Unearthed from old soils: New records of Antarctic tardigrades, nematodes, and rotifers in the Prince Charles Mountains based on partial sequences of Cytochrome c oxidase subunit I*

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Open Research Statement

Parts of the data are already published, with those publications cited in this article. All data were provided as in-confidence for peer review and have been revised during peer review. Data associated with this work are located via DOI 10.5281/zenodo.13919278 (always linking to latest version. Code is available via https://github.com/macrobiotus/454_invertebrates (pre-release v0.1 alpha)

Abstract

Despite only 0.3% of Antarctica being ice-free, those areas harbor diverse small organisms such as tardigrades, nematodes, and rotifers. The habitats of these cryptic organisms face threats from human activity, climate change, and pollution. Biodiversity surveys are essential for managing their protection and such surveys have been proven well possible in Antarctica using environmental DNA (eDNA) analysis. Early eDNA research had greatly benefited from high-throughput sequencing using the now obsolete 454 pyrosequencing technique, which at the time offered superior read length to the Illumina platform. In a 2011-12 expedition to the

remote Prince Charles Mountains we had collected soil samples, from which we amplified and described 18S Ribosomal 1 RNA gene sequences. Recently we reanalysed concomitantly generated 454-derived Cytochrome Oxidase Subunit 1 ("COI") amplicons from those soils, making use of now updated reference data. From our "historic" 454 data we now can provide additional records for some Antarctic taxa, including tardigrades *Acutuncus antarcticus* and *Macrobiotus hufelandi*, the nematodes *Plectus frigophilus* and *Plectus murrayi*, and rotifers such as *Habrotrocha angularis*. We show that reanalysing past eDNA projects can yield novel information as reference data collections grows, at the same highlighting the need to curate and further expand those data collections using voucher specimen.

Main text

Although only 0.3% of continental Antarctica are ice-free, many organisms including bacteria, unicellular eukaryotes, fungi, lichen, cryptogamic plants and invertebrates are scattered across the continent in island-like terrestrial habitats, soil-like substrates, lakes, and cryoconite holes (Convey *et al.* 2014). Antarctic biodiversity is threatened by human activity, climate change, pollution, and invasive species. Mitigation of these threats relies on well-tailored management strategies across the continent's bioregions, including surveying its biodiversity (Wauchope *et al.* 2019). Genetic material shed by organisms into their habitats is called environmental DNA (eDNA). Analysis of eDNA is a well-established molecular technique for multispecies surveys (reviewed in Cristescu and Hebert 2018). Environmental DNA analysis is often advertised as a good choice for biodiversity surveys, because sampling and laboratory work can be easily standardized across multiple taxa, and the method scales well to analyse many samples at once (Cristescu and Hebert 2018). These benefits are particularly tangible for surveying terrestrial Antarctica, where fieldwork is challenging, and sample transport is extremely expensive (Czechowski *et al.* 2017). At the same time, reliable low-level taxonomic annotation, particularly at the species level, as a prerequisite for useful biodiversity and natural resource surveillance, is challenging with eDNA–related methods, and even more so for the many small and inconspicuous taxa of terrestrial Antarctica (Wauchope *et al.* 2019; Czechowski *et al.* 2022, 2024).

Environmental DNA research has been greatly empowered by the development of high-throughput DNA sequencing technology (Cristescu and Hebert 2018). The ability to ligate artificial, unique "labelling" sequences to purified DNA stemming from many individual environmental samples allowed DNA from those samples to be read out at once, making high throughput sequencing cost-efficient for eDNA projects (Binladen *et al.* 2007; Meyer *et al.*

2008) The first commercially available high-throughput DNA sequencing technology was 454 pyrosequencing (Margulies *et al.* 2005). Considered obsolete in 2016, the technique has now been superseded by the more powerful Illumina platforms. In general, the workflow has remained the same for the last 15 years – samples are collected from the environment, and DNA is isolated from those samples. Then, in polymerase chain reaction (PCR, Saiki *et al.* 1988), selected genome regions of the isolated nucleic acids are copied over and over ("amplified") so that their sequences reach sufficient quantities to be read out by a machine. For taxonomic annotation it is important to amplify a correct genome region ("marker", such as the small subunit 18S rRNA gene, "RNA18S1"; or the Cytochrome c oxidase gene, "COI"), or parts thereof, so that taxa can be meaningfully discerned by comparison to already existing taxonomically annotated sequences ("reference data"; Hebert *et al.* 2003). Today, the field of eDNA research is very active in developing biodiversity monitoring strategies, which are perhaps somewhat underappreciated in Antarctic terrestrial science (Czechowski *et al.* 2017).

Refence data for environmental DNA sequences are growing steadily, thus potentially enabling re-annotation of newly available taxonomic information to samples obtained in past field campaigns (Czechowski *et al.* 2022). In 2011-12, we collected soil samples in one of the most remote places on earth, the Prince Charles Mountains, situated southeast of the Amery Ice Shelf, and first mapped by Australian National Antarctic Research Expeditions between 1954-61 (Fig. 1). While we investigated the resulting Illumina-derived RNA18S1 sequences thoroughly, recovering well known Antarctic phyla (tardigrades, nematodes, rotifers, and mites, e.g. Czechowski *et al.* 2022) we did not do so yet for COI sequences concomitantly generated at the time using the 454 platform. Recently, we converted those 454 data into modern formats, and annotated them with recent (2023) reference data from NCBI's Genbank using MEGAN's well established LCA algorithm (Huson *et al.* 2007; Sayers *et al.* 2019). We thus can now provide more detailed COI-derived species records for invertebrate phyla of the Prince Charles Mountains. Those include the tardigrades *Acutuncus antarcticus* (Fig. 2), and *Macrobiotus hufelandi,* the nematodes *Plectus frigophilus*, *Plectus murrayi* (Fig. 3), rotifers related to the *Adineta vaga* species complex (Iakovenko *et al.* 2015) as well as the endemic *Habrotrocha angularis* (Murray, James 1910). Due to lacking reference data, the small size of invertebrates, and a constant flux in taxonomic classification (Convey and Stevens 2007; Hleap *et al.* 2021), our species-level assignments can still only be considered close estimates, and some mite and rotifer taxa are still not resolved to species level. Additionally, some species level identifications are questionable – for instance some nematode sequences resolved to

Bursaphelenchus cocophilus, a palm parasite from high latitudes. At the same time we deem Antarctic presence of the at least some of the recovered species identifications to be reasonably certain based on our previous work in the area and with the same samples, firstly based on work involving morphologic taxonomy and our previous work with RNA18S1 (Velasco-Castrillón *et al.* 2014; Czechowski *et al.* 2022), and secondly based on additional ClustalW alignments and neighbour joining trees generated using R packages *msa* and *phangorn* (Ma *et al.* 2007; Schliep 2011; Bodenhofer *et al.* 2015 – see supplementary online materials).

We encourage the reanalysis of past eDNA projects, and funding of initiatives seeking to generate reference data particularly for cryptic soil taxa. Furthermore, we are also convinced that environmental DNA analysis holds tremendous potential for surveying biodiversity even in the remotest and harshest regions of our planet.

Author contributions

Authorship assignment followed the Contributor Roles Taxonomy (https://casrai.org/credit). Conceptualization: PC and MS; Data Curation and Formal Analysis: PC; Funding Acquisition: MS; Investigation: PC; Methodology: PC; Project Administration: PC; Resources: PC, MS; Software: PC; Supervision: PC; Validation: PC, IN, KZ, Visualization: PC, IN, KZ; Writing – Original Draft PC; Writing – Review & Editing: all authors.

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References

127 Binladen J, Gilbert MTP, Bollback JP, *et al.* 2007. The use of coded PCR primers enables high-
128 throughput sequencing of multiple homolog amplification products by 454 parallel
129 sequencing. *PLoS ONE* **2**: e197.

130 Bodenhofer U, Bonatesta E, Horejš-Kainrath C, and Hochreiter S. 2015. msa: an R package
131 for multiple sequence alignment. *Bioinformatics* **31**: 3997–9.

132 Convey P and Stevens MI. 2007. Antarctic Biodiversity. *Science* **317**: 1877–8.

133 Cristescu ME and Hebert PDN. 2018. Uses and Misuses of Environmental DNA in Biodiversity
134 Science and Conservation. *Annual Review of Ecology, Evolution, and Systematics* **49**:
135 209–30.

136 Czechowski P, Clarke LJ, Cooper A, and Stevens MI. 2017. A primer to metabarcoding surveys
137 of Antarctic terrestrial biodiversity. *Antarctic Science* **29**: 3–15.

138 Czechowski P, Lange M de, Heldsinger M, *et al.* 2024. Comparison of traditional and molecular
139 surveys of fish biodiversity in southern Te Wāhipounamu/Fiordland (Aotearoa/New
140 Zealand). *Environmental DNA* **6**: e514.

141 Czechowski P, Lange M de, Knapp M, *et al.* 2022. Antarctic biodiversity predictions through
142 substrate qualities and environmental DNA. *Frontiers in Ecology and the Environment*
143 **20**: 550–7.

144 Hebert PDN, Cywinska A, Ball SL, and DeWaard JR. 2003. Biological identifications through
145 DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* **270**: 313–21.

146 Hleap JS, Littlefair JE, Steinke D, *et al.* 2021. Assessment of current taxonomic assignment
147 strategies for metabarcoding eukaryotes. *Molecular Ecology Resources* **21**: 2190–203.

148 Huson DH, Auch AF, Qi J, and Schuster SC. 2007. MEGAN analysis of metagenomic data.
149 *Genome Res* **17**: 377–86.

150 Iakovenko NS, Smykla J, Convey P, *et al.* 2015. Antarctic bdelloid rotifers: diversity, endemism
151 and evolution. *Hydrobiologia* **761**: 5–43.

152 Ma L, G B, Np B, *et al.* 2007. Clustal W and Clustal X version 2.0. *Bioinformatics (Oxford,*
153 *England)* **23**.

154 Margulies M, Egholm M, Altman WE, *et al.* 2005. Genome Sequencing in Open
155 Microfabricated High Density Picoliter Reactors. *Nature* **437**: 376–80.

156 Meyer M, Stenzel U, and Hofreiter M. 2008. Parallel tagged sequencing on the 454 platform.
157 *Nature Protocols* **3**: 267–78.

158 Murray, James (Ed). 1910. British Antarctic Expedition 1907-9, Reports on the Scientific
159 Investigations. London: W. Heinemann.

160 Saiki RK, Gelfand DH, Stoffel S, *et al.* 1988. Primer-directed enzymatic amplification of DNA
161 with a thermostable DNA polymerase. *Science (New York, NY)* **239**: 487–91.

162 Sayers EW, Cavanaugh M, Clark K, *et al.* 2019. GenBank. *Nucleic Acids Research* **48**: D84–
163 6.

164 Schliep KP. 2011. phangorn: phylogenetic analysis in R. *Bioinformatics* **27**: 592–3.

165 Velasco-Castrillón A, Gibson J a. E, and Stevens MI. 2014. A review of current Antarctic limno-
166 terrestrial microfauna. *Polar Biology* **37**: 1517–31.

167 Wauchope HS, Shaw JD, and Terauds A. 2019. A snapshot of biodiversity protection in
168 Antarctica. *Nature Communications* **10**: 946.

Figures

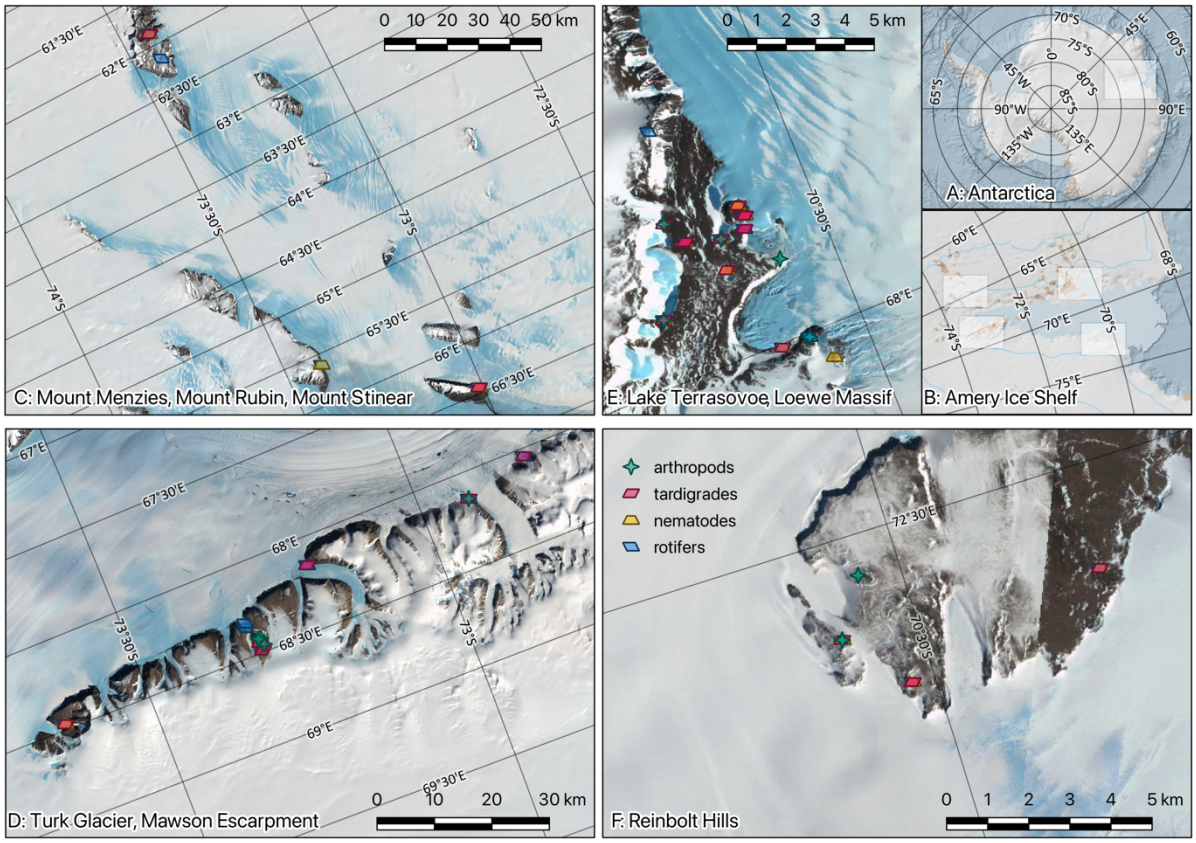

Fig. 1: Sampling locations yielding invertebrate phylotypes in the Prince Charles Mountains, East Antarctica. A and B: Overview map of Antarctica and the Prince Charles Mountains (PCMs) with sampling locations, respectively. C: Mount Menzies, Mount Rubin, and Mount Stinear. D: Turk Glacier and Mawson Escarpment. E: Lake Terrasovoe and the Lowe Massif; F: Reinbolt Hills. Note that additional taxon occurrences at the given locations had previously been reported in Czechowski *et al.* (2022) based on 18S rDNA amplification. CIRREF Imagery courtesy of the US Geological Survey, distributed with Quantarctica. Quantarctica package courtesy of the Norwegian Polar Institute, visit www.quantarctica.org.

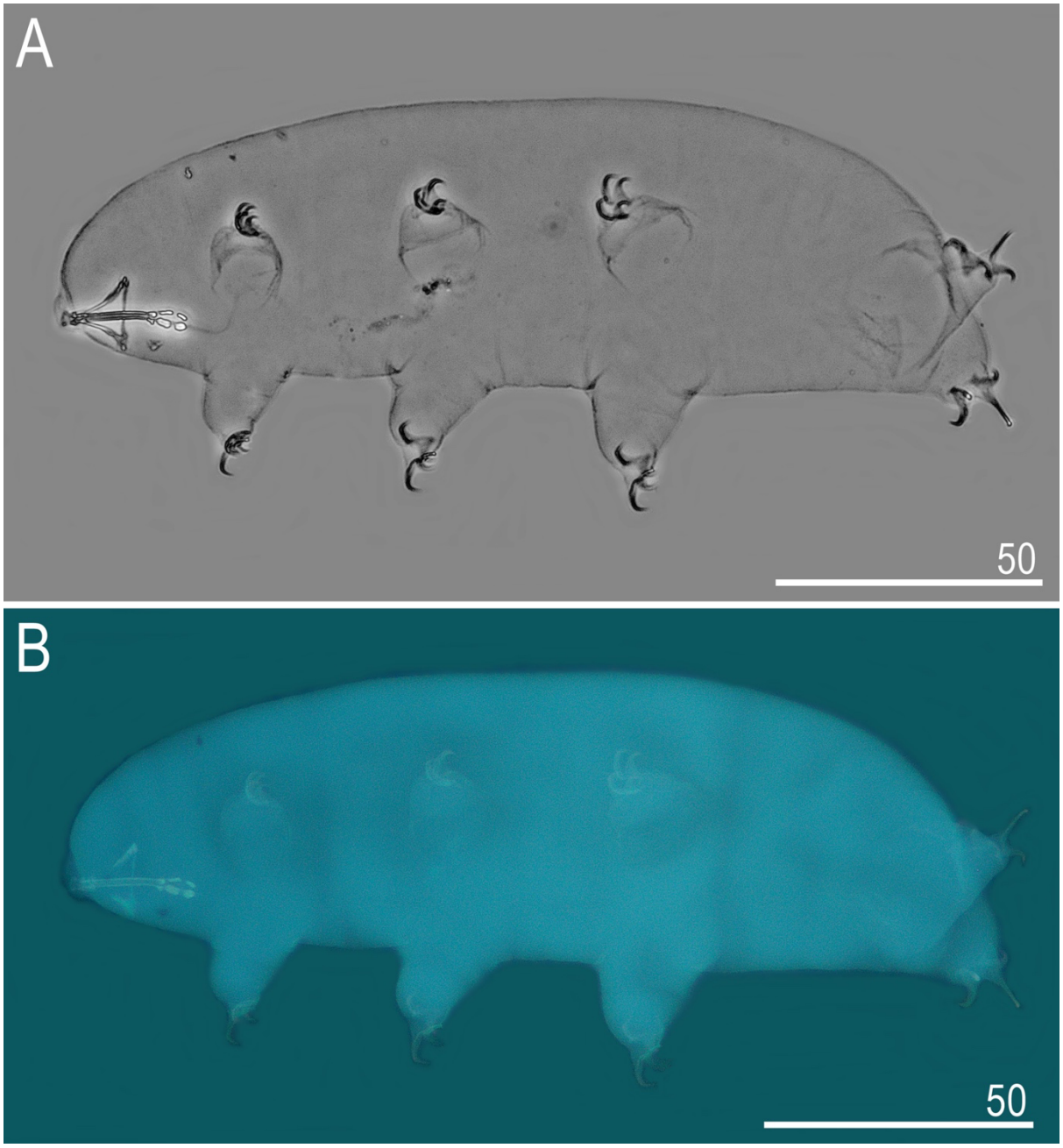

Fig. 2: Phase contrast (A) and UV light excitation image (B) of *Acutuncus antarcticus*. Image courtesy of Krzysztof Zawierucha, Adam Mickiewicz University, Poznań, PL-PO (2024).

Fig. 3: Bright-field microscopic image of *Plectus murrayi*. Image courtesy of John Gibson, Australian Antarctic Division, Kingston, AU-TAS (2024).