

“Everything is not everywhere”: Time-calibrated phylogeography of the genus *Milnesium* (Tardigrada)

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Abstract

There is ample evidence that macroscopic animals form geographic clusters termed as zoogeographic realms, 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 the eutardigrade genus *Milnesium*, seem to question the general applicability of the EiE hypothesis to tardigrade species. Nevertheless, all *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 test the EiE hypothesis more confidently, we considerably enlarged the *Milnesium* data set both taxonomically and geographically, and 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 have influenced the evolutionary history of *Milnesium*. In conclusion, phylogenetic relationships within the genus seem to be determined mainly by paleogeography.

KEYWORDS

Apochela, climate type, EiE, long-distance dispersal (LDD), molecular clock, reproductive mode

1 | 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 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 et al., 2008; Incagnone et al., 2015). Earlier studies based on morphological characters seemed to confirm the EiE hypothesis across different taxonomic groups such as ciliated protozoa, microorganisms or diatoms (e.g. Fenchel et al., 1997; Heino et al., 2010; Martiny et al., 2006). However, with the development of molecular tools, many species of microscopic animals, for example, rotifers or tardigrades, previously thought to be cosmopolitan, were demonstrated to actually be species complex (Cesari et al., 2009; Fontaneto et al., 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. Baltanás & Danielopol, 2013; Fontaneto et al., 2008; Garraffoni & Balsamo, 2017; Gąsiorek et al., 2021; Worsaae et al., 2019), 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) with 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 et al., 2018). Many tardigrade species have been considered cosmopolitan, but the knowledge on the distribution of particular species is usually extremely fragmentary (Gąsiorek, Jackson, et al., 2019). In recent years, the discussion on whether tardigrades support or undermine the EiE hypothesis has been debated (e.g. Guil, 2011; Pilato & Binda, 2001), but only a limited number of geographically and/or taxonomically restricted studies on this subject have so far been conducted (e.g. Cesari et al., 2016; Gąsiorek, Jackson, et al., 2019; Gąsiorek et al., 2020; Guil et al., 2009; Jørgensen et al., 2007; Kaczmarek et al., 2020; Morek & Michalczyk, 2020; Morek et al., 2019). 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 et al., 2011; Morek, Stec, et al., 2019; Stec et al., 2018), 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 (Guidetti et al., 2017; McInnes & Pugh, 1998), which would not be the case if tardigrade species were

generally cosmopolitan. In parallel, anthropogenic dispersal is likely to obscure the natural distribution of tardigrade species (Gąsiorek et al., 2019; Morek, Suzuki, et al., 2019) 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 et al. (2012a), Michalczyk et al. (2012b) and further questioned by Morek, Suzuki, et al. (2019). 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 the phylogenetic position of any given *Milnesium* species than the morphological characters traditionally used in apochelan taxonomy.

Importantly, however, with small sample sizes, 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 sizes may produce artefactual patterns. In other words, the correlation of geography with phylogeny reported by Morek and 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 and Michalczyk (2020), we considerably enlarged the phylogenetic *Milnesium* data set from 34 in Morek and 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).

2 | MATERIALS AND METHODS

2.1 | 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 realms: Afrotropical, Palaearctic, Oriental, Neotropical, Australian, Madagascan, Panamanian, Nearctic and Oceanian (approximate localities are shown in Figure 1; for detailed information see Table S1).

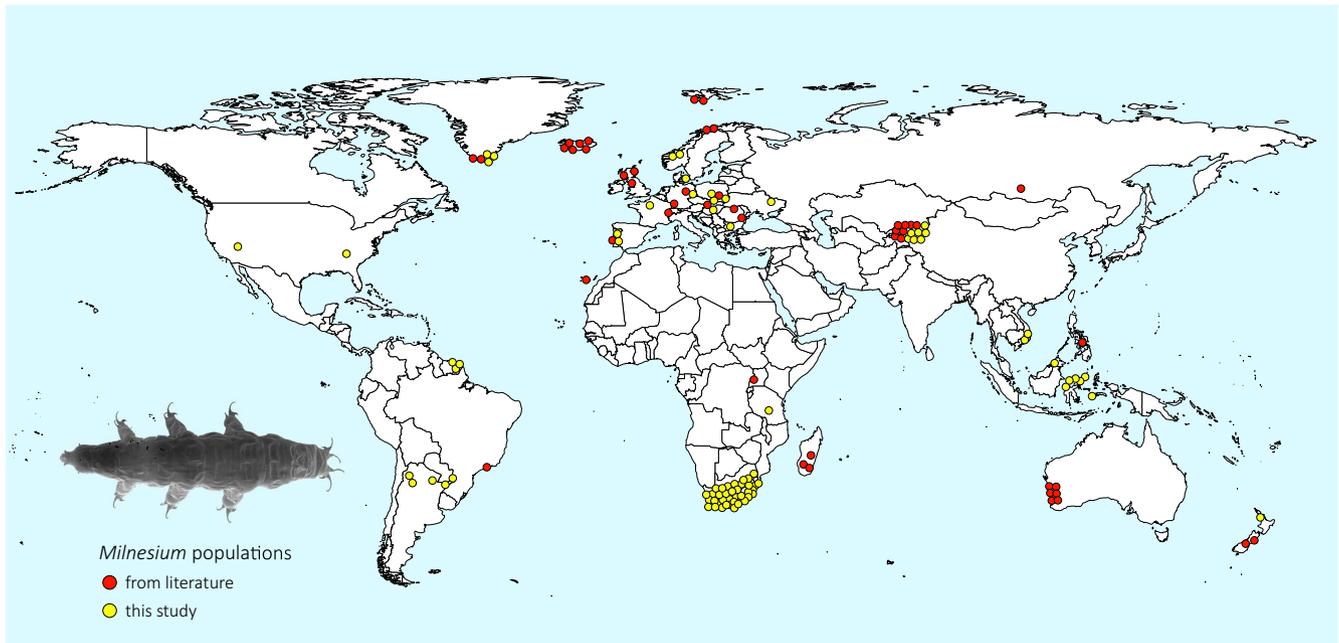


FIGURE 1 Approximate localities of the 127 *Milnesium* populations analysed in this study (see Tables S1 and S2 for details). Yellow circles indicate the newly analysed populations ($N = 83$), whereas red circles stand for data retrieved from the literature ($N = 44$)

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

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source	PCR programme
18S rRNA	18S_Tar_Ff1	Forward	AGGCGAAACCGGAATGGCTC	Stec et al. (2017)	Zeller (2010)
	18S_Tar_Rr1	Reverse	GCCGCAGGCTCCACTCCTGG		
28S rRNA	28S_Eutar_F	Forward	ACCCGCTGAACCTAAGCATAT	Gąsiorek et al. (2018)	Mironov et al. (2012)
	28SR0990	Reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)	
ITS-2	ITS2_Eutar_Ff	Forward	GCATCGATGAAGAACGCAGC	Stec et al. (2018)	Stec et al. (2018)
	ITS2_Eutar_Rr	Reverse	TCCTCCGCTTATTGATATGC		
COI	COI_Mil.tar_Ff	Forward	TATTTATTTTGGTATTGATGTGC	Morek, Stec, et al. (2019)	Morek, Stec, et al. (2019)
	COI_Mil.tar_Rr	Reverse	CCTCCCCTGCAGGATC		
	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	Folmer et al. (1994)
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA		
	LCO1490_JJ	Forward	CHACWAAYCATAAAGATATYGG	Geller et al. (2013)	Folmer et al. (1994)
	HCO2198_JJ	Reverse	AWACTTCVGGRTGVCCAAARAATCA		

2.2 | DNA sequencing

DNA was extracted individually from 1 to 12 specimens (in most cases four) per population following the Chelex 100 resin (Bio-Rad) extraction method by Casquet et al. (2012), with modifications by Stec et al. (2015). After extraction, exoskeletons, if found in the vial, were mounted on permanent microscope slides 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 et al. (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 et al., 2018). All sequences are deposited in GenBank (accession numbers are listed in Table S2).

2.3 | 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 Tables S1–S2), were aligned independently for each marker, using MAFFT version 7 (Katoh et al., 2002; Katoh & 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 1,159 (18S rRNA), 887 (28S rRNA), 841 (ITS-2) and 569 bp (COI). Next, the four obtained alignments were concatenated in SequenceMatrix (Vaidya et al., 2011). We used the Bayesian Information Criterion in PartitionFinder version 2.1.1 (Lanfear et al., 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 and Michalczyk (2020) in MrBayes v3.2 (Ronquist & Huelsenbeck, 2003). Additional Bayesian Inference analyses were carried out in BEAST 1.10.4 (Drummond et al., 2012) using different clock (strict, log-normal relaxed, and exponential relaxed) and tree (coalescent with constant population size vs. 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: (a) we constrained the divergence between Hetero- and Eutardigrada using a normal distribution according to the estimates by Guidetti et al. (2017), and (b) we set a log-normal distribution for the origin of *Milnesium* using the estimated age of the only known fossil assigned to this genus, *Milnesium swolenskyi* Bertolani and Grimaldi (2000), as lower boundary.

The analyses ran for 100 million generations in the CIPRES Science Gateway (Miller et al., 2010), sampling every 10,000 steps. The best combination of clock and tree priors was the 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.

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

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}
Standard deviation	3.00×10^{-5}	1.49×10^{-4}	4.65×10^{-4}	8.78×10^{-4}

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 values 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>).

2.4 | Species delineation

To estimate the number of species present in the data set, we used a molecular species delimitation method, the Poisson tree process (PTP) (Zhang et al., 2013), conducted on the bPTP webserver (<http://species.h-its.org/ptp>), and GYMC, using both single and multithreshold approaches (Fujisawa & Barraclough, 2013; Pons, 2006). We applied the same methodology as in Morek and Michalczyk (2020), constructing the tree based on concatenated COI+ITS-2 sequences. The results were then compared with morphological data and genetic distances to verify the putative species identified by bPTP and GMYC. In parallel to genetic delineation, we attempted to identify species using the latest diagnostic key to the genus (Morek et al., 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 the phylogeny, all species identified and delineated in the present study are given running numbers, from no. 1 to no. 64.

2.5 | 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 nonexclusive ways: (a) by the comparison of geographic localities of populations representing individual species, and (b) 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: (a) 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 (b) closely related species will probably 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: (a) populations representing the same species will be found exclusively in a single zoogeographic realm, and (b) 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 et al., 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. Zoogeographic realms were specified according to Holt et al. (2013), whereas main climate categories follow Peel et al. (2007). The RASP analysis provided information on the geographic origin of the major clades, which allowed for an identification of “inclusion species”, that is, species 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 dispersal abilities (Maynard Smith, 1978), this information (dioecy or parthenogenesis) was also mapped on the phylogenetic tree. The mode was determined with PCM and individual culturing whenever possible (Rebecchi & Nelson, 1998); mode of reproduction was classified as (a) dioecy, when a male specimen was found in the population; (b) parthenogenesis, if the population was cultured and no males appeared, or there were at least 10 adult specimens available and all of them were females; and (c) unknown, when culturing failed and there were fewer than 10 adult females available, as the probability of not detecting males in such cases was too high. Although this approach does not allow for distinguishing between facultative and obligatory parthenogenesis, even facultative parthenogenesis should increase dispersal potential, thus parthenogenesis, whether obligatory or facultative, should be considered when investigating biogeography.

3 | RESULTS

3.1 | 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 Figures 2 and 3, whereas MrBayes

topology is available in Figure S1). As multiple basal nodes in the MrBayes tree are poorly supported/unsupported (PP = 0.56–0.78; see Figure S1), only the topology obtained with BEAST is shown and discussed in detail (Figures 2 and 3).

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 Figure 3a. 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; Figure 3b). Clade C consists of 31 populations representing 19 species and clade D include 8 populations representing seven 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; Figure 3c) is related to a small clade F (PP = 1.00; three populations, classified as two species Figure 3c) but with low support.

The phylogeny largely corresponds to the relationships presented by Morek and Michalczyk (2020) (Figure 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 Figure 2). Clades C and E herein correspond to clades E and C in Morek and Michalczyk (2020), but their positions are different (dashed and dotted arrows in Figure 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 and 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/species.

3.2 | 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 Figure 2), indicating considerable uncertainty in node dating (see also Table 3), which was expected considering 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 probably 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

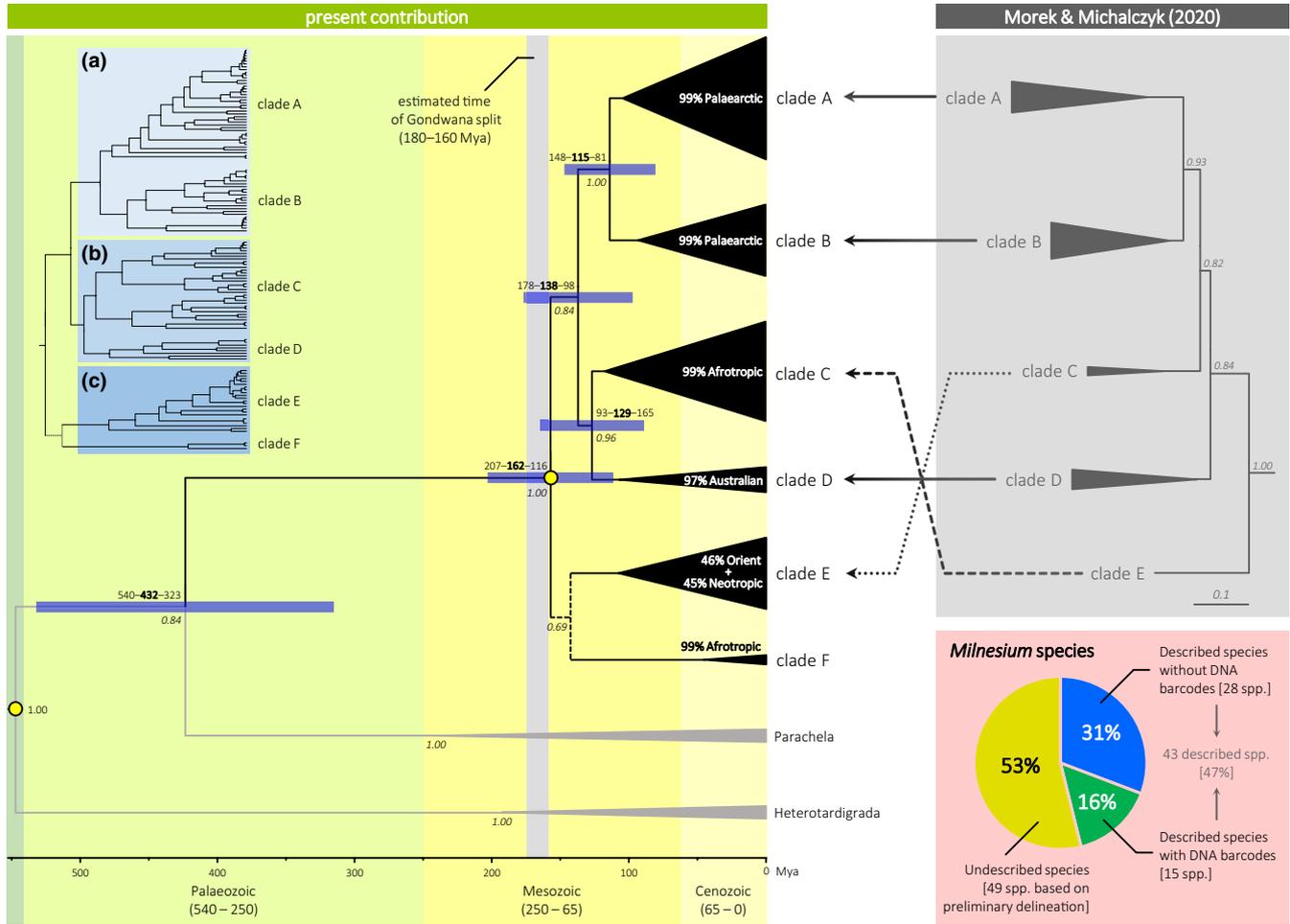


FIGURE 2 Simplified Bayesian Inference time calibrated phylogenetic tree based on concatenated 18S rRNA+28S rRNA+ITS-2+COI nucleotide sequences obtained with BEAST (left) and its relation to the most recent published phylogeny of *Milnesium* by Morek and Michalczyk (2020) (right). **The left side tree:** the upper values above nodes indicate the 95% highest posterior densities, which are graphically presented as horizontal blue bars, whereas the values under the nodes and in italics represent 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. Mya, 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 Figure 3a–c 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 relationships between clades obtained in this contribution and in the phylogeny by Morek and 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, that is, of which phylogenetic position is unknown; yellow – undescribed putative new species detected in this study)

from 116 to 207 Mya, thus possibly correlating with the initial stages of the Gondwana breakdown (160–180 Mya, Jokat et al., 2003; partly transparent vertical grey bar in Figure 2). Due to the polytomy

present in the basal part of the tree (Figure 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

FIGURE 3 *Milnesium* phylogeny reconstructed using the time calibrated Bayesian Inference based on concatenated 18S rRNA +28S rRNA +ITS-2 + COI nucleotide sequences obtained with BEAST: A – clades A and B, B – clades C and D, C – clades E and F (see Figure 2 for the simplified tree). Values at nodes represent posterior probability (PP) supports and the scale indicates the time before present in Millions of years. Black vertical bars to the right of 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 last tree

TABLE 3 Estimated ages for the main divergence events in the phylogenetic tree (Figure 2). Abbreviations: HPD, highest posterior density; Mya, million years ago

Most recent common ancestor	Estimated age (95% HPD) (Mya)		
	Minimal	Mean	Maximal
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

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 (Figure S2), 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 (Figures 2 and 3c).

There are a total of 11 species (17% of all species delineated in our data set) collected exclusively in a different zoogeographic realm than the majority of species in a given clade (Figures 2 and 3b and Table 4). Such species are termed here as "inclusion species" (analogously to inclusions in minerals) 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 (Figure 3a); *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 (Figure 3a); *M. matheusi* (Kaczmarek et al., 2019: species #33) and *M. wrightae* (Kaczmarek et al., 2019: species #46), from the Madagascan realm, both embedded in the overwhelmingly Afrotropic clade C (Figure 3b); *Milnesium* sp. nov. ID.947 (putative species #53), from the Oriental realm, embedded in the chiefly Australian clade D (Figure 3b). Given that clade E (Figure 3c) 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; *M. 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 Figure 3c 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 seven (26%) species were recorded from more than one realm (Figure 3 and Table 5). Such species with wide geographic ranges, termed here as "widespread species", signify recent LDD events: *M. tardigradum* (species #1) and *M. berladnicorum* Ciobanu et al., 2014 (species #3), both present in the Palaeartic and the Afrotropic (both in clade A; Figure 3a); *M. inceptum* (species #26),

TABLE 4 "Inclusion species", that is, 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)	Inclusion species	Species origin	Clade origin
16 (A)	<i>Milnesium</i> sp. nov.	Nearctic (USA)	Palaeartic
19 (B)	<i>Milnesium</i> sp. nov.	Orient (Malaysia)	Palaeartic
23 (B)	<i>Milnesium</i> sp. nov.	Afrotropic (Uganda)	Palaeartic
33 (C)	<i>Milnesium matheusi</i>	Madagascan (Madagascar)	Afrotropic
46 (C)	<i>Milnesium wrightae</i>	Madagascan (Madagascar)	Afrotropic
53 (D)	<i>Milnesium</i> sp. nov.	Orient (Indonesia)	Australian
54 (E)	<i>Milnesium</i> sp. nov.	Orient/Oceanic (Indonesia, Philippines, Vietnam)	Orient/Neotropic
55 (E)	<i>Milnesium</i> sp. nov.	Neotropic/Panamanian (Argentina, French Guiana)	Orient/Neotropic
56 (E)	<i>Milnesium</i> sp. nov.	Afrotropic (RSA)	Orient/Neotropic
57 (E)	<i>Milnesium bohleberi</i>	Nearctic (USA)	Orient/Neotropic
61 (E)	<i>Milnesium</i> sp. nov.	Neotropic (Brazil)	Orient/Neotropic

TABLE 5 Species recorded from more than one zoogeographic realm, that is evidence for recent LDD (● – the most probable realm of origin; ○ – most probably natural dispersion; ◻ – most probably anthropogenic dispersion)

#Species (clade)	Species	Afrotropic	Palearctic	Madagascar	Neotropic	Australian	Orient	Panamanian	Oceanic
1 (A)	<i>Milnesium tardigradum</i>	○	●						
3 (A)	<i>Milnesium berladorum</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.				●				

found in the Palearctic, Afrotropic and the Australian realm (clade B; Figure 3a); a preliminarily delineated new species #40 recorded from the Madagascar and Afrotropic realms (clade C; Figure 3b); putative new species #54 found in the Oriental and the Oceanic realm (clade E; Figure 3c); putative new species #55 present in the Neotropic and the Panamanian realm (clade E; Figure 3c), and putative new species #58 found in the Neotropic and the Afrotropic (clade E; Figure 3c).

3.4.2 | 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% populations; 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 (Figure 3). Species representing both arctic and cool climates were present exclusively in clades A and B, those dwelling in the 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 the 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 the temperate climate (23/31, 74%) and four populations from dry and tropical climates (13% each). Clade D consist chiefly of populations found in the dry climate (5/8, 63%), with an addition of temperate (2/8, 25%) and tropical types (1/8, 12%). In clade E, the majority of populations represent the tropical climate (11/21, 52%) with additional populations dwelling in the temperate (9/21, 43%) and one in the dry climate (1/21, 5%). Finally, clade F consists of populations originating from the dry climate. The RASP analysis (Figure S3) 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 the cool climate for clades A and B, temperate for clades C, D and E (with 98%, 94% and 84% support, respectively), and the dry climate for clade F (97% support).

With respect to 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 nonsingleton species (i.e., species represented by more than one population in our data set), 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.

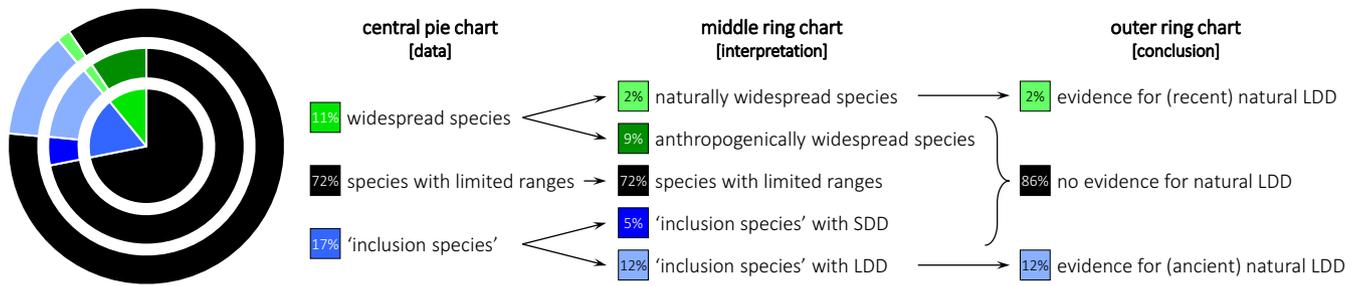


FIGURE 4 The summary and interpretation of distribution patterns of *Milnesium* species recovered in this study. Species with limited ranges = species found only in a single zoogeographic realm; widespread species = species found in more than one zoogeographic realm (i.e., suggesting recent long distance 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)

#2, #6, #8, #26), tropical+temperate (spp. #28, #40, #55), dry+cool (spp. #3, and 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 that is, arctic (8) and tropical (18) climate, whereas all combinations with the most sampled type (temperate) were present. The probability of obtaining such results by chance, as a consequence of unequal sample sizes, was tested by permutation using 10,000 random assignments of climate type to the populations. The obtained *p*-values indicate the probability of sharing no species between a given pair of climate types under the null hypothesis ("climate type has no effect on species distribution"). The results of the tests indicated significant *p*-values for tropical+dry ($p=0.002$) and tropical+cool ($p<0.001$). Higher probabilities of the null hypothesis were indicated in the dry+arctic ($p=0.074$) and tropical+arctic ($p=0.135$) combinations, as a consequence of the lowest sample size in the arctic zone. Thus, we may conclude that observed patterns are most probably not sampling artefacts (although it must be taken into account that spatial autocorrelation of sampling sites may inflate the statistical significance).

3.4.3 | Reproductive mode

The majority of the analysed populations were identified as most probably (at least facultatively) parthenogenetic (72; 57% populations), whereas dioecious populations added up to 13% (17 populations) and they were scattered across all clades. Moreover, there were two cases of species with both dioecious and putatively parthenogenetic populations: *M. eurystomum*/species #18 (GL.045, NO.398 and GL.043, GL.048, GL.052, GB.005, respectively) and an undescribed species #28 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 was too low to reliably assess the reproductive mode.

4 | DISCUSSION

4.1 | *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 (a) the homogeneous geographic character of almost all major clades along their evolutionary history (Figure 2), (b) the low percentage of inclusion species (Figures 3 and 4 and Table 4), and (c) the low percentage of species with geographic ranges including more than one zoogeographic realm, with at least some probably being examples of ecademy caused by human activity (ecademy = the opposite of endemic, that is, an alien addition to a local fauna; Figures 3 and 4, and Table 5; see also Gąsiorek, Vončina, et al., 2019). The following paragraphs discuss these three lines of evidence in detail.

4.1.1 | 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 (Figure 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, that is, 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 (Figure 3c), seems to support the EiE hypothesis to some extent.

Clade E (Figure 3c) 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 following realms: Oriental (7 populations), Neotropical (6), Afrotropical (3), Panamanian (3), and Oceanic (1). 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 subsection 4.1.5 below 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 Figure 3c). The pantropical character of clade E may be explained by LDD mediated by trade winds that tend to channel both northern and southern subtropical air masses, which 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 et al., 2013). 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 of the continents and islands along the equator provided they had a chance to arrive 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 et al., 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 Figure 3b vs. Figure 3c). 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 for the species to disperse pantropically. Thus, South Africa and Australia may be more effectively isolated than central Africa and the 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, where trade winds operate, 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 subsection 4.3 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 the 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.

4.1.2 | 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, similarly to inclusions in otherwise monomorphic minerals; Table 4). Given that all these species diverged from their closest relatives (as identified in our data set) 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 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. Other examples of a potential SDD could be the two Madagascan species (#33 and #46) embedded in the otherwise South African clade C.

4.1.3 | 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 (Figure 3 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 probably 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 Palearctic 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 together in one of the samples (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, Suzuki, et al., 2019; Australia in Morek & Michalczyk, 2020; North America in Nelson et al., 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 it could be 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 do three other populations (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, that is, 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 the potential of human mediated dispersion. However, populations representing species #40 and #55 were found in adjacent zoogeographic realms: the Afrotropic and the Madagascan realms (ca. 2,000 km apart), and the Neotropic and the Panamanian realms (ca. 3,500 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 (Gašiorek, Vončina, et al., 2019), thus mechanisms behind recent dispersal events of all seven species discussed above (Table 5) should be treated as working hypotheses.

4.1.4 | *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, Blagden, et al., 2020). Although tardigrades shrink when they enter the cryptobiotic tun stage, milnesiids are still on average larger than other tardigrade genera, which may reduce dispersal potential of apochelan species. Moreover, *Milnesium* deposit their eggs inside the shed cuticle, so it is the size of the exuviae, which are only slightly smaller than the adult female, instead of the egg itself that determines 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 Macrobiotioidea [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 (Fontaneto, 2011; Hörandl, 2009; 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 et al., 2004; Wang et al., 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. are compared in a common garden experiment, the effect of this trait on dispersal cannot be evaluated. Furthermore, in the great majority of investigated populations represented by at least 10 adults, no males have been found (72/89; 81%), which suggests (at least facultative) parthenogenesis. If this reproductive mode indeed prevails in the genus, the lack of evidence for a widespread LDD suggests that this trait is not a key factor that shapes geographic distributions of *Milnesium* species.

4.1.5 | Everything is not everywhere (Figure 4)

In summary, our analyses 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, Neotropic and Panamanian zones in South America, and 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 probably 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 (Figure 4).

The fact that we found multiple climate types within the majority of the main clades (Figure 3) 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 EIE predicts (that is, 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 reinforces the hypothesis 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 Palearctic and the other to the Orient (Gąsiorek et al., 2020). Furthermore, Gąsiorek et al. (2021), who analysed 64 populations representing 25 species of another echiniscid, *Pseudechiniscus* (Thulin, 1911), from six continents (eight realms) found that the great majority of species are limited to a single

zoogeographic zone and only two were found to have pantropical distributions. Finally, among the several known species of the *Echiniscus virginicus* (Riggin, 1962) complex, only one has a wide distribution and, again, it is pantropical in character (Gąsiorek, Jackson, et al., 2019). 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 data sets 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 data set 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 both monogonont and bdelloid rotifers have been conducted (e.g., Fontaneto et al., 2008; Mills et al., 2007, 2016). The seminal study by 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 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, Jackson, et al., 2019; Gąsiorek et al., in review) rather than disjunct temperate ranges in the northern and southern hemisphere (Gąsiorek, Vončina, et al., 2019; Kaczmarek et al., 2020), the precedence of pantropical distributions has not been found in rotifers (see Fontaneto et al., 2008; Jaturapruet et al., 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 4.1.1 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. 700–1,000 μm) and oviposition strategy (eggs laid freely into the environment vs. eggs laid in clutches into exuviae).

4.2 | Molecular clock dating

Molecular clock estimations of the first split within *Milnesium* lineages present in our data set is dated for the Jurassic, 162 Mya. Therefore, the analysis confirms the previously hypothesised ancient origin of the genus (Bertolani & Grimaldi, 2000; Guil & Giribet, 2012; Morek & Michalczyk, 2020), which may be older than the first split observed in our analysis. Moreover, this suggests that the remaining genera within Milnesiidae (Ramazzotti, 1962) could be even older, possibly >200 million years 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 split between the Apochela and the Parachela is estimated to be 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 fossil data available 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 et al., 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 probably characterised by slower pace of evolution. 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 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 with a larger number of calibration points should be performed to assess the values of these rates for the entire phylum, or independently for each of the classes and/or orders.

4.3 | 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 Figure 2 herein). Three out of the five main clades obtained in the previous study 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 in composition and slightly changed in 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 (Figure 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 the most extensive to date, the considerable number of species that were sequenced for the first time (see subsection 4.4. below for details) suggests that new phyletic lineages could be out there, but have not been detected yet. In addition to new species of *Milnesium*, 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 Figure 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 than what our analysis suggests. 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. A possible explanation for the putative rapid diversification is the breakdown of Gondwana as these two processes seem to coincide (see Figure 2). Under this scenario, the force behind the diversification was geographic isolatione apparent low dispersal abilities of apochelans, as demonstrated in the present study, seem to support this hypothesis. On the other hand, our molecular clock analysis is burdened with considerable error, that is, the time correlation between geology and phylogeny observed in the present study may be accidental. Thus, the Gondwana breakdown hypothesis may require fossil evidence or more molecular data to be verified and therefore it is likely to remain untested for a long time.

4.4 | *Milnesium* species diversity

The addition of new populations to the data set 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 advanced from 25 to 64 (2.5 times), and the number of species represented by single populations decreased (from 84% to 56%). Although species delineation in this study is preliminary and taxonomic identities of the analysed populations require further investigation, including a detailed morphological and ontogenetic analyses, the 49 identified putative new species constitute a greater number than the number of the currently described species in the genus *Milnesium*

(i.e., 43; Morek, Ciosek, et al., 2020). 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 et al., 2019).

4.5 | 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 Palaearctic, within clades A and B, or whether they constitute their own separate clade(s). Given that the Nearctic and the Palaearctic 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 Palaearctic 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 improvements in the molecular clock analysis, and help us better understand the evolution of this phylum. Last but not least, 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.

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AUTHOR CONTRIBUTIONS

W. M. conceived the study, collected part of molecular data, ran phylogenetic analyses, prepared figures, drafted the manuscript and provided part of the funding. B. S. collected the remaining molecular data, ran initial phylogenetic analysis, prepared figures and drafted the manuscript. A. L.-L. ran phylogenetic analyses, prepared figures and drafted the manuscript. Ł. M. conceived the study, supervised the study, drafted the manuscript, prepared figures and provided part of the funding.

DATA AVAILABILITY STATEMENT

The DNA sequences obtained for the purpose of this study have been deposited in the NCBI GenBank database and the accession numbers are provided in Table S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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