

1 **Cryptic diversity constrains biogeographical inference in microscopic animals: evidence**
2 **from bdelloid rotifers in Greenland**

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18 **Abstract:** Species are fundamental units of biodiversity, yet their delimitation remains challenging in many
19 organismal groups. The increasing use of DNA data has revealed widespread cryptic diversity, in which genetically
20 distinct lineages are morphologically indistinguishable. Consequently, many morphology-based biogeographical
21 inferences have likely overestimated species ranges, particularly in microscopic animals traditionally considered
22 ubiquitous. We investigated bdelloid rotifer diversity and biogeographical patterns in small aquatic habitats across
23 Greenland, a poorly explored and geographically isolated region. Using morphological identification combined with
24 mitochondrial COI sequencing and molecular species delimitation, we analysed Greenlandic populations in a global
25 context. Seven morphologically defined species were recorded, all new for Greenland, and each represented a complex
26 of cryptic lineages. We identified 24 putative species, most lacking close matches in public databases. Species richness
27 estimates exceeded observed values, indicating extensive hidden diversity. Most putative species exhibited restricted
28 distributions, whereas only a minority were shared with other Holarctic regions. Our results suggest that apparent
29 ubiquity in bdelloid rotifers largely reflects unresolved cryptic diversity and limited sampling. Additional barcoding
30 of tardigrades from the same samples revealed similar gaps in reference libraries for aquatic meiofauna. Overall, this
31 study highlights the importance of DNA-based taxonomy for robust biogeographical inference in microscopic animals.

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33 **Key words:** Bdelloidea, cryptic species, DNA taxonomy, Holarctic, Nearctic, Tardigrada.

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35

36 **Introduction**

37 Historically, the biogeographical ranges of microscopic aquatic animals have been assumed to extend
38 across far broader spatial scales than those of macroscopic animals, giving rise to the “Everything is
39 Everywhere” hypothesis, also referred to as the ubiquity hypothesis (Fenchel & Finlay, 2004). According
40 to this view, high dispersal ability and large population sizes should result in weak geographical structuring
41 and widespread distributions. However, despite its long-standing influence, empirical support for this
42 hypothesis remains equivocal, largely because several confounding factors may obscure true
43 biogeographical patterns and generate artefactual signals of cosmopolitanism (Martiny et al., 2006; Bass &
44 Boenigk, 2011). Among these factors, the widespread occurrence of cryptic species complexes has been
45 identified as one of the most important (Cerca et al., 2020; Vanreusel et al., 2023; Zhu et al., 2026). If
46 morphologically defined species actually comprise multiple genetically distinct lineages with more
47 restricted distributions, apparent geographic ubiquity may largely reflect taxonomic underestimation rather
48 than genuine patterns (Hending, 2025). Consequently, resolving cryptic diversity represents a crucial
49 prerequisite for robust inference in the biogeography of microscopic animals (Morek et al., 2019; Martínez
50 et al., 2025).

51 As model organisms for testing whether apparently broad geographical distributions are genuinely
52 widespread or instead reflect unresolved cryptic diversity, we focused on bdelloid rotifers. This group is
53 among the most intensively studied microscopic metazoans and is characterised by a high incidence of
54 morphologically conserved species complexes and extensive reports of wide geographical ranges (Mills et
55 al., 2017; Walczyńska et al., 2024; Zhu et al., 2026). Despite this attention, knowledge of rotifer diversity
56 remains geographically uneven (Fontaneto et al., 2012). Most available records originate from Europe,
57 whereas large parts of the Northern Hemisphere remain poorly surveyed and information from the Southern
58 Hemisphere is scarce (Donner, 1965; Lukashanets et al., 2025). Even within the Northern Hemisphere,
59 major gaps persist. In particular, Greenland represents one of the least explored regions for bdelloid rotifers,
60 with only a limited number of species reported so far, most of them shared with European faunas
61 (Lukashanets et al., 2025). In contrast, other Arctic and subarctic regions, such as Svalbard, have been
62 comparatively well studied, providing a valuable reference framework for biogeographical comparisons
63 (Kaya et al., 2010).

64 Arctic and subarctic organisms often exhibit broader geographical distributions than their temperate
65 counterparts, reflecting historical connectivity, postglacial recolonization processes, and relatively
66 homogeneous climatic conditions across high latitudes (Ruggiero & Werenkraut, 2007; Radomski, 2025).
67 For example, several well-known macroscopic taxa, including for example the reindeer *Rangifer tarandus*
68 (Linnaeus, 1758) and the shrimps *Branchinecta paludosa* (O.F.Müller, 1788) and *Lepidurus arcticus*

69 (Pallas, 1793), display Holarctic distributions. Nevertheless, even in such widely distributed species,
70 phylogeographical analyses frequently reveal pronounced genetic structuring and spatially segregated
71 lineages (Flagstad & Røed, 2003; Hessen et al., 2004; Lindholm et al., 2016). In other cases, closely related
72 sister species replace one another between the Nearctic and Palearctic regions, as observed in beavers
73 (*Castor fiber* Linnaeus, 1758 and *C. canadensis* Kuhl, 1820) and magpies of the genus *Pica* Brisson, 1760
74 (Horn et al., 2011; Song et al., 2018). These patterns illustrate that broad geographical ranges do not
75 preclude the existence of more nuanced regional differentiation. Similar processes may therefore be
76 expected in microscopic aquatic taxa, even in groups traditionally considered to be broadly distributed.

77 According to recent faunistic surveys, a total of 26 bdelloid species from limno-terrestrial habitats
78 have been reported from Greenland, all of them also known from other regions, mainly Europe and the
79 Palearctic (Segers, 2007; Lukashanets et al., 2025). On the basis of morphological identification alone,
80 these data support the view of largely widespread distributions. However, no DNA sequence information
81 has so far been available for Greenlandic bdelloids, preventing an assessment of cryptic diversity and
82 phylogeographical structure within the fauna of this region. Consequently, two alternative scenarios can be
83 envisaged. Under the ubiquity hypothesis, Greenlandic populations of widespread species are expected to
84 belong to the same genetic lineages as those occurring in other parts of the world, at least in the Holarctic
85 region. Alternatively, if cryptic speciation and limited effective dispersal are more common than expected
86 also in bdelloids, Greenland may harbour genetically distinct and potentially regionally restricted lineages.

87 The aim of the study is to evaluate which of these scenarios could fit reality better. To achieve this
88 goal, we sampled bdelloid rotifers from aquatic habitats in Greenland, identified specimens
89 morphologically, and generated mitochondrial COI sequences for molecular species delimitation and
90 comparative analyses with global datasets. In addition, we opportunistically obtained COI sequences from
91 co-occurring tardigrades, another neglected meiofaunal phylum. Approximately 100 tardigrade species
92 have been reported from Greenland, of which only about 20% are aquatic (Kaczmarek et al., 2016).
93 Although the limited number of recovered tardigrade specimens allowed only restricted comparative
94 analyses, these data contribute to improving the DNA reference library for Arctic meiofauna and provide a
95 valuable baseline for future faunistic and biogeographical studies (Coulson et al., 2024; Surmacz et al.,
96 2026).

97

98 **Material and Methods**

99 *Sampling*

100 We collected samples from small water bodies across different areas of Greenland (Online Resource 1).
101 Sampling sites were selected based on the criteria that they were small, contained submerged moss
102 vegetation, and were isolated from other water catchments. The sampled habitats included small bogs,
103 *Sphagnum* bogs, and the shores of ponds and lakes. At each site, approximately 250 ml of water was
104 collected using a plastic bottle directly from the shoreline, including floating detritus, vegetation,
105 submerged mosses, and surface sediment.

106 *Species identification from morphology*

107 Bdelloid rotifers were sorted and isolated in a field laboratory using a portable dissecting microscope. All
108 isolated individuals were identified to species level whenever possible, or to genus level when species-level
109 identification was not feasible, based on morphological characters examined under a compound
110 microscope. Because diagnostic characters in bdelloids are only visible in active individuals (Donner,
111 1965), specimens were processed immediately after sample collection. Tardigrades were sorted and isolated
112 alongside bdelloid rotifers under the dissecting microscope in the field but were subsequently fixed and
113 stored in 95% ethanol. They were examined only after returning from the field, and morphological
114 identification was not attempted because preservation conditions were unsuitable for reliable taxonomic
115 assessment (Nelson, et al., 2020). Consequently, tardigrade specimens were identified later exclusively
116 using DNA-based methods.

117 *DNA extraction and sequencing*

118 DNA was extracted from 180 specimens (127 bdelloid rotifers and 53 tardigrades) using Chelex (InstaGene
119 Matrix; Bio-Rad, Hercules, CA, USA). Individual animals were placed at the bottom of a 0.5-mL
120 microcentrifuge tube, and 45 μ L of 6% Chelex solution and 1 μ L of proteinase K were added. Samples
121 were incubated in a shaker at 56 °C for 30 min, followed by a final incubation step at 70 °C for 10 min.
122 After incubation, samples were cooled, briefly centrifuged, and stored at -20 °C until further use. For each
123 individual, a partial fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was
124 sequenced following the protocol of (Tang et al., 2014). Fragments of 661 bp (bdelloid rotifers) and 658 bp
125 (tardigrades) were amplified using the primer pair LCOI-1490 (Folmer et al., 1994) and HCO-2198-*jj*
126 (Astrin & Stüben, 2008). PCR cycling conditions consisted of an initial denaturation at 95 °C for 5 min,
127 followed by 35 cycles of 95 °C for 30 s, 51 °C for 90 s, and 72 °C for 60 s, with a final extension at 72 °C
128 for 10 min. PCR product purification and sequencing were performed by an external commercial service.
129 Raw chromatograms were edited and assembled in MEGA 12 (Kumar et al., 2024). COI sequences were
130 translated into amino acids in MEGA 12 to screen for stop codons and potential pseudogenes. All newly
131 generated sequences were deposited in GenBank; accession numbers for each vouchered specimen are
132 provided in the Online Resource 1.

133 *DNA-based species delimitation – Greenland sequences*

134 To gain deeper insight into the number of putative species represented in the newly generated DNA
135 sequence dataset, we applied the ASAP species delimitation method (Assemble Species by Automatic
136 Partitioning; Puillandre et al., 2021) separately for the bdelloid rotifer and tardigrade datasets. Sequence
137 alignments were generated using MAFFT v7 (Katoh et al., 2002; Katoh & Standley, 2013) with the “auto”
138 strategy. ASAP analyses were performed under default settings using the JC69 substitution model, and the
139 species partition with the lowest ASAP score was selected as the best-supported solution. Results of the
140 species delimitation analyses are provided in the Online Resource 1. To assess whether the COI sequences
141 obtained in this study correspond to sequences already available in public databases, we conducted BLAST
142 searches using the NCBI Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990). For each
143 putative species identified by ASAP, a representative COI sequence (the longest available one) was queried
144 against the NCBI core_nt database (Sayers et al., 2026) using blastn (Camacho et al., 2009) with default
145 parameters. Matches were considered reliable when pairwise sequence identity exceeded 97%, a threshold
146 commonly applied as an approximate species boundary in animals (Hebert et al., 2003). Such a distance is
147 confidently applicable also to rotifers and tardigrades, for which even larger within-species distances exist
148 and 97% represents a very conservative approach (Fontaneto et al., 2009; Cesari et al., 2013; Surmacz et
149 al., 2026). In case of tardigrades, genus level ID was assigned to the putative species if the closest BLAST
150 match had at least 85% similarity to the reference query sequence (Surmacz et al., 2026); no attempt to
151 identify genus from COI sequences was performed for bdelloid rotifers, because COI is already known not
152 to be able to recover monophyletic genera (Tang et al., 2014). A list of the best BLAST matches is provided
153 in the Online Resource 1. Additionally, to quantify intra- and interspecific divergence within the analysed
154 species complexes, pairwise p-distance matrices were calculated in MEGA. The resulting matrices, together
155 with the R script used to compute summary statistics, are provided in the Online Resource 2.

156 *Distribution of bdelloid rotifer species complexes*

157 Putative bdelloid rotifer species were assigned to each of the species complexes identified as morphological
158 species. To refine the identification of putative cryptic species within each complex, we implemented our
159 dataset by downloading all COI sequences available in GenBank that were assigned to the same
160 morphological species found and identified in Greenland. These datasets were supplemented with
161 information on the country of origin for each downloaded sequence. As “country”, we identified actual
162 countries, in addition to Svalbard and Greenland, considered as separate geographical units that will be
163 used at the same level of countries. For each species complex, GenBank-derived sequences were merged
164 with the corresponding COI sequences generated in this study, resulting in a combined dataset Greenland
165 + GenBank for each morphological species, to search for evidence of species complexes. The complete

166 datasets are provided in the Online Resource 1. To obtain reliable putative cryptic species partitions, we
167 used two DNA taxonomy approaches applying distance-based species delimitation methods: previous
168 studies have shown that such approaches outperform tree-based methods, which tend to over split biological
169 species (Mills et al., 2017; Dellicour & Flot, 2018; Magoga et al., 2021; Stec et al., 2022). Specifically, we
170 used ASAP, as described above, and ABGD (Automatic Barcode Gap Discovery; Puillandre et al., 2012).
171 ABGD analyses were performed using default settings, except that the JC69 substitution model was selected
172 to ensure consistency with ASAP. For ASAP, the first partition with the lowest ASAP score was considered
173 the best-supported solution. For ABGD, both initial and recursive partitions were examined across a range
174 of prior intraspecific divergence values (P). To minimize potential over splitting associated with recursive
175 partitioning, species hypotheses were based on stable initial partitions, defined as those consistent across
176 multiple P values and closely corresponding to recursive partitions, either matching them exactly or
177 differing only by minor splits. To further avoid over splitting, the final number of putative species and the
178 corresponding sequence groupings for each of the analysed datasets were selected using the most
179 conservative scenario, defined as the lowest number of putative species supported by one or both
180 delimitation approaches. The numbers of putative species inferred using ASAP and ABGD are provided in
181 the Online Resource 1. In order to visualize geographic occurrence patterns and sampling coverage of
182 molecularly delimited species within each bdelloid rotifer species complex, heatmaps were used. For each
183 species complex, the presence and number of DNA sequences for each combination of putative species and
184 country were summarised.

185 *Diversity analyses*

186 Species accumulation curves were constructed separately for each species complex to assess the
187 relationship between sampling effort and observed and expected levels of diversity of putative species
188 (Ugland et al., 2003). These metrics were used to assess the level of completeness in the knowledge of
189 cryptic species within each species complex. Each DNA sequence was treated as an individual sampling
190 unit, and each molecularly delimited putative species was treated as a species. Individual-based species
191 accumulation curves were generated using the function `specaccum()` in the R version 4.3.3 (R Core Team,
192 2024) package *vegan* version 2.6-8 (Oksanen et al., 2025), with “random” method and 500 permutations.
193 This approach randomly permutes the order of individuals and calculates the mean accumulated species
194 richness and its standard deviation across permutations. Ninety-five per cent confidence intervals were
195 calculated as ± 1.96 standard deviations around the mean. Additionally, to approximate total species
196 richness under incomplete sampling, asymptotic species richness was estimated for each complex using
197 abundance-based estimators implemented in the function `estimateR()` in *vegan*. In addition to observed
198 richness (S_{obs}) and species accumulation curves, we estimated the level of completeness for each species

199 complex using Chao1 and ACE estimators treating each DNA sequence as an individual (Magurran, 2003).
200 For Chao1 and ACE, 95% confidence intervals were computed from the corresponding standard errors
201 ($\pm 1.96 \times SE$). Differences in asymptotic species richness among species complexes were evaluated using a
202 bootstrap resampling approach based on Chao1 estimates. Within each complex, individuals (DNA
203 sequences) were resampled with replacement, and Chao1 richness was recalculated for each bootstrap
204 replicate. This procedure was repeated 5000 times, generating bootstrap distributions of richness estimates
205 for all complexes. Pairwise comparisons between complexes were performed by calculating the distribution
206 of differences in Chao1 estimates across matched bootstrap iterations. Two-sided bootstrap p-values were
207 computed based on the proportion of resampled differences crossing zero, using a +1 correction to account
208 for finite resampling. Resulting p-values were adjusted for multiple testing using the Benjamini–Hochberg
209 false discovery rate procedure. Median differences and 95% bootstrap confidence intervals were used to
210 quantify the magnitude and uncertainty of richness contrasts.

211 All analyses were conducted in R using the packages *tidyverse* (Wickham et al., 2019), *ggplot2*
212 (Wickham, 2016), *vegan*, and *patchwork* (Pedersen, 2025). Custom R scripts used for data processing,
213 visualisation, and resampling-based inference are available in Online Resource 3.

214

215 **Results**

216 *Summary of the Greenland dataset*

217 Based on morphological identification of bdelloid rotifer species from Greenland seven morphological
218 species, each of them being a potential species complex, could be identified (GBIF dataset:
219 <https://doi.org/10.15468/trud2e>): *Dissotrocha aculeata* (Ehrenberg 1832), *Dissotrocha macrostyla*
220 (Ehrenberg, 1838), *Otostephanos donneri* Bartoš 1959, *Philodina citrina* Ehrenberg 1832, *Rotaria macrura*
221 (Ehrenberg 1832), *Rotaria rotatoria* (Pallas 1766), and *Rotaria tardigrada* (Ehrenberg 1830). *Dissotrocha*
222 *aculeata* and *Dissotrocha macrostyla* were kept together in the same species complex for the following
223 analyses, given that it has already been suggested that COI cannot resolve them as two monophyletic
224 separate groups and any similarity search in COI may lead to a misleading identification of the other
225 morphological species (Fontaneto et al., 2019). Other seven bdelloid individuals from Greenland could be
226 identified only at the genus level, *Philodina* sp., and other five never revealed any taxonomic feature and
227 were thus kept unidentified.

228 Out of the 180 animals isolated from 13 samples and sequenced in this study, we successfully
229 obtained 140 COI sequences, of which 114 belonged to Rotifera Bdelloidea and 26 to Tardigrada (GenBank
230 accession numbers PZ144880-PZ145019). Initial ASAP analyses conducted on the Greenland-only dataset

231 identified 24 putative bdelloid rotifer species. Among the Greenlandic bdelloid rotifer species delimited in
232 this study, three putative species could not be confidently assigned to any species complex, as neither
233 morphological identification nor BLAST results were conclusive in species identification: two of them
234 corresponded to *Philodina* sp. and one to the unidentified bdelloid. The 21 remaining putative species were
235 assigned to species complexes as follows: ten putative species to the *Rotaria rotatoria* complex; four
236 putative species to *Dissotrocha aculeata–macrostyla* complex, three to the *Philodina citrina* complex; two
237 putative species to the *Rotaria macrura* complex; and one putative species each to the *Rotaria tardigrada*
238 and *Otostephanos donneri* complexes. Occupancy, defined as the number of distinct sampling sites /
239 samples in which a given putative species was detected, was generally low. One bdelloid putative species
240 was detected in three analysed samples, three putative species occurred in two distinct samples, and all
241 remaining putative species were found in a single sample each.

242 ASAP analyses conducted on the tardigrade dataset from Greenland identified nine putative
243 species. Only one of the nine putative species exhibited a high-similarity match, indicating limited
244 representation of Greenlandic lineages in public reference databases. Specifically, our sequence matched
245 with unidentified *Mesobiotus* Vecchi et al., 2016 species found in freshwater habitat in Far East Russia
246 (Tumanov, 2025). One tardigrade putative species was recorded in five different samples, another putative
247 species in four samples, one putative species in two samples, and the six remaining putative species were
248 detected in only one sample each. Given that morphological identification of tardigrades was not possible
249 and BLAST searches did not allow reliable taxonomic classification for eight out of nine putative species,
250 tardigrade data were not subjected to further comparative analyses.

251 *Global species delimitation within bdelloid rotifer species complexes*

252 After merging the newly generated sequences with the corresponding species complex datasets, the total
253 number of sequences in each of the six datasets was as follows: *Dissotrocha aculeata–macrostyla* (92
254 sequences), *Otostephanos donneri* (10), *Philodina citrina* (106), *Rotaria macrura* (197), *Rotaria rotatoria*
255 (1,318), and *Rotaria tardigrada* (35). Both dataset size and inferred putative species richness varied
256 markedly among species complexes, with the *Rotaria rotatoria* complex comprising by far the largest
257 number of sequences and the highest number of delimited putative species. Molecular species delimitation
258 analyses using the most parsimonious results between ABGD and ASAP on these datasets revealed the
259 following numbers of putative species: *Dissotrocha aculeata–macrostyla* (22 putative species),
260 *Otostephanos donneri* (7), *Philodina citrina* (11), *Rotaria macrura* (6), *Rotaria rotatoria* (111), and *Rotaria*
261 *tardigrada* (7). Importantly, all sequences belonging to putative species delimited in the initial ASAP
262 analysis based solely on the Greenland dataset were retained as single, coherent putative species in the
263 subsequent analyses incorporating global datasets, indicating congruence between local and global species

264 delimitation results. Uncorrected COI distances within putative species were generally low across all
265 analysed species complexes. Mean genetic distances within each putative species was around 1-2% and
266 reached 3.0% in *Philodina citrina* and *Rotaria tardigrada* complexes. Maximum within-species distances
267 varied among complexes, reaching up to 9.9% in the *Dissotrocha aculeata–macrostyla* and 10.0% in
268 *Philodina citrina* complexes, 9.0% in *Rotaria tardigrada*, 8.3% in *Rotaria rotatoria*, 5.7% in *Rotaria*
269 *macrura*, and 3.7% in *Otostephanos donneri* (Online Resource 1). Interspecific distances within species
270 complexes were substantially higher and well separated from intraspecific variation, with mean values of
271 15.9% in *Dissotrocha aculeata–macrostyla* (range: 9.6–25.0%), 13.6% in *Otostephanos donneri* (9.9–
272 16.6%), 11.0% in *Philodina citrina* (6.6–17.3%), 9.2% in *Rotaria macrura* (5.2–18.5%), 14.9% in *Rotaria*
273 *rotatoria* (6.5–22.7%), and 13.3% in *Rotaria tardigrada* (7.2–18.9%) (Online Resource 1).

274 *Geographic distribution*

275 Each of the six species complexes is known to have a broad geographical distribution. This is confirmed
276 by the analysed dataset with GenBank sequences. On the contrary, each putative species within each
277 complex reveals a more limited distribution. Several putative species within a given complex seem to occur
278 only in one country (Figure 1).

279 For *Dissotrocha aculeata–macrostyla*, two of the four putative species recorded in Greenland have
280 previously been reported from Sweden. Among the remaining putative species within the complex, eight
281 were found in more than one country, whereas ten are restricted to a single country.

282 For *Otostephanos donneri*, the putative cryptic species recorded in Greenland represents a
283 previously unknown taxonomic entity. Among the remaining six putative species within the complex, only
284 one occurs in more than one country, whereas the others are restricted to a single country.

285 For *Philodina citrina*, three putative species were recorded in Greenland, of which one have
286 previously been reported from Svalbard. Among the remaining putative species in the complex, three occur
287 in more than one country, whereas five are restricted to a single country.

288 For *Rotaria macrura*, the two putative species recorded in Greenland have not been reported
289 elsewhere and therefore represent geographically unique lineages within the dataset. Among the remaining
290 putative species in the complex, two are restricted to a single country, whereas two occur in more than one
291 country.

292 For *Rotaria rotatoria*, five of the ten putative species recorded in Greenland were previously known
293 from other countries: Italy, Svalbard, Sweden, and the United Kingdom. Among the remaining 101 putative
294 species in the complex, 70 are restricted to a single country, whereas 31 have been recorded from more than
295 one country.

296 For *Rotaria tardigrada*, the putative cryptic species recorded in Greenland was already known from
297 Italy. Among the remaining putative species within the complex, four are restricted to a single country,
298 whereas two occur in more than one country.

299 *Diversity estimates*

300 Species accumulation curves indicated variable levels of sampling completeness among species complexes
301 with extreme richness concentrated in *R. rotatoria*, intermediate diversity in *Dissotrocha*, and
302 comparatively low but incompletely sampled diversity in the other species complexes (Figure 2). In
303 *Dissotrocha aculeata–macrostyla* and *Rotaria macrura*, observed and estimated richness were nearly
304 identical, suggesting close-to-asymptotic sampling. In contrast, *Otostephanos donneri* and *Rotaria*
305 *tardigrada* showed substantially higher estimated richness relative to observed values, indicating
306 incomplete sampling. *Philodina citrina* and *Rotaria rotatoria* exhibited intermediate levels of
307 underestimation.

308 Bootstrap resampling of Chao1 estimates revealed pronounced differences in asymptotic richness
309 among complexes. *Rotaria rotatoria* exhibited significantly higher estimated richness than all other
310 complexes (all BH-adjusted $p = 0.001$). *Dissotrocha aculeata–macrostyla* showed intermediate richness
311 and differed significantly from *Philodina citrina*, *Rotaria macrura*, and *Rotaria tardigrada* (BH-adjusted
312 $p \leq 0.001$), as well as from *Otostephanos donneri* (BH-adjusted $p = 0.009$). In contrast, *Otostephanos*
313 *donneri*, *Philodina citrina*, *Rotaria macrura*, and *Rotaria tardigrada* did not differ significantly from one
314 another after correction for multiple testing (Online Resource 1).

315

316 **Discussion**

317 Our survey of aquatic habitats in Greenland revealed seven morphologically defined species of bdelloid
318 rotifers, all representing new records for the region (Lukashanets et al., 2025). However, molecular analyses
319 demonstrated that each of these morphological species constitutes a complex of deeply divergent cryptic
320 lineages. This pervasive hidden diversity provides an opportunity to test whether the apparent broad
321 geographical distributions traditionally attributed to bdelloid rotifers reflect genuine ubiquity or instead
322 result from taxonomic underestimation of independently evolving lineages (Cerca et al., 2020; Stec et al.,
323 2021; Vanreusel et al., 2023; Martínez et al., 2025).

324 Three main patterns emerge from our analyses: (1) extensive levels of cryptic diversity existed
325 within all examined species complexes; (2) estimated diversity substantially exceeded observed richness;
326 and (3) some putative species occur also outside Greenland in other regions of the Holarctic.

327 *Observed diversity within complexes*

328 All seven morphologically defined bdelloid species analysed in this study were revealed to represent
329 complexes of cryptic species. Such pervasive hidden diversity has already been documented in rotifers in
330 general and in bdelloids in particular, for which most nominal species are considered to comprise multiple
331 independently evolving lineages (Gabaldón et al., 2017; Cakil et al., 2021). To date, only one morphological
332 species, *Rotaria socialis* (Kellicott 1888), has been consistently suggested to represent a genetically
333 coherent species using different DNA taxonomy approaches (Fontaneto et al., 2009). Our results extend the
334 observation of high levels of cryptic diversity to Greenlandic species and reinforce the view that cryptic
335 diversity is a fundamental feature of bdelloid diversity.

336 The interpretation of species boundaries in bdelloids could be considered unsupported, given their
337 peculiar ancient asexuality, described as an “evolutionary scandal” (Birky, 2004; Hayden, 2008; Fontaneto
338 & Barraclough, 2015). Under strict biological species concepts with species identity due to reproductive
339 isolation and interruption of gene flow (Coyne et al., 2004; Matute & Cooper, 2021), the absence of
340 conventional sexual recombination in bdelloids would challenge the applicability of the biological species
341 concepts to bdelloids. Regardless of the species concept that can be adopted for bdelloids (De Queiroz,
342 2007), and of the possibility that alternative forms of genetic exchange may exist in bdelloids (Simion et
343 al., 2021; Terwagne et al., 2022), our molecular data clearly reveal deeply divergent mitochondrial lineages
344 within each morphologically defined species.

345 Across the full dataset, COI distances between putative species within each complex ranged from
346 5.2% to 25.0%, with the distribution strongly centred around approximately 15% (mean: 14.8%; median:
347 14.9%). Ninety percent of pairwise comparisons between putative species fell between 11.0% and 18.5%
348 (5th–95th percentiles), indicating that both lower (~5%) and higher (~25%) divergence values represented
349 relatively rare extremes. These divergence levels are substantially higher than typical interspecific values
350 reported for most animal taxa (Hebert et al., 2003; Meyer & Paulay, 2005; Magoga et al., 2021), yet they
351 are consistent with previous findings in rotifers (Gabaldón et al., 2017). Such pronounced genetic
352 structuring within morphological species strongly supports the presence of independently evolving cryptic
353 lineages within each species complex.

354 Importantly, species delimitation in this study was not based on fixed genetic distance thresholds,
355 but on algorithmic approaches (ASAP and ABGD) that infer putative species from the structure of genetic
356 variation within each dataset (Puillandre et al., 2012, 2021). Nevertheless, we acknowledge that limited
357 sampling may affect the resolution of molecular clusters, and that increased geographical and population-
358 level sampling could reveal intermediate haplotypes or alter current species boundaries (Bergsten et al.,
359 2012). Consequently, the putative species identified here should be regarded as working hypotheses that

360 require expanded sampling, further validation using multilocus data and ideally integrative species
361 delimitation that would involve detailed morphological and experimental analyses (Pante et al., 2015).
362 From an evolutionary perspective, the presence of deeply divergent lineages within morphologically
363 defined species raises broader questions about the processes generating diversity in bdelloid rotifers. While
364 diversification in asexual organisms is increasingly recognised, the mechanisms driving the emergence and
365 persistence of these lineages remain poorly understood (Felsenstein, 1981; Tang et al., 2014; Simion et al.,
366 2021). These processes involve ecological differentiation, historical and geographical factors, as well as
367 molecular mechanisms underlying genome evolution.

368 In addition, several of the morphological species examined here are known to exhibit considerable
369 phenotypic variability, with subspecies, forms, or variants described in the taxonomic literature. For
370 example, two subspecies have been recognised within *Dissotrocha macrostyla*, approximately 20 within
371 *Dissotrocha aculeata*, and morphological variants are also documented for *Rotaria rotatoria* (Donner,
372 1965; Bērziņš, 1982). It is plausible that at least some of these morphologically defined entities could
373 correspond to genetically distinct cryptic species. However, comprehensive integrative taxonomic revisions
374 are currently lacking for bdelloids. Comparable efforts in monogonont rotifers, such as in the *Brachionus*
375 *calyciflorus* and *Brachionus plicatilis* complexes (Mills et al., 2017; Michaloudi et al., 2018), illustrate the
376 idea that some of the previously known morphological variants could indeed represent separate species.
377 Therefore, similar integrative approaches are urgently needed for bdelloid rotifers to comprehensively
378 describe diversity and enable robust biogeographical inference.

379 *Expected versus observed diversity*

380 The estimated number of putative species within each complex substantially exceeded the observed richness
381 across all analysed datasets. In all complexes, several putative species were represented by singletons and
382 were detected in only one country (Figure 1). Such patterns reflect high underlying diversity coupled with
383 limited sampling intensity. Indeed, estimated richness consistently exceeded observed values. In some
384 complexes, particularly *Otostephanos donneri* and *Rotaria tardigrada*, the estimated diversity was much
385 higher than the observed richness. In the most extensively sampled species complex, *Rotaria rotatoria*,
386 observed richness was already high, with 111 putative species, but the estimated one is even higher. If these
387 estimates approximate true diversity, bdelloid rotifers harbour an exceptionally large amount of hidden and
388 still undescribed genetic diversity. The large number of putative cryptic species relative to morphologically
389 recognised species indicates that bdelloid diversity is likely substantially underestimated when based solely
390 on morphological surveys. This has important implications for biodiversity assessments, as morphospecies
391 may mask multiple independently evolving lineages that differ in ecological preferences and geographical
392 distributions. Consequently, cryptic diversity may influence our understanding of community composition,

393 biogeographical patterns, and potentially even conservation priorities in poorly explored ecosystems. Most
394 importantly for the aims of this study, extensive undersampling within each species complex could produce
395 artefactual evidence of restricted geographical distributions of putative species within broadly distributed
396 species complexes: by sampling only a very small proportion of the genetic diversity within each species
397 complex, the chances of finding the same putative species in different geographical areas are small.

398 *Biogeographical inference*

399 The high levels of cryptic diversification revealed in this study supports the idea that traditional
400 morphology-based assessments could overestimate the actual geographical ranges of bdelloid species. As
401 previously suggested for other meiofaunal groups (Cerca et al., 2020; Martínez et al., 2025), reliable
402 biogeographical inference in bdelloids requires the explicit incorporation of DNA-based species
403 delimitation to avoid conflating taxonomic and spatial information. Most putative species identified here,
404 namely 108 out of 164 (~66%), exhibited restricted distributions and were detected in single countries.
405 Such a pattern could be the result of more restricted geographical ranges of putative species than those of
406 the whole complex, or substantial undersampling. Notwithstanding the possibility to obtain the same pattern
407 as the consequence of a biological process or due to sampling bias, a subset of putative species within the
408 Greenlandic species complexes, 8 out of 21 (38%), was shared between Greenland and other parts of the
409 Holarctic, particularly in Northern Europe at high-latitude regions such as Svalbard, Sweden, and the United
410 Kingdom, but also at lower latitudes like Italy. This pattern suggests that broad distributions may occur also
411 in bdelloids putative species within a complex, against the hypothesis that cryptic diversity masks apparent
412 broad distributions. Yet, such apparent broad distributions are not a peculiarity of microscopic organisms
413 (Fenchel & Finlay, 2004): the biogeographical patterns of species with occurrence in Greenland and in
414 Palearctic temperate areas mirrors biogeographical structures observed also in some larger animals
415 (Brunner et al., 2001; Lindholm et al., 2016; Valenzuela-Turner et al., 2026).

416 Overall, our results do not support a simple dichotomy between ubiquitous and geographically
417 restricted distributions in microscopic animals like bdelloid rotifers. Instead, they indicate a mixed pattern,
418 in which several cryptic species appear to have narrow ranges, while others achieve broad, at least Holarctic
419 distributions. Similar patterns have been reported in monogonont rotifers and other microscopic
420 invertebrates (Mills et al., 2017; Surmacz et al., 2025; Collins et al., 2026; Vecchi et al., 2026), although
421 direct comparisons remain limited by differences in sampling intensity and geographical coverage. In our
422 Greenlandic dataset, approximately 38% of the putative species were detected outside the Nearctic region,
423 whereas 62% were apparently restricted to Greenland. Previous studies on bdelloids in other parts of the
424 world, using DNA taxonomy to couple accurate identification of units of diversity and biogeography,
425 revealed narrower distributions of putative cryptic species or unclear biogeographical patterns, for example

426 on *Adineta beysunae* Örstan 2018, *R. rotatoria*, and *Rotaria sordida* (Western 1893) in China (Wang et al.,
427 2026; Zhu et al., 2026). The only comparison for the whole community of rotifers and not for single species
428 can be performed on what was done for monogonont rotifers in New Zealand, where 6% of the sequenced
429 putative species, five out of 81, resulted widespread and the other 94% apparently limited to New Zealand
430 (Collins et al., 2026). Given the scarcity of comparable datasets and the limited sampling effort, these
431 proportions should be interpreted cautiously. Expanded geographic sampling and population-level studies
432 will be essential to determine whether these patterns reflect genuine biogeographical structure or incomplete
433 knowledge.

434 *Tardigrades and DNA reference data*

435 Although the primary focus of this study was bdelloid rotifers, the opportunistic sequencing of co-occurring
436 tardigrades provides an additional perspective on the current limitations of molecular reference data for
437 aquatic meiofauna. Similar to the patterns observed in bdelloids, tardigrade barcoding revealed substantial
438 gaps in available COI reference libraries. Only one of the nine putative species identified in this study was
439 represented in public databases. Although available barcode libraries appear relatively comprehensive for
440 European tardigrades, particularly for limno-terrestrial taxa (Surmacz et al., 2026), this coverage is much
441 more limited for hydrophilous and aquatic species (Vecchi et al., 2026; Camarda et al., in press). Aquatic
442 and hygrophilous tardigrades are generally less diverse and less abundant than their terrestrial counterparts:
443 reviews of freshwater tardigrade diversity indicate that such taxa constitute only about 10% of known non-
444 marine species (McFatter et al., 2007; Garey et al., 2008; Kayastha et al., 2021), a pattern that is also
445 reflected in Greenland, where approximately only one out of five recorded species are hygrophilous
446 (Kaczmarek et al., 2016). Previous studies have focused primarily on lotic and lentic systems, yet freshwater
447 tardigrades often belong to morphologically conservative and taxonomically challenging groups, with
448 frequent convergence and limited diagnostic characters (e.g., Gąsiorek et al., 2019; Zawierucha et al., 2020;
449 Guidetti et al., 2022; Camarda et al., 2025b, 2025a; Gąsiorek, 2025). These features hinder reliable
450 identification and contribute to the slow accumulation of molecular reference data. Although our dataset
451 does not allow robust comparative analyses between tardigrades and bdelloid rotifers, it highlights a shared
452 pattern of limited sampling and incomplete reference libraries for aquatic taxa. In contrast, limno-terrestrial
453 tardigrades are currently better represented in public databases than bdelloid rotifers (Surmacz et al. 2026).
454 Our results therefore emphasize the need for targeted sequencing efforts also on freshwater and
455 hygrophilous tardigrades to improve molecular resources and enable more reliable biogeographical and
456 ecological inference. This pattern mirrors the situation observed for bdelloid rotifers and further illustrates
457 how the lack of reliable molecular reference datasets remains a major limitation for biodiversity assessment
458 and biogeographical inference in microscopic aquatic animals.

459 *Conclusions*

460 Our study demonstrates that, except for a few unidentifiable individuals, most of the bdelloids found in
461 Greenland belonged to morphological species that were already known in other parts of the world, including
462 Europe. Bdelloid diversity in Greenland is dominated by high levels of cryptic diversity, altering traditional
463 morphology-based interpretations of species distributions, but in line with what is known for bdelloids
464 elsewhere. By integrating DNA-based species delimitation with biogeographical data, we support that at
465 least one out of three putative species within a complex from Greenland could achieve broad, Holarctic
466 distributions. These findings undermine the hypotheses that unresolved cryptic diversity can distort
467 biogeographical inference in bdelloid rotifers: a mixed pattern of restricted and widespread cryptic species
468 within a complex suggests that not all apparent ubiquity can be solely attributable to potentially unresolved
469 cryptic diversity. As highlighted for other meiofaunal groups (Vanreusel et al., 2023), resolving such
470 patterns in the distribution of cryptic species will require intensified sampling in poorly explored regions
471 and the routine application of DNA-based taxonomy. Even in comparatively well-sampled complexes such
472 as *Rotaria rotatoria*, taxonomic diversity and geographic structure remain far from being fully understood.
473 The integration of extensive, geographically explicit molecular datasets will therefore be essential for
474 advancing a robust and realistic understanding of biogeography in bdelloids as well as in other meiofaunal
475 groups.

476

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485

486 **Statements and Declarations**

487 **Author contribution**

488 All authors contributed to the study conception and design, to material preparation, data collection, data management,
489 and analyses. The first draft of the manuscript was written by DF and DS and all authors commented on previous
490 versions of the manuscript. All authors read and approved the final manuscript.

491 **Competing interest**

492 The authors have no competing interests to declare that are relevant to the content of this article.

493 **Ethical approval**

494 All authors consent to the publication of this study.

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502 **Data availability**

503 The DNA sequences obtained for the study are available in GenBank under accession number PZ144880-PZ145019;
504 georeferenced data is available in GBIF under <https://doi.org/10.15468/trud2e>; the dataset and R scripts underlying
505 the analyses in this study are available in the FigShare repository: <https://doi.org/10.6084/m9.figshare.31650424> (DOI
506 link will become inactive until the MS is published).

507 **Declaration of generative AI and AI-assisted technologies in the manuscript preparation process**

508 During the preparation of this work, the authors used ChatGPT in order to improve the readability and language of
509 the manuscript. After using this tool, the authors reviewed and edited the content as needed and took full responsibility
510 for the content of the published article.

511 **Permits**

512 All samples were collected under the “Licence No. G24-109 for Utilization of Greenland Genetic Resources” (03 July
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516

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785 **Supplementary Materials**

786 **Online Resource 1.** Excel file with tabs containing the following information: (1) metadata about the file, (2) sample
787 data, (3) information Greenlandic mOTUs delimited from the data collected in this study, (4) information about DNA
788 sequences obtained in this study, (5) final number of species delimited by ABGD and ASAP for each analysed species
789 complex, (6) information about individuals / sequences of the global data set, (7) species richness estimates for each
790 analysed species complex, (8) bootstrap-based pairwise comparison of Chao1 richness in between analysed species
791 complexes (9) summary of p-distances within and between putative species.

792 **Online Resource 2.** Matrices of p-distances calculated for each species complex and R script used for the analyses
793 (zip).

794 **Online Resource 3.** Individual-level data on species present across different countries for and R script for species
795 richness analyses and visualization.

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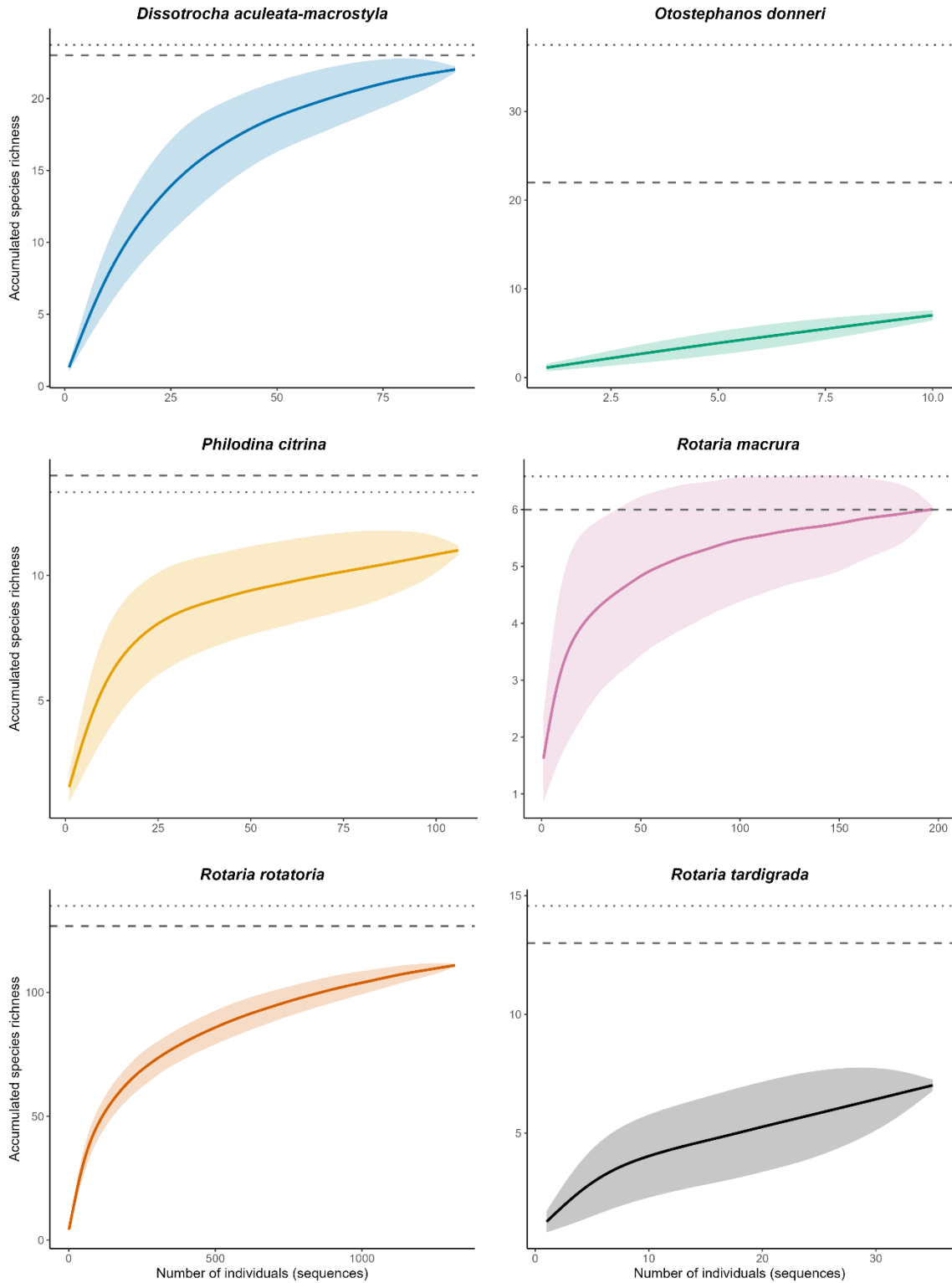
798 **Figure captions**

799 **Figure 1.** Geographic distribution of molecularly delimited putative species within six rotifer species complexes.
800 Heatmaps show species occurrence across countries, listed in alphabetical order. Filled cells indicate presence, and
801 numbers inside cells represent the number of DNA sequences of each putative species in each country. For *R. rotatoria*
802 complex the numbers from cells and the numbers of putative species are not shown to enhance visibility of the
803 occurrence pattern.

804 **Figure 2.** Species accumulation curves and richness estimates for the six rotifer species complexes analysed in this
805 study. Solid lines show mean accumulated richness, shaded areas indicate 95% confidence intervals, and horizontal
806 lines represent Chao1 (dashed line) and ACE estimates (dotted line) (See Supplementary Table S2 for numerical
807 details on estimates).

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Figure 2. Species accumulation curves and richness estimates for the six rotifer species complexes analysed in this study. Solid lines show mean accumulated richness, shaded areas indicate 95% confidence intervals, and horizontal lines represent Chao1 (dashed line) and ACE estimates (See Supplementary Table S2 for numerical details on estimates).