e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 73 (2025), No 1
https://doi.org/10.3409/fb_73-1.01

Integrative approach reveals the first record of *Milnesium guanyinensis* in the Palearctic along with two other *Milnesium* (Tardigrada: Apochela) species from Poland

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Accepted March 03, 2025 *Published online March 07, 2025* *Issue online April 29, 2025*

Original article

Surmacz B., Stec D. 2025. Integrative approach reveals the first record of *Milnesium guanyinensis* in the Palearctic along with two other *Milnesium* (Tardigrada: Apochela) species from Poland. Folia Biologica (Kraków) **73**: 1-9.

We present the first record of *Milnesium guanyinensis* in the Palearctic region (S Poland), along with two other *Milnesium* species (*M. berladnicorum* and *M. inceptum*) that are reported as new for Poland. While recent studies have provided empirical evidence that tardigrades belonging to the genus *Milnesium* exhibit zoogeographical patterns, and only some taxa are recognised as truly cosmopolitan, all of the species reported in our study have broad distribution ranges. We contextualise our findings within the current understanding of tardigrade phylogeography and discuss the potential for anthropogenic long-distance dispersal. The current study increases the number of known *Milnesium* species in Poland from four to seven and raises the total number of tardigrades in Poland to 118. All of the observations are based on an integrative taxonomy approach, which combines a morphological analysis with DNA barcoding.

Key words: phylogeography, biogeography, meiofauna, COI barcoding, microinvertebrates, everything is everywhere.

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Tardigrades are a phylum of microscopic animals inhabiting diverse terrestrial and aquatic environments that are at least occasionally moist, since tardigrades require water to remain active. The species richness observed in the phylum consists of around 1,500 described species (Degma & Guidetti 2024), with many more estimated to be discovered. The tardigrade fauna of Poland has a long history of research, from early studies completed in the 20th century (e.g. Jakubski 1915; Dastych 1988) to recent intensive faunistic inventories (e.g. Kaczmarek *et al*. 2018; Bartylak *et al*. 2024). So far, 115 species of tardigrades have been recorded from Poland (Erdmann *et al*. 2024). Among them, only four species represent the order Apochela, and all belong to the genus *Milnesium* Doyère, 1840. *Milnesium* species are considered to be large (usually 500 µm – 1 mm of length) carnivorous tardigrades, which can feed on other tardigrades, nematodes, rotifers and amoebas (Roszkowska *et al*. 2016). The genus *Milnesium* had been considered as monotypic (represented by a single widespread species – *Milnesium tardigradum* Doyère, 1840), until advances in tardigrade taxonomy allowed for the detection of previously unobserved diversity within the genus (Michalczyk *et al*. 2012). Especially in the last two decades, modern approaches (e.g. morphometry, ontogenetic variability tracking and DNA sequencing) have allowed for the discovery of many species new to science in the genus (e.g. Tumanov 2006; Meyer *et al*. 2013;

Surmacz *et al*. 2019; Sugiura *et al*. 2020; Rocha *et al*. 2022). Currently, the total number of nominal taxa of the genus *Milnesium* is 51 (Degma & Guidetti 2024, excluding fossil species and *nomina dubia*). Apart from these, multiple *Milnesium* species have not yet been formally described, but have been analysed in a phylogeographic context (Morek *et al*. 2021; Guil *et al*. 2022). However, prior to now, the Polish tardigrade fauna was comprised of only four nominal species, namely: *Milnesium tardigradum tardigradum* (e.g. Morek *et al*. 2019a); *Milnesium granulatum* Ramazzotti, 1962 (Kaczmarek *et al*. 2018); *Milnesium dornensis* Ciobanu, Roszkowska & Kaczmarek, 2015 (Kaczmarek *et al*. 2018; Bartylak *et al*. 2024); and *Milnesium beasleyi* Kaczmarek, Jakubowska & Michalczyk, 2012 (Kaczmarek *et al*. 2018; Bartylak *et al*. 2024). Besides these, one population representing an undescribed species was analysed by Morek *et al*. (2021).

Herein, we provide three novel records of *Milnesium* taxa in Poland, confirmed by the classical morphological approach and supplemented with DNA barcodes. Our results contribute to a better understanding of the tardigrade biogeography, as the genus *Milnesium* has been utilised as a model in recent global-scale phylogeographic studies (Morek *et al*. 2021; Guil *et al*. 2022).

Methods

Sample collection and tardigrade extraction

Two moss and two lichen samples were collected between 2023 and 2024 from different localities in southern Poland (Table 1). The material was stored at room temperature within paper envelopes until the tardigrade extraction. The extraction from a portion of each sample was conducted using the standard technique, as described in detail in Stec *et al*. (2015). The tardigrades were manually picked up with a glass pipette under a stereomicroscope. Then, isolated individuals were divided and prepared for different analyses: (i) morphological examination with a light microscope, and (ii) DNA extraction and sequencing (for details, please see the 'Materials examined' section provided below for each new *Milnesium* record). In the sample B23_K.U.8, two *Milnesium* exuviae with eggs were found and stored for two weeks in distilled water at room temperature, allowing the individuals to hatch. Then, we mounted the animals and exuviae on microscope slides to verify the claw configuration changes, by comparing the claw morphology of the exuviae and hatchlings (Surmacz *et al*. 2019).

Microscopy and morphological identification

Specimens for the light microscopy were mounted on microscope slides in a small drop of Hoyer's medium and secured with a cover slip, following the protocol by Morek *et al*. (2016). The slides were then dried for five to seven days at 60 °C. The dried slides were sealed with a transparent nail polish and examined under a Leica DMLB light microscope with phase contrast, associated with a digital camera. The permanent microscope slides have been deposited in the Tardigrada Collection of the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016, Kraków, Poland. All of the figures were assembled in Inkscape 1.0.2-2 (Inkscape Project 2020). For structures that could not be satisfactorily focused on in a single photograph, a stack of 2-3 images was taken with an equidistance of ca. 0.2 µm and assembled manually into a single deep-focus image in GIMP 2.10.36.

In order to confirm the species identification, the newly prepared materials were carefully compared

Table 1

Details on the samples of *Milnesium* spp. used in the study

Sample code	Coordinates	Collection date	Substrate	Species	GenBank accession number (COI)
TUCH.01	49.911°N 21.067°E	2.07.2024	lichen on tree	*M. berladnicorum*	PQ821864 PQ821865
TUCH.07	49.911°N 21.067°E	2.07.2024	lichen on tree	*M. berladnicorum*	PQ821866 PQ821867
PL.048	50.067°N 19.957°E	31.03.2023	moss on tree	*M. inceptum*	PQ821868 PQ821869
B23_K.U.8	49.882°N 20.086°E	7.06.2023	moss on tree	*M. guanyinensis*	PQ821870 PQ821871

with the original descriptions of respective *Milnesium* species (Morek *et al*. 2019b; Ciobanu *et al*. 2014; Yuan *et al*. 2023). To describe the claw configurations, we used the notation introduced by Michalczyk *et al*. (2012).

Genotyping and occurrence data

DNA was extracted from individual animals following the *Chelex® 100* resin (Bio-Rad) extraction method by Casquet *et al*. (2012), with modifications as described in detail in Stec *et al*. (2020). The 658 base pair long fragment of the COI gene was amplified using the LCO1490-JJ and HCO2198-JJ primer set (Astrin & Stüben 2008). The COI fragment was amplified and sequenced, according to the protocols described in Stec *et al*. (2020). Sequencing products were read with the *ABI 3130xl* sequencer at the Genomed company (Warsaw, Poland). The sequences were processed in *BioEdit* ver. 7.2.5 (Hall 1999) and were submitted to GenBank. Prior to the submission, all obtained COI sequences were translated into protein sequences in *AliView* 1.28 (Larsson 2014) to check against pseudogenes. The obtained sequences were also used in an NCBI BLAST (Altschul *et al*. 1990) search, to assess their similarity to sequences already present in GenBank (we analysed the BLAST results with at least a 97% identity), confirming the species identification and finding additional georeferenced occurrences of the species not mentioned in the literature. In addition, to investigate the effect of climatic conditions on the distributions of *M. guanyinensis*, we extracted the mean annual temperature and annual precipitation data from the Worldclim2 dataset (Fick & Hijmans 2017) corresponding to the coordinates of their occurrences.

Results

The materials prepared for the morphological examination indicated the presence of three species of the genus *Milnesium* that were new to Polish fauna. The two analysed lichen samples (TUCH.01, and TUCH.07) contained individuals of *Milnesium berladnicorum*, which is characterised by the presence of pseudopores in the cuticle, as well as a [2-3]-[2-2] claw configuration (CC) in larger specimens (Fig. 1) and [2-2]-[2-2] in the smallest animals. The sample B23_K.U.8 was comprised of individuals of *Milnesium guanyinensis*, which exhibits a strong epicuticular reticulate sculpture visible in the PCM (especially prominent in smaller individuals), with CC [2-3]-[3-2] and CC [2-2]-[2-2] in hatchlings (Figs 2-3). The sample PL.048 contained a population of *Milnesium* having a smooth cuticle and CC [3-3]-[3-3] in all the animals, matching the diagnosis of *M. inceptum* (Fig. 4). Good-quality COI

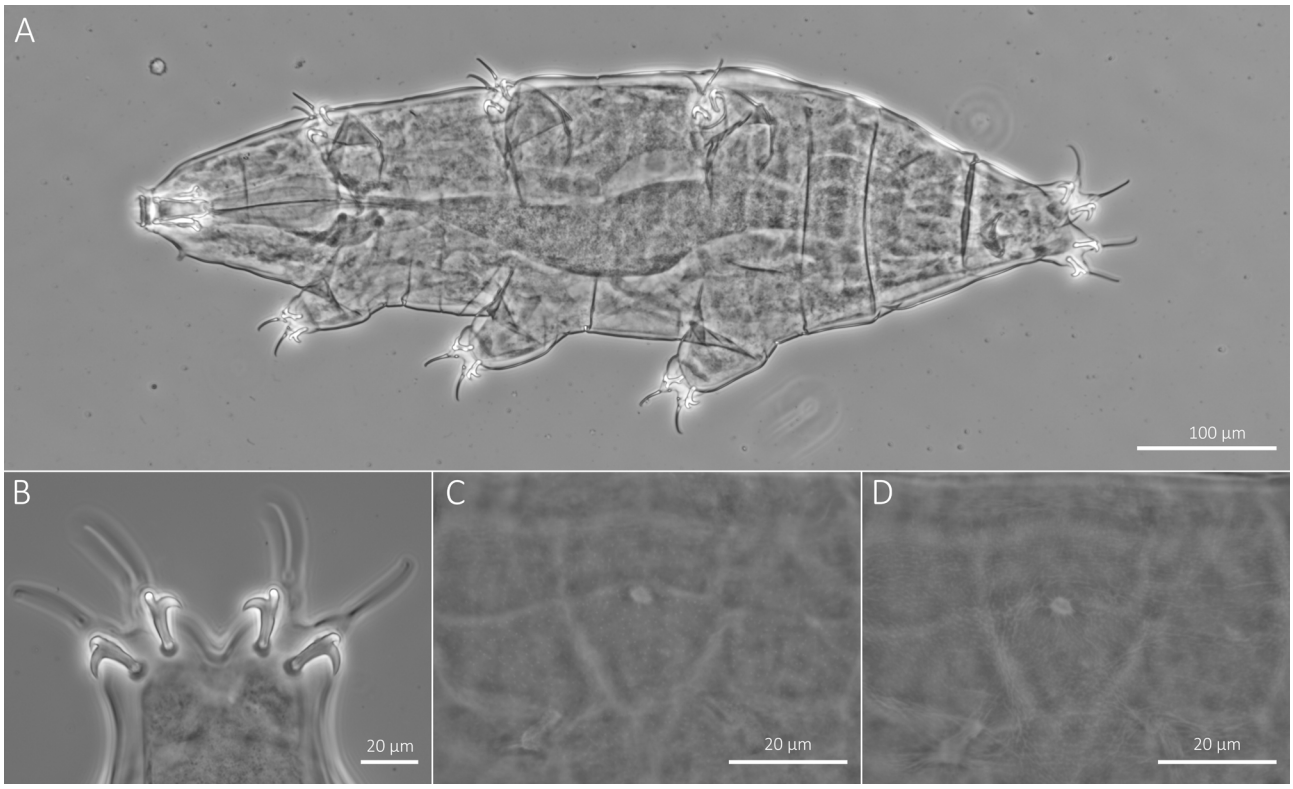

Fig. 1. *Milnesium berladnicorum* from Poland (adult, PCM): A – habitus, dorso-ventral projection; B – claws on the hind legs with two points on secondary branches; C – pseudopores in the dorsal cuticle; D – reticulate sculpture on the dorsal cuticle.

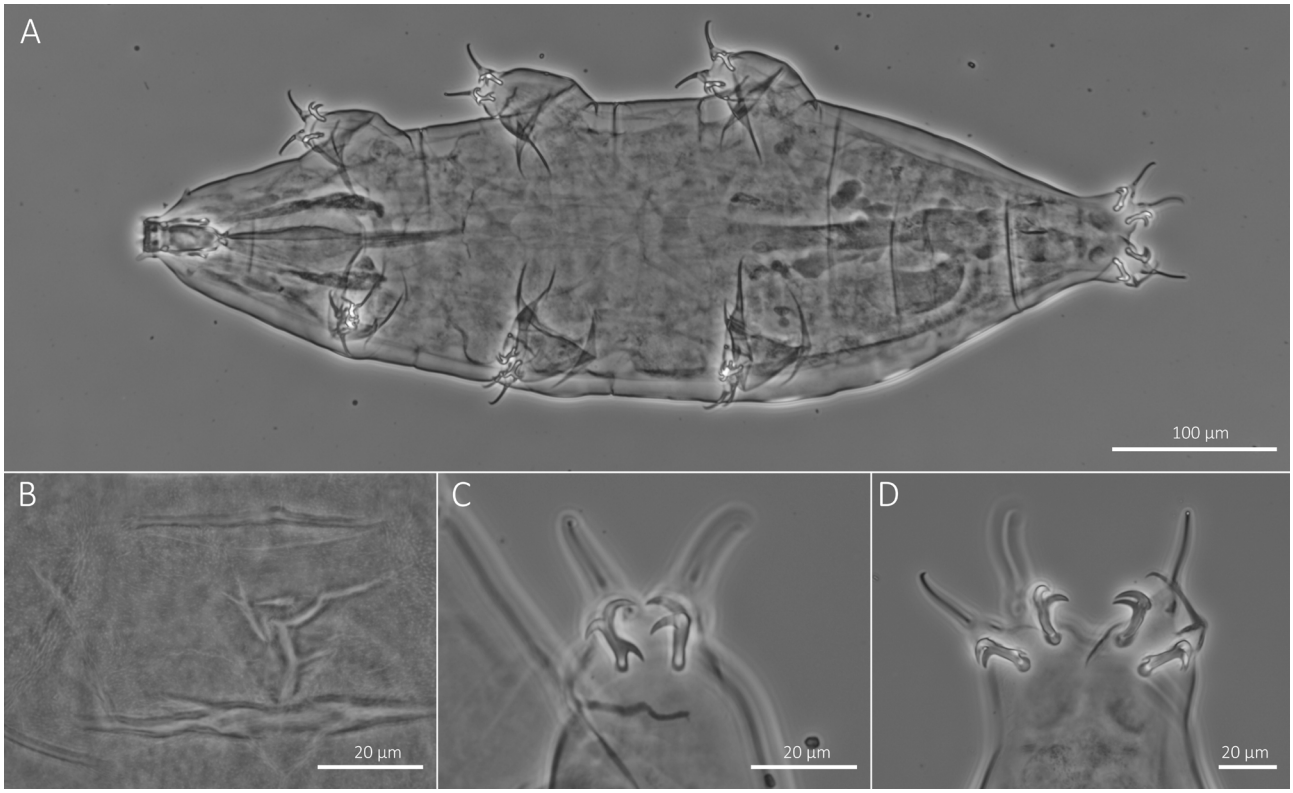

Fig. 2. *Milnesium guanyinensis* from Poland (adult, PCM): A – habitus, dorso-ventral projection; B – reticulate sculpture on the dorsal cuticle; C – claws II with two points on the external secondary branch and three points on the internal secondary branch; D – claws on the hind legs with three points on the anterior secondary branches and two points on the posterior secondary branches.

sequences were obtained for two individuals from each sample. The DNA sequences confirmed the morphological identifications, and the results of the BLAST search are provided below for each new species record, respectively.

Taxonomic accounts of the species found in the study

Phylum: Tardigrada (Spallanzani, 1777)

Class: Eutardigrada Richters, 1926

Order: Apochela Schuster, Nelson, Grigarick and Christenberry, 1980

Family: Milnesiidae Ramazzotti, 1962

Genus: *Milnesium* Doyère, 1840

Species: *Milnesium berladnicorum* Ciobanu, Zawierucha, Moglan & Kaczmarek, 2014

Locality: 49.911°N 21.067°E, Furmaniec hill, Tuchów, Poland, lichen on a tree trunk, coll. Bartłomiej Surmacz and Daniel Stec, collected on 2 July 2024. The sampling locality is an abandoned post-agricultural area, with species-rich xerothermic grasslands undergoing a forest succession. Materials examined: 28 animals mounted on microscope slides in Hoyer's medium, and four animals were genotyped. Slide codes: TUCH.01.01, TUCH.01.02, TUCH.07.01 and TUCH.07.02.

GenBank accession numbers: PQ821864, PQ821865, PQ821866 and PQ821867.

BLAST search: The closest match was to the COI sequences of *M. berladnicorum* from Russia (OP009212; Tumanov *et al*. 2022), Slovakia (MN847749, Guil *et al*. 2022), Ukraine (MW560656; Morek *et al*. 2021) and South Africa (MW560689, MW560657; Morek *et al*. 2021) (all with more than a 99% sequence identity).

Remarks: The original description of the species reports the presence of pseudopores in the cuticle, which were also evident in the specimens examined in this study (Figure 1C). However, upon closely focusing on the external dorsal cuticle, the specimens of the newly discovered population also exhibited a subtle reticulate sculpture that was not previously reported for this species (Figure 1D).

Fig. 3. *Milnesium guanyinensis* from Poland (hatchling, PCM): A – habitus, dorso-ventral projection; B – reticulate sculpture on the dorsal cuticle.

Species: *Milnesium guanyinensis* Yuan, Q. Liu, Y. Wang, L. Liu, Y. Chen & X. Li, 2023

Locality: 49.882°N 20.086°E, Park Władysława Kowalskiego, Dobczyce, Poland, moss on a tree, urban environment, coll. Bartłomiej Surmacz, collected on 7 June 2023.

Materials examined: 10 individuals and two exuviae were preserved on the slides, and two individuals were genotyped. Slide codes: B23.KU8.01, B23.KU8.02, B23.KU8.03, B23.KU8.04, B23.KU8.05 and B23.KU8.06.

GenBank accession numbers: PQ821870 and PQ821871.

BLAST search: The highest similarity was to COI sequences from the original description of *M. guanyinensis* from China (98% identity, ON754953; Yuan *et al*. 2023), and to sequences from Argentina (MW560663) and South Africa (MW560697) (97% and 100% identity, respectively), classified as *Milnesium* sp. in Morek *et al*. (2021), before the description of *M. guanyinensis*.

Remarks: The morphology of the hatched individuals confirmed a claw configuration change: [2-2]-[2-2] CC of the hatchlings and [2-3]-[3-2] of the adults (exuviae).

Species: *Milnesium inceptum* Morek, Suzuki, Schill, Georgiev, Yankova, Marley & Michalczyk, 2019

Locality: 50.067°N 19.957°E, Lubomirskiego Street, Kraków, Poland, moss on a tree trunk, urban environment, coll. Bartłomiej Surmacz and Jakub Godziek, collected on 31 March 2023.

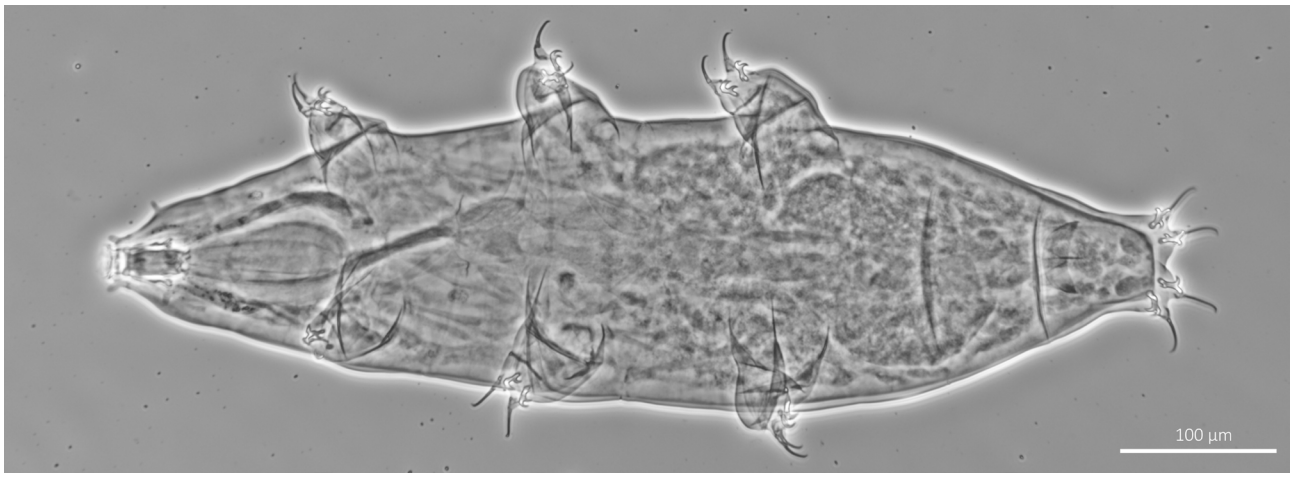

Fig. 4. *Milnesium inceptum* from Poland (adult, PCM) – habitus, dorso-ventral projection.

Materials examined: 7 individuals preserved on the slides, and two individuals were genotyped for COI. Slide codes: PL.048.01 and PL.048.02.

GenBank accession numbers: PQ82186 and, PQ821869.

BLAST search: The closest matches (all of them > 99% identity) were to the COI sequences from multiple populations of *Milnesium,* including records from South Africa (MW560661), New Zealand (MW560660), Japan (MK628723, EU244604), Germany (EU244603, KU513422) and the USA (MT502117) (Kosztyła *et al*. 2016; Morek *et al*. 2019b, 2021; Nelson *et al*. 2020). Some of these records were previously misidentified, but their status has been clarified by Morek *et al*. (2019b).

Discussion

While the early paradigm on the distributions of meiofauna followed the rule 'Everything is Everywhere but the environment selects' (Baas-Becking 1934), the recent studies on tardigrades have indicated that tardigrades form regionally distinct faunas, with only a fraction of the species having worldwide distributions (Morek *et al*. 2021). Nevertheless, all three species analysed in our study have been recorded from other biogeographic realms. *Milnesium berladnicorum*, which was originally described from Romania (Ciobanu *et al*. 2014), has also been reported from Ukraine, Slovakia, Russia and South Africa (Morek *et al*. 2021). *Milnesium guanyinensis* was described from southeast China (Yuan *et al*. 2023), yet occurrences of conspecific populations (based on the similarity of the COI sequences and morphology) were previously recorded from Argentina and South Africa (Morek *et al*. 2021). *Milnesium inceptum* has been recorded from USA, Australia, New Zealand, South Africa, Japan and several localities in Europe (Morek *et al*. 2019b; Morek *et al*. 2021). In contrast, multiple other *Milnesium* species have been observed in only one biogeographic realm, e.g. *M. eurystomum* Maucci, 1991 and *M. reductum* Tumanov, 2006 (Morek *et al*. 2021).

The species analysed in our study are representatives of three highly-divergent clades, whose divergence time is estimated as more than 100 million years before the present (Clades A, B, and E following the nomenclature of Morek *et al*. 2021). *Milnesium berladnicorum* represents Clade A, which consists mostly of Palearctic species. *Milnesium inceptum* is positioned within Clade B, which also encompasses Palearctic species, with a few exceptions (notably, including *M. inceptum* itself, recorded from multiple localities worldwide). Interestingly, *M. guanyinensis* is positioned within Clade E, in which no species has ever been recorded in the Palearctic (but it includes species found in the Afrotropic, Nearctic, Neotropic, Oceanic, Oriental and Panamanian geographic realms). The fact that no species from this clade has been found in the Palearctic, despite the intensive tardigrade research that has taken place in Europe, may suggest that the distribution of *M. guanyinensis* in Europe may be a result of a recent long-distance dispersal (possibly anthropogenic, since it was found in an urban area). This hypothesis is also supported by the fact that the known distribution of this species is neither linked to geography nor to climatic conditions (Figure 5). Otherwise, the species could have been undetected in Europe, because it has been described only recently (Yuan *et al*. 2023). However,

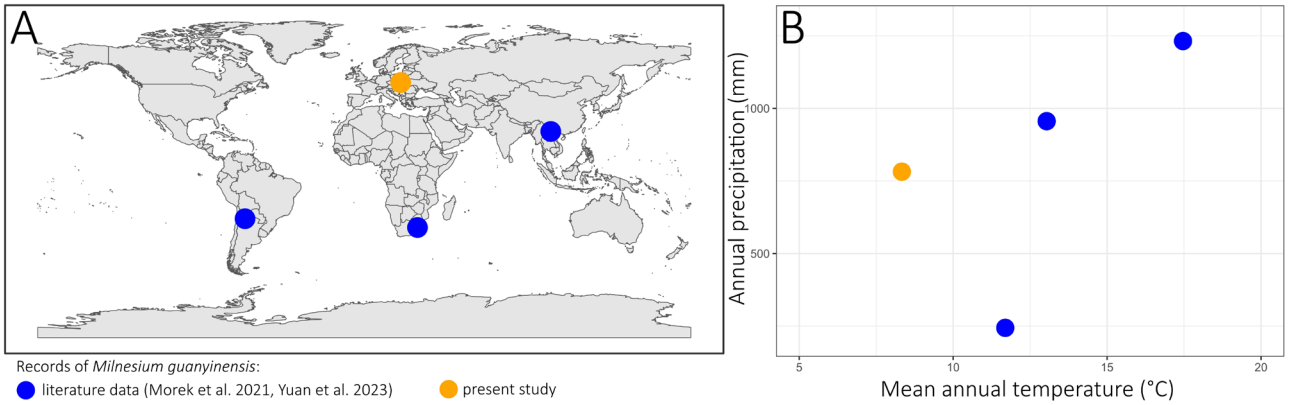

Fig. 5. Known localities of *Milnesium guanyinensis*: A – geographical localities; B – main climatic parameters of the localities.

the unique morphological features of *M. guanyinensis* (fine reticulation pattern on the cuticle and a [2-3]-[3-2] claw configuration of mature individuals) make it distinct from all other *Milnesium* species recorded from the Palearctic.

Our findings confirm the observations that anthropogenic areas can be a source of new tardigrade species records (e.g. McCarthy & delBarco-Trillo 2020; Rocha *et al*. 2024), but also suggest that anthropogenic activities can have an impact on the distributions of tardigrade species, which can disturb the existing phylogeographic patterns. Interestingly, no males were ever recorded in all the three species found in the present study. This supports the hypothesis that parthenogenetic reproduction may be linked to wide distributions of some tardigrade species (Guidetti *et al*. 2019; Kayastha *et al*. 2023).

The use of an integrative approach, encompassing both a morphological investigation and DNA barcoding, allowed us to confidently identify the examined populations, and as a result considerably improves the knowledge of the Polish fauna of *Milnesium*. Including the contributions presented in our study, the known number of *Milnesium* species in Poland is seven, with three of them (43% of the species) being recorded herein for the first time. Such a large increase in the species number of a tardigrade genus in Poland (which is considered one of the most intensively studied regions by tardigradologists) shows that our current knowledge of the distributions of tardigrade species is extremely limited due to insufficient sampling efforts (Vuori *et al*. 2020). Our results, especially the first record of *M. guanyinensis* in the Palearctic, provide evidence that the link between micrometazoan phylogeny and biogeography is weak (Figure 5). Recent advances in tardigrade phylogeography have provided general clues (like the regional distinctiveness of fauna and a group of cosmopolitan species), but the mechanisms shaping tardigrade distributions are still virtually unknown. Therefore, large-scale biogeographic studies based on a low number of samples (e.g. confirming or disproving the cosmopolitanism of tardigrades, assessing the distribution of species or climatic niche modelling) should be treated with caution, especially if they include species recorded from anthropogenically altered habitats.

Acknowledgements

We extend our gratitude to Jakub Godziek (Jagiellonian University, Kraków, Poland) for his assistance during sample collection and to two anonymous reviewers whose comments and suggestions improved our work.

Funding

The study was supported by the Preludium programme of the Polish National Science Centre (grant no. 2022/45/N/NZ8/01992 to B.S.).

Author Contributions

Research concept and design; Collection and/or assembly of data; Data analysis and interpretation; Writing the article; Critical revision of the article; Final approval of article: B.S., D.S.

Conflict of Interest

The authors declare no conflict of interest.

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