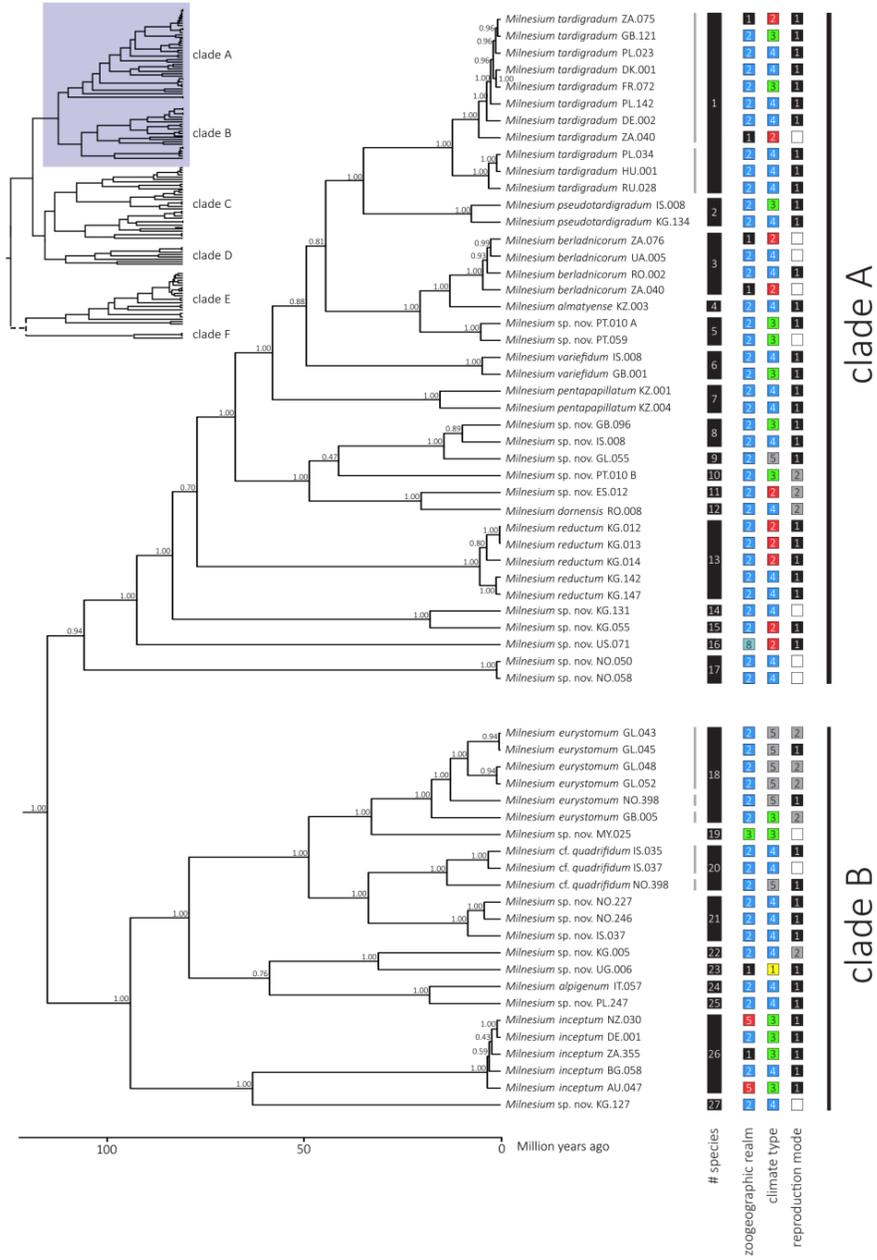
SM.03. The Bayesian Inference phylogenetic tree based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with MrBayes. Values at nodes represent posterior probability (PP) supports. Scale bar shows the number of substitutions per site.

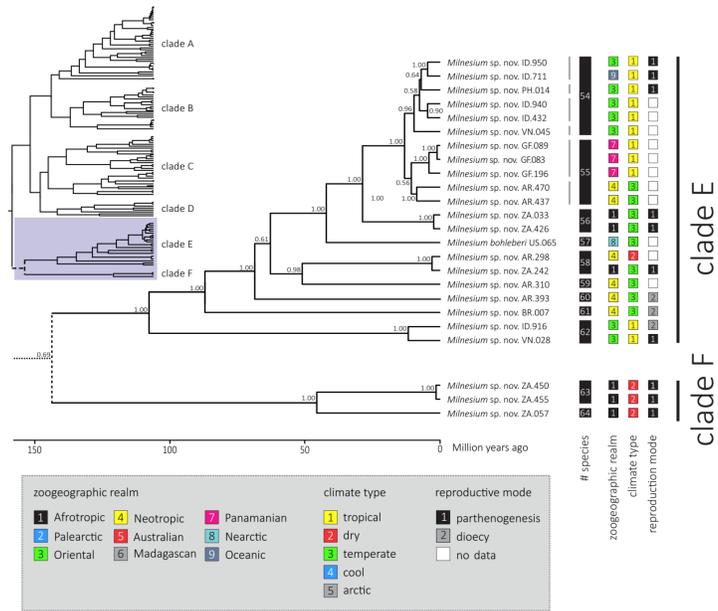
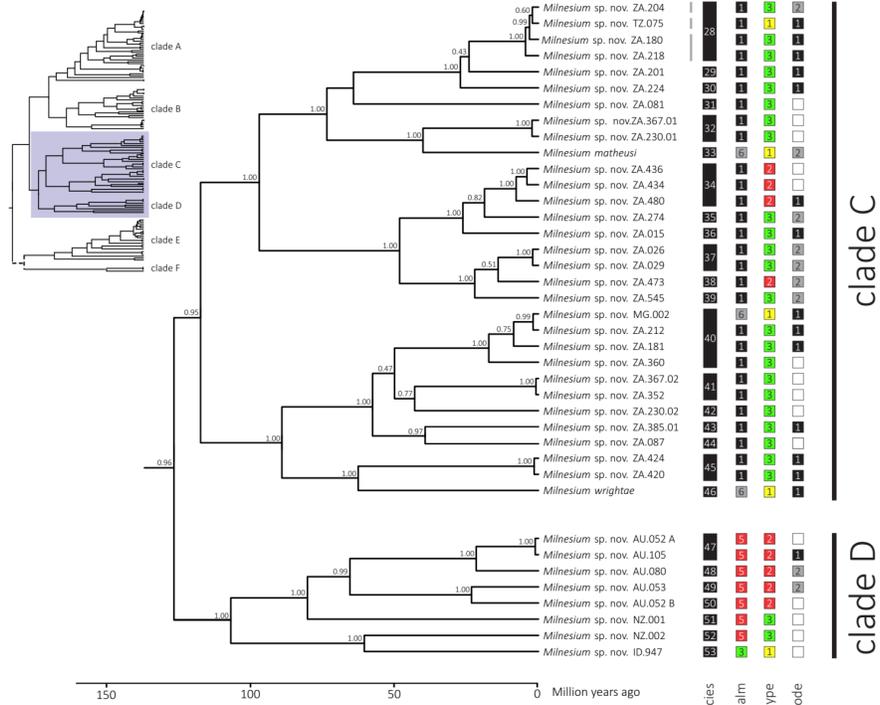
SM.04. The results of the RASP analysis of geographic origin of the ancestor, based on the Bayesian Inference phylogenetic tree based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST.

SM.05. The results of the RASP analysis of climate type of the main clades, based on the Bayesian Inference phylogenetic tree based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST.









zoogeographic realm			climate type			reproductive mode	
1	4	7	1	2	1	1	2
2	5	8	3	4	2	1	1
3	6	9	5	5	1	1	1
			6	6	1	1	1
			7	7	1	1	1
			8	8	1	1	1
			9	9	1	1	1

