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An ancient, Antarctic-specific species complex: large divergences between multiple Antarctic lineages of the tardigrade genus *Mesobiotus*

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A B S T R A C T

Antarctica has been isolated and progressively glaciated for over 30 million years, with only approximately 0.3 % of its area currently ice-free and capable of supporting terrestrial ecosystems. As a result, invertebrate populations have become isolated and fragmented, in some cases leading to speciation. Terrestrial invertebrate species currently found in Antarctica often show multi-million year, and even Gondwanan, heritage, with little evidence of recent colonisation. *Mesobiotus* is a globally distributed tardigrade genus. It has commonly been divided into two "groups", referred to as *harmsworthi* and *furciger*, with both groups currently considered cosmopolitan, with global reports including from both the Arctic and the Antarctic. However, some authors considered that *Meb. furciger,* as originally described, may represent an Antarctic-specific lineage. Using collections of tardigrades from across the Antarctic continent and publicly available sequences obtained from online databases, we use mitochondrial and nuclear ribosomal sequence data to clarify the relationships of Antarctic *Mesobiotus* species. Our analyses show that all Antarctic members belong to a single lineage, evolving separately from non-Antarctic representatives. Within this Antarctic lineage there are further deep divisions among geographic regions of the continent, consistent with the presence of a species complex. Based on our data confirming the deep divisions between this Antarctic lineage, which includes representatives of both groups, we recommend that the use of *furciger* and *harmsworthi* group terminology is now abandoned, as it leads to systematic and biogeographical confusion.

1. Introduction

Antarctica has been geographically isolated and progressively glaciated for at least 30 million years. Only 0.3% of the land surface is currently ice-free and suitable for terrestrial life, with most ice-free areas small, fragmented and isolated from each other (Bergstrom and Chown, 1999; Convey et al., 2008; Convey and Stevens, 2007). This long-term isolation has led to evolutionary divergence and speciation, creating the high levels of endemism characterising multiple terrestrial invertebrate groups currently present in Antarctica, including mites, springtails, rotifers and nematodes (Cakil et al., 2021; Convey et al., 2020, 2008; Pugh and Convey, 2008; Stevens et al., 2021; Velasco-Castrillón et al., 2014).

Another terrestrial group that is well represented across the Antarctic continent and surrounding islands is the Tardigrada (Cesari et al., 2016; Convey and McInnes, 2005; Guidetti et al., 2017; Velasco-Castrillón et al., 2015). Of particular interest is *Mesobiotus furciger* Murray, 1907 and the relationships between the groups of tardigrades that are included within the relatively new genus *Mesobiotus* Vecchi, Cesari, Bertolani et al., 2016. Tardigrade taxonomists use the term "group" in two different contexts, as a "species group" or a "morphogroup". A "species group" is a taxonomic place holding device. At the species level, morphological characters of these tiny animals are often exceedingly subtle and what is initially described as a good species, over time becomes a cluster of species each with its own growing list of increasingly subtle differences. As a testament to the skills of morphological taxonomists, many of these differences are now being supported by molecular data and, over time, the group may be elevated to genus. For example, within the genus *Macrobiotus* the "*richtersi*" and "*areolatus*" groups have recently become the genus *Paramacrobiotus* Guidetti, Schill, Bertolani et al., 2009. A "morphogroup" is a group of species that share a morphological character or characters but lacks systematic support. Two such groups that share morphological features are the "*harmsworthi*" and "*furciger*" groups. Using molecular analyses they were demonstrated to form a monophyletic group and have been merged to become the genus *Mesobiotus* (Vecchi et al., 2016). Similarly *Macrobiotus hufelandi* Schultz, 1834 formed the basis of the "*hufelandi*" group that unites a suite of widespread species that share similar morphological characters (Kaczmarek and Michalczyk, 2017; Stec et al., 2021). However, these groups of "*harmsworthi*" and "*furciger*" are non-monophyletic and, although

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explicitly referred to as "species groups" in recent literature (Kaczmarek et al., 2020, 2018), which implies systematic support, should be correctly considered morphogroups (see Stec et al., 2021 for context). Indeed, the use of groups in this context with no clarity of "group" definition may have led to misidentification of lineage evolution and biogeographic structure. This is evident when considering the study of Binda et al. (2005) who were the first to suggest that *Macrobiotus furciger* (now *Mesobiotus furciger*) was likely to be an Antarctic-specific lineage. They also recongnised the existence of other species showing similarities to *Mesobiotus furciger*, suggesting that these had been erroneously attributed to that species. That there could be a specific Antarctic lineage of Tardigrada implies a long continuous presence on the continent, from which one would expect *in situ* evolution and thus diversification to have occurred.

Several species of *Mesobiotus* (accepted abbreviation is *Meb.* to prevent confusion with the closely related genus *Macrobiotus,* abbreviated as *Mac.* (Perry et al., 2019)) currently assigned to either of the global "*harmsworthi"* or "*furciger"* groups have been reported from various Antarctic locations. However, there is no strong direction as to how each overall group is defined (Binda et al., 2005; Kaczmarek et al., 2020), although egg morphology is an important character. Described species include *Meb. aradasi* Binda, Pilato & Lisi, 2005, *Meb. blocki* Dastych, 1984, *Meb. furciger* Murray, 1907, *Meb. hilariae* Vecchi, Cesari, Bertolani et al., 2016, *Meb. krynauwi* Dastych & Harris, 1995, *Meb. montanus* Murray, 1910, *Meb. mottai* Binda and Pilato, 1994 and *Meb. polaris* Murray, 1910. However, it is possible, or even likely, that some of these taxa should be synonymised – for example, the recently described *Meb. hilariae* Vecchi, Cesari, Bertolani et al., 2016 and the taxonomically dubius *Meb. polaris* (Kaczmarek et al., 2020) – and it is also possible that within any of these taxa there may be further as yet unrecognised diversity. As species-level morphological taxonomy of tardigrades deals with subtle differences in tiny characters only visible under high power or electron microscopy, accurately identifying specimens collected from field studies that are then to be used for molecular studies is exceedingly difficult, as morphological taxonomy requires mounting in media under slides for oil immersion that preclude the use of the specimen for DNA extraction, while the DNA extraction methods we use are destructive leaving nothing behind for taxonomy. Systems are in place to reconcile morphology and molecular results based on cultures (Cesari et al., 2009) but these have proved difficult to apply to our Antarctic field based collections. Irrespective of precise taxonomic placement, the evolution of these tardigrades in Antarctica is of particular interest as it may have taken place over the breakup of Gondwana and through the successive extensive periods of Antarctic glaciation. Using the "group" term may be a useful tool that sacrifices precision but retains accuracy, as long as the group represents a systematically united collection of species, or clade. An important practical consideration is that it is possible to assign individual tardigrades to a group under low powered microscopy prior to DNA extraction and molecular analyses (Sands et al., 2008a).

The nominate species *Mesobiotus furciger* is a limno-terrestrial eutardigrade originally described from the maritime Antarctic South Orkney Islands (Murray, 1907), and suggested to have a wide distribution across and beyond Antarcrica (Binda et al., 2005; Binda and Rebecchi, 1992). Studies examining Antarctic material referred to as *Meb. furciger* have identified levels of morphological and genetic divergence supporting a "group" of species from around the continent (Binda et al., 2005; Czechowski et al., 2012; Dastych, 1984; Sands et al., 2008b; Vecchi et al., 2016). With the more recent application of molecular markers there even appears to be very fine spatial partitioning of genetic variation, as Czechowski et al. (2012) identified molecular operational taxonomic units (MOTUs) belonging to distinct lineages between isolated nunataks within the Dronning Maud Land region of continental Antarctica. The existence of distinct spatially explicit lineages suggests a long history of existence and evolution on the Antarctic continent, which leads to important questions: How does *Meb. furciger sensu stricto* and the "Antarctic group" align with the global "*furciger*" group? How are these related to the Antarctic members of the "*harmsworthi*" group? If the existence of these "groups" can be confirmed, are they cosmopolitan as some have suggested (Dastych, 1984), which would imply multiple independent successful colonization events into the Antarctic? Alternatively, are the Antarctic representatives part of a single long-isolated lineage?

In this study we set out to clarify the structure of diversity within the genus *Mesobiotus*. Through this we hope to develop a better understanding of the origins, evolution and diversification of tardigrades in Antarctica. Two independently evolving gene regions were chosen for phylogenetic analyses, the mitochondrial cytochrome *c* oxidase sub-unit 1 (COX1) that is a proven marker for investigating both intra- and interspecific relationships (Ballard and Whitlock, 2004), and the ribosomal small sub-unit (18S) that tends to be less variable within species but is very useful for examining deep evolutionary histories (Field et al., 1988; Rajendhran and Gunasekaran, 2011). A further advantage of these unlinked genes is that they are the most used gene regions in phylogenetic reconstruction and species discrimination making their use much more likely to be comparable between studies (Blaxter et al., 2005; Floyd et al., 2002; Hajibabaei et al., 2007; Hebert et al., 2003). Specimens assigned to *Mesobiotus* were collected from diverse locations across Antarctica, combined with publicly available sequences of Antarctic *Mesobiotus*, and were compared with *Mesobiotus* sequences from around the world.

2. Methods

2.1. Sample and sequence collection

Fresh collections of moss were sampled from multiple locations in the maritime Antarctic (Antarctic Peninsula and Scotia Arc archipelagos) during the period 30th November 2018 to 28th February 2019 (Fig. 1). Moss samples of approximately 10 g (dry mass) were carefully removed using a small trowel, which was cleaned with ethanol between sampling to prevent cross-contamination. Each sample of moss was air dried before being placed in an individual paper herbarium bag and sealed in a plastic box for transport to the British Antarctic Survey, Cambridge, UK under all required quarantine protocols. Mosses from continental Antarctica (Dronning Maud Land, Mac. Robertson Land, Victoria Land) were sampled from the collections stored at −20 °C in the South Australian Museum, Adelaide, South Australia (Fig. 1). These samples were processed *in situ* as required by Australian quarantine protocols and individual tardigrades were stored in RNAlater (ThermoFisher) for transport to the British Antarctic Survey, Cambridge, UK. Further information on the collection of this material can be found in Velasco-Castrillón et al., (2015). All other available sequence data relating to Antarctic *Mesobiotus* (for example: Czechowski et al., 2012; Kaczmarek et al., 2020, 2018; Vecchi et al., 2016) and relevant outgroups were downloaded from NCBI GenBank. The sample locations are shown in Fig. 1 and a complete list of ingroup species and their sample details can be found in Table 1.

2.2. Extraction and identification of tardigrades

The technique used for extracting individuals from the material was a density gradient, flotation technique modified from Sands et al. (2008a). Samples of material were re-hydrated in reverse osmosis (RO) water for 24 h at room temperature before being lightly homogenised by hand in a small beaker. A 1 mL layer of pure OptiPrep™ (SigmaAldrich) density gradient medium was added to a standard test tube with a 2 mL layer of a 50/50 OptiPrep™ and RO water mixture added to the top to create a double layer. The test tube was then filled to the top with the homogenised material to form a third layer. The tubes were then centrifuged on full power (110 × *g*) for 1 min. The top layer was then carefully removed and passed through a 32 μm sieve, which was then rinsed into a Petri dish. The dish was examined under a Wild M5

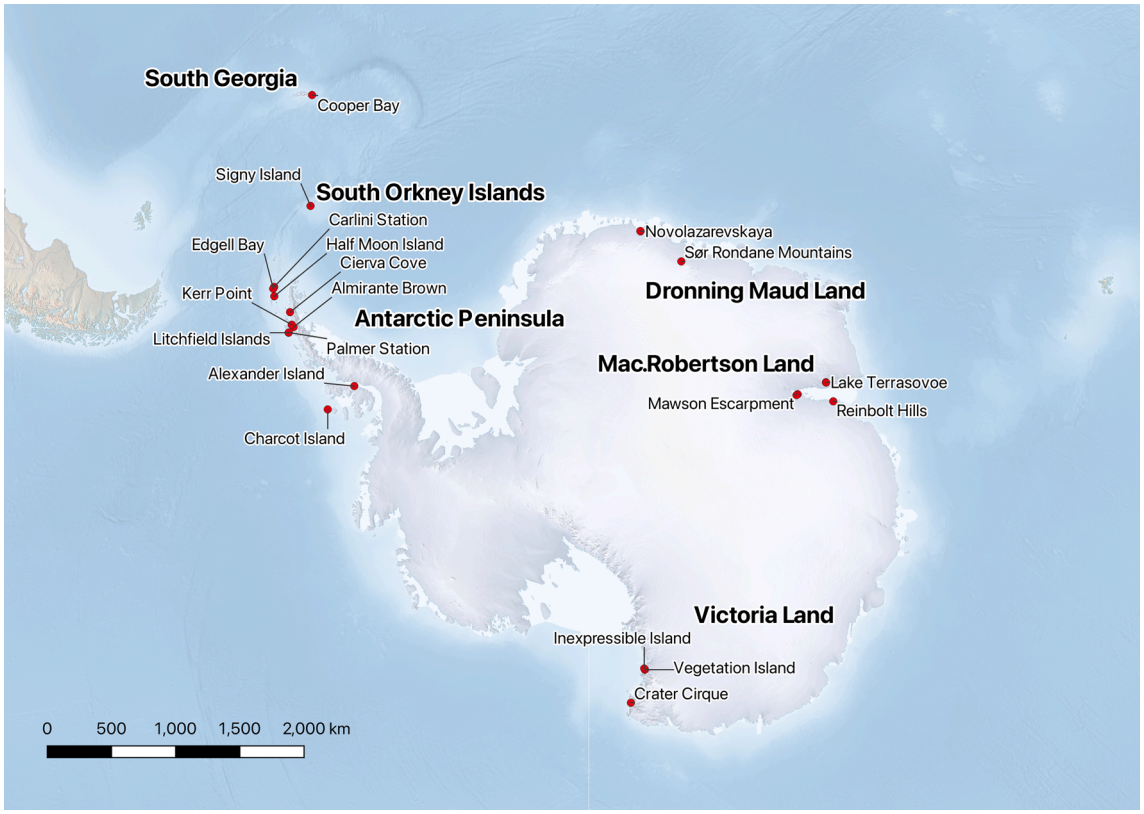

Fig. 1. Map of the Antarctic continent with surround regions. Locations where samples originated from are indicated, with details of each sample and sequence listed in Table 1.

dissection microscope and, if individual tardigrades or eggs were present, they were removed using an Irwin loop. Each individual collected was then placed into a drop of RO water in a cavity slide for identification under 40 × magnification with an Olympus BX30 microscope. Those identified as resembling *Mesobiotus* were placed individually into 0.5 mL micro-centrifuge tubes containing 10 μL of RNA/DNA-free water for molecular analyses. Where eggs were available, these were either mounted and photographed, or sequenced to assist in assigning sequences to groups based on morphogroup. Photographs of egg exemplars are presented in Supplementary Figure 1.

2.3. DNA extraction, amplification, and sequencing

DNA extraction protocols and application followed Sands et al. (2008a). In brief this involved adding 40 μL of 5% Chelex 100 solution to each individual tardigrade tube (to give 50 μL total). Each tube was then subjected to six freeze–thaw cycles using dry ice for freezing and a heating block set at 99 °C for the thaw with a short vortex after each cycle. After the cycles were complete the tubes were heated to 99 °C for 20 min, then vortexed and centrifuged for 2 min at 110 × g. Samples were stored at −20 °C until DNA amplification.

Two microlitres of the DNA extraction were added to 19 μL of the master mix and the following primers for amplification of the COX1 gene region used: LCO_1490 (forward) (GGTCAACAAATCATAAAGATATTGG) (Folmer et al., 1994) and HCOoutout (reverse) (GTAAATATATGRTGDGCTC) (Prendini et al., 2005), and amplified using the protocol described in Sands et al. (2008a). For amplification of the 18S gene region the same procedure was used with the following primers: 18S_Tar_Ff1 (forward) (AGGCGAAACCGCGAATGGCTC) (Stec et al., 2017) and 18S_Tar_Rr2 (reverse) (CTGATCGCCTTCGAACCTCTAACTTTCG) (Gąsiorek et al., 2017) and amplified using the first 18S amplification protocol described in Sands et al. (2008a). Products were sequenced commercially by Macrogen Ltd (Netherlands).

2.4. Sequence alignment, summary statistics and phylogenetic analyses

Trace files of both the 18S and COX1 sequences were imported to CodonCode Aligner ver. 5.1.5 (CodonCode Corp., Dedham, MA), where they were base-called and quality assessed using the PHRED function in CodonCode Aligner (Ewing et al., 1998; Ewing and Green, 1998). The forward and reverse fragments of each sequence for each individual were paired using the Advanced Assembly function of CodonCode Aligner to form a contig for each gene region. Every contig was then checked by eye.

Both 18S and COX1 sequences were aligned using MAFFT v7.45 (Katoh et al., 2002; Katoh and Standley, 2013) within the software GENEIOUS v11.1.5 (Biomatters). Outgroups included *Murrayon pullari* Murray, 1907, *Murrayon dianeae* Kristensen, 1982, *Dactylobiotus* Schuster, Nelson, Grigarick et al., 1980 species sampled from the Arctic and Antarctic members of the *Macrobiotus hufelandi* group. Alignment of COX1 was trivial with no ambiguities and all sequences were checked for an open reading frame. The 18S alignment required some editing around the arbitrary placements of gaps. As arbitrary placement of gap columns may impact sequence homology the ambiguous gap regions were removed to reduce alignment artefacts.

Substitution saturation in COX1 can decrease the amount of phylogenetic signal, but the point at which sequence similarities could be the result of chance alone rather than homology is not homogenous across the entire phylogeny (Yang, 1998). We tested three datasets (1) Entire taxon dataset of 92 sequences (64 unique) corresponding to Table 1 using all codons, and separately for each codon; (2) all *Mesobiotus* taxon dataset of 59 sequences (49 unique); and (3) all Antarctic *Mesobiotus* taxon dataset of 39 sequences (30 unique). Saturation of substitutions was evaluated by plotting the number of transitions (s) and transversions (v) against genetic distance, as implemented in DAMBE7 (ver. 7.0.13) (Xia, 2018) using only unique sequences (as required in DAMBE7). We used the GTR substitution model that was found to be the

Table 1
Metadata associated with individual sequences used in this study, including precise locations where available, GenBank accession numbers, whether the sequence was obtained from an egg, and the citation of where the sequence was first published.

Region	Location	Latitide	Longitude	Accession # 18S	Accession # CO1	Individual Ref	Idenification	Source
Dronning Maud Land	Sør Rondane Mountains	−72.018	23.095		JX296220	BCOIAD023	*Meb*. sp	Czechowski et al. 2012
Dronning Maud Land	Sør Rondane Mountains	−72.017	23.094		JX296250	BCOIAD043	*Meb*. sp	Czechowski et al. 2012
Dronning Maud Land	Sør Rondane Mountains	−72.017	23.094		JX296240	BCOIAD049	*Meb*. sp	Czechowski et al. 2012
Dronning Maud Land	Sør Rondane Mountains	−72.017	23.094		JX296228	BCOIAD083	*Meb*. sp	Czechowski et al. 2012
Dronning Maud Land	Sør Rondane Mountains	−72.017	23.094		JX296257	BCOIAD056	*Meb*. sp	Czechowski et al. 2012
Dronning Maud Land	Sør Rondane Mountains	−72.017	23.094	JX296290		A18S054	*Meb*. Sp	Czechowski et al. 2012
Dronning Maud Land	Novolazarevskaya	−70.776	11.814	KT226068		C3610_A1	*Meb. hilariae*	Vecchi et al. 2016
Dronning Maud Land	Novolazarevskaya	−70.776	11.814	KT226069		C3610_A2	*Meb. hilariae*	Vecchi et al. 2016
Dronning Maud Land	Novolazarevskaya	−70.778	11.818	KT226070		C3620_B1	*Meb. hilariae*	Vecchi et al. 2016
Dronning Maud Land	Novolazarevskaya	−70.759	11.7817	KT226071	KT226108	C3623_C1	*Meb. hilariae*	Vecchi et al. 2016
Victoria Land	Vegetation Island	−74.784	163.646	KT226075		C3431_1	*Meb. polaris*	Vecchi et al. 2016
Victoria Land	Vegetation Island	−74.784	163.646	KT226076		C3431_2	*Meb. polaris*	Vecchi et al. 2016
Victoria Land	Inexpressible Island	−74.884	163.718	KT226077		C3434_1	*Meb. polaris*	Vecchi et al. 2016
Victoria Land	Inexpressible Island	−74.884	163.718	KT226078		C3434_2	*Meb. polaris*	Vecchi et al. 2016
Victoria Land	Crater Cirque	−72.603	169.349	KT226072		C3324	*Mac*. cf *mottai*	Vecchi et al. 2016
South Georgia	Cooper Bay	−54.788	−35.82		JX865310	Macro07_037	*Meb*. sp stellate egg	Czechowski et al. 2012
South Georgia	Cooper Bay	−54.788	−35.82	EU266926		Macro07_039	*Meb*. sp stellate egg	Sands et al. 2008b
South Georgia	Cooper Bay	−54.788	−35.82	MW751940		Macro07_040	*Meb*. sp stellate egg	This study
South Georgia	Cooper Bay	−54.788	−35.82	MW751941		Macro07_041	*Meb*. sp stellate egg	This study
South Georgia	Cooper Bay	−54.788	−35.82	MW751942	MW727957	Macro07_042	*Meb*. sp stellate egg	Czechowski et al. 2012
South Orkney Islands	Signy Island	−60.709	−45.595		JX865308	Macro06_282	*Meb. furciger*	Czechowski et al. 2012
South Orkney Islands	Signy Island	−60.709	−45.595	MW751936	MW727958	Macro06_296	*Meb. furciger*	This study
South Orkney Islands	Signy Island	−60.709	−45.595		MW727959	Macro06_309	*Meb. furciger*	This study
South Orkney Islands	Signy Island	−60.709	−45.595	MW751937	MW727961	Macro06_310	*Meb. furciger*	This study
South Orkney Islands	Signy Island	−60.709	−45.595	EU266929		Macro06_311	*Meb. furciger*	Sands et al. 2008b
South Orkney Islands	Signy Island	−60.709	−45.595	MW751938		Macro06_312	*Meb. furciger*	This study
South Orkney Islands	Signy Island	−60.709	−45.595	MW751939	MW727960	Macro06_313	*Meb. furciger*	This study
Antarctic Peninsula	Carlini, King George Island	−62.238	−58.668	EU266297		Macro07_014	*Meb.furciger*	Sands et al. 2008b
Antarctic Peninsula	Litchfield Islands	−64.767	−64.1	MW751963		LI_MF_4	*Meb.furciger*	This study
Antarctic Peninsula	Litchfield Islands	−64.767	−64.1	MW751964		LI_MF_6	*Meb.furciger*	This study
Antarctic Peninsula	Litchfield Islands	−64.767	−64.1	MW751965		LI_MF_7	*Meb.furciger*	This study
Antarctic Peninsula	Litchfield Islands	−64.767	−64.1		MW727982	LI_MF_12	*Mac*. sp cf *hufelandi*	This study
Antarctic Peninsula	Cierva Cove	−64.165	−60.895		MW727942	CC_MF_1	*Meb. furciger*	This study
Antarctic Peninsula	Cierva Cove	−64.165	−60.895	MW751948		CC_MF_3	*Meb. furciger*	This study
Antarctic Peninsula	Cierva Cove	−64.165	−60.895	MW751949	MW727933	CC_MF_4	*Meb. furciger*	This study
Antarctic Peninsula	Cierva Cove	−64.165	−60.895		MW727979	CC_MF_5	*Mac*. sp cf *hufelandi*	This study
Antarctic Peninsula	Cierva Cove	−64.165	−60.895		MW727943	CC_MF_7	*Meb. furciger*	This study
Antarctic Peninsula	Charcot Island	−69.47	−75.185	EU266298		Macro05_147	*Meb. furciger*	Sands et al. 2008b
Antarctic Peninsula	Charcot Island	−69.47	−75.185		JX865306	Macro05_148	*Meb. furciger*	This study
Antarctic Peninsula	Almirante Brown	−64.902	−62.858		MW727931	ABDC_MF_1	*Meb. furciger*	This study

(*continued on next page*)

Table 1 (*continued*)

Region	Location	Latitide	Longitude	Accession # 18S	Accession # CO1	Individual Ref	Idenification	Source
Antarctic Peninsula	Almirante Brown	−64.902	−62.858	MW751943		ABDC_MF_2	*Meb. furciger*	This study
Antarctic Peninsula	Almirante Brown	−64.902	−62.858	MW751944	MW727932	ABDC_MF_3	*Meb. furciger*	This study
Antarctic Peninsula	Almirante Brown	−64.902	−62.858		MW727936	ABDC_MF_8	*Meb. furciger*	This study
Antarctic Peninsula	Kerr Point	−64.706	−62.637	MW751962	MW727934	KPRI_MF_1	*Meb. furciger*	This study
Antarctic Peninsula	Kerr Point	−64.706	−62.637		MW727981	KPRI_MF_5	*Mac*. sp cf *hufelandi*	This study
Antarctic Peninsula	Kerr Point	−64.706	−62.637		MW727935	KPRI_MF_8	*Meb. furciger*	This study
Antarctic Peninsula	Half Moon Island	−62.593	−59.918	MW751957	MW727941	HMI_MF_1	*Meb. furciger*	This study
Antarctic Peninsula	Half Moon Island	−62.593	−59.918	MW751958		HMI_MF_5	*Meb. furciger*	This study
Antarctic Peninsula	Half Moon Island	−62.593	−59.918		MW727980	HMI_MF_8	*Mac*. sp cf *hufelandi*	This study
Antarctic Peninsula	Edgell Bay	−62.248	−58.987	MW751951		EBNI_MF_1	*Meb. furciger*	This study
Antarctic Peninsula	Edgell Bay	−62.248	−58.987	MW751952	MW727937	EBNI_MF_2	*Meb. furciger*	This study
Antarctic Peninsula	Edgell Bay	−62.248	−58.987	MW751953		EBNI_MF_3	*Meb. furciger* egg	This study
Antarctic Peninsula	Edgell Bay	−62.248	−58.987	MW751954	MW727938	EBNI_MF_4	*Meb. furciger* egg	This study
Antarctic Peninsula	Palmer Station	−64.774	−64.054	MW751966		PSAI_MF_1	*Meb. furciger*	This study
Antarctic Peninsula	Palmer Station	−64.774	−64.054	MW751967	MW727939	PSAI_MF_2	*Meb. furciger*	This study
Antarctic Peninsula	Palmer Station	−64.774	−64.054	MW751968		PSAI_MF_3	*Meb. furciger*	This study
Antarctic Peninsula	Palmer Station	−64.774	−64.054	MW751969		PSAI_MF_4	*Meb. furciger* egg	This study
Antarctic Peninsula	Palmer Station	−64.774	−64.054		MW727940	PSAI_MF_7	*Meb. furciger* egg	This study
Antarctic Peninsula	Duthiers Point	−64.807	−62.818	MW751950		DPL_MF_1	*Meb. furciger*	This study
Antarctic Peninsula	Duthiers Point	−64.807	−62.818		MW727983	DPL_MF_3	*Mac*. sp cf *hufelandi*	This study
Antarctic Peninsula	Alexander Island	−70.815	−68.493	MW751933		Macro06_159	*Meb. furciger*	This study
Antarctic Peninsula	Alexander Island	−70.815	−68.493		JX865314	Macro06_161	*Meb. furciger*	Czechowski et al. 2012
Antarctic Peninsula	Alexander Island	−70.815	−68.493	MW751934	MW727955	Macro06_162	*Meb. furciger*	This study
Antarctic Peninsula	Alexander Island	−70.815	−68.493	MW751935	MW727956	Macro06_171	*Meb. furciger* egg	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927	MW751945		AF01_MF_1	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927		MW727944	AF01_MF_2	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927	MW751946		AF01_MF_3	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927	MW751947		AF01_MF_4	*Meb. furciger* egg	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927		MW727948	AF01_MF_6	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927		MW727949	AF01_MF_7	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.517	67.927		MW727950	AF01_MF_8	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.518	68.004	MW751959	MW727951	JN07_MF_1	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.518	68.004		MW727946	JN07_MF_2	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.518	68.004		MW727952	JN07_MF_3	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.518	68.004	MW751960	MW727953	JN07_MF_4	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.518	68.004		MW727954	JN07_MF_5	*Meb. furciger*	This study
Mac Robertson Land	Lake Terrasovoe	−70.518	68.004	MW751961	MW727947	JN07_MF_8	*Meb. furciger*	This study
	Mawson Escarpment	−72.82	68.042		MW727962	AP01_MF_3	*Mac*. sp cf *hufelandi*	This study

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Table 1 (*continued*)

Region	Location	Latitide	Longitude	Accession # 18S	Accession # CO1	Individual Ref	Idenification	Source
Mac Robertson Land								
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727963	AP01_MF_4	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727964	AP01_MF_5	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727965	AP01_MF_8	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727966	AP01_MF_9	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727967	AP01_MF_13	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727968	AP01_MF_21	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727969	AP01_MF_25	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727970	AP01_MF_28	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.82	68.042		MW727971	AP01_MF_30	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727972	FP03_MF_1	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727973	FP03_MF_4	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727974	FP03_MF_7	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727975	FP03_MF_8	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727976	FP03_MF_12	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727977	FP03_MF_13	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Mawson Escarpment	−72.924	68.142		MW727978	AP03_MF_26	*Mac*. sp cf *hufelandi*	This study
Mac Robertson Land	Reinbolt Hills	−70.487	72.493	MW751955	MW727945	FN01_MF_6	*Meb. furciger* egg	This study
Mac Robertson Land	Reinbolt Hills	−70.487	72.493	MW751956		FN01_MF_7	*Meb. furciger* egg	This study
Europe				HQ604967			*Meb. harmsworthi*	Bertolani et al. 2014
Europe				HQ604968			*Meb. harmsworthi*	Bertolani et al. 2014
Europe				HQ604969			*Meb. harmsworthi*	Bertolani et al. 2014
Europe				HQ604970			*Meb. harmsworthi*	Bertolani et al. 2014
Europe	Italy			KT226073			*Meb. harmsworthi* group	Vecchi et al. 2016
Europe	Italy			KT226074			*Meb. harmsworthi* group	Vecchi et al. 2016
Russia				MH197149	MH195154		*Meb. harmsworthi* group	Kaczmarek et al. 2018
Norway				MH197148	MH195153		*Meb. furciger* group	Kaczmarek et al. 2018
Svalbard	Hornsund	77.01333	15.55139	MH197147	MH195152		*Meb. occultas*	Kaczmarek et al. 2018
Svalbard	Phippsøya	80.68694	20.84444	MH197146	MH195154		*Meb. harmsworthi*	Kaczmarek et al. 2018
Philippines				MF441488	MF441491		*Meb. insanis*	Mapalo et al. 2017
Philippines				KX129793	KX129796		*Meb. philippinicus*	Mapalo et al. 2016
Philippines				MN257048	MN257047		*Meb. dilimanensis*	Itang et al. 2020
Vietnam				MK584659	MK578905		*Meb. datanlanicus*	Stec 2019
Ethiopia				MF678793	MF678794	Stec and Kristensen 2017	*Meb. ethiopicus*	Stec and Kristensen, 2017
Kenya				MH197153	MH195148		*Meb. radiatus*	Stec et al., 2018a
Ecuador				MH197158	MH195149		*Meb. romani*	Roszkowska et al., 2018
Madagascar				MH681585	MH676056		*Meb. fiedleri*	Kaczmarek et al., 2020
Canada	Banff National Park	51.40583	−116.2408	MW680642	MW656257	CN8.115/S	*Meb. storackii*	Kayastha et al., 2021
South Africa	Table Mountain	–33.96222	18.41056	MT903468	MT904513		*Meb. anastasiae*	Tumanov, 2020
Columbia				MF568532	MF568534			Stec et al., 2018b

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Table 1 (*continued*)

Region	Location	Latitide	Longitude	Accession # 18S	Accession # CO1	Individual Ref	Idenification	Source
							Paramacrobiotus lachowskae	
Europe	Italy			MK041023	MK040994		*Paramacrobiotus richtersi*	Guidetti et al., 2019
United States of America				MH664946	MH676018		*Paramacrobiotus tonollii*	Stec et al., 2020
Europe	Spain			FJ435737	FJ435801		*Murrayon dianeae*	Guil and Giribet, 2012
Europe	Italy				AY598772		*Murrayon pullari*	Guidetti et al., 2005
				HQ604983			*Murrayon pullari*	Bertolani et al., 2014
Europe				MT373695	MT373804		*Dactylobiotus parthenogeneticus*	Pogwizd and Stec, 2020
Antarctica	King George Island			EF632436	EF632525	Dacty_078	*Dactylobiotus* sp	Sands et al., 2008a

most appropriate model (see model selection below). Plots are provided in Supplementary Figure 2.

COX1 haplotype relationships were visually assessed using the TCS (Clement et al., 2000) network method in POPART v1.7 (Leigh and Bryant, 2015). Summary statistics for COX1 alignment were generated in DNAsp v6.12.3 (Rozas et al., 2017, 2003). Within- and between-group distances were calculated using MEGA X (Kumar et al., 2018).

Model selection was conducted using JModelTest 2 (Darriba et al., 2012) which identified a GTR + I + Γ model as best fit for both datasets. Due to strong correlation between invariant sites "I" and gamma distribution "Γ" (Yang, 2014) we used a simplified GTR + Γ model in our analyses. Phylogenetic inference of the 18S and COX1 gene regions were performed using both Maximum Likelihood (ML) and Bayesian methods. In both analyses the COX1 alignment was partitioned into codon positions. ML analyses were performed using RAxML v8.2.11 (Stamatakis, 2014; Stamatakis et al., 2012) using rapid bootstrapping method searching for the best scoring ML tree and including 1000 bootstrap pseudoreplicates. Bayesian phylogenetic reconstruction was conducted in Mr Bayes v3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Molecular clock analyses are only a very rough estimate in single gene non-model taxa. In order to estimate approximate dates of relevant divergences a range of proposed rates, from a conservative 0.01564 to a more rapid 0.05 mutations per site per million years were used. The conservative rate was suggested by Guidetti et al. (2017), (0.001264 for nuclear genes, converted to 0.01564 to account for a ten times faster mitochondrial rate), and the faster rate falls within those suggested by Loeza-Quintana et al. (2019) for Arctic marine arthropods. Analyses on both gene alignments ran four replicates of $2x10^7$ generations of four heated chains, sampling every 1000th generation. Convergence was assessed using TRACER v1.7.1 (Rambaut et al., 2014) and a burn-in of 25 % applied to ensure all sampled trees were post convergence. Trees were initially viewed in FigTree v1.4.4 and exported to Graphic v3.1 (Picta Inc.). The consensus tree was used from Bayesian analyses, with tip branches collapsed to the first moderately supported node (pp > 0.8). The most likely trees were used from the likelihood analyses and nodes collapsed where bootstrap support was less than 50. Details of the node collapse strategy are presented in Supplementary Figures 3–6.

Species delimitation used general mixed yule coalescent (GMYC, Fujisawa and Barraclough, 2013) using both the single and multiple threshold methods in the Splits package implemented in R, and multi-rate Poisson tree process (mPTP, Kapli et al., 2016; Zhang et al., 2013), also using single and multiple thresholds. Datasets were generated using only Antarctic *Mesobiotus* species, and *Meb. insanis* as the outgroup. Ultrimetric trees used for GMYC were generated using Mr Bayes following the protocol described above for molecular clock analysis, Phylogenies for mPTP were generated in RAxML.

3. Results

Sequences of COX1 from 43 Antarctic *Mesobiotus* specimens were included in a population-based assessment. Haplotype network analysis indicated 6 genetically and geographically disparate groups, representing specimens from South Georgia, South Orkney Islands, Antarctic Peninsula (including the South Shetland Islands), Alexander Island, Dronning Maud Land and Mac. Robertson Land (Fig. 2). Genetic distances between groups were large (between 18 % uncorrected P between South Georgia and Dronning Maud Land (30 % corrected) and 24.7 % uncorrected P between Mac. Robertson Land and South Orkney Islands (47.9 % corrected, see Table 2), consistent with these groups representing different species.

The ratios between haplotype and nucleotide diversity (H_D and π) were particularly high among Antarctic Peninsula, Mac Robertson Land and Dronning Maud Land individuals (Table 3), indicating considerable divergence or variation within each of these groups, more than would be expected within a species (Goodall-Copestake et al., 2012). Summary statistics investigating population growth and selection were all non-significant.

Phylogenetic analyses of COX1 further clarified the spatial genetic groups, with strong support (all posterior probabilities (pp) = 1) for each of these clades (Figs. 3 and 4). Furthermore, the maximum likelihood analysis shows that all Antarctic *Mesobiotus* specimens all grouped together in a single clade to the exclusion of all other (non-Antarctic) *Mesobiotus*. The Bayesian COX1 analysis shows similar grouping of Antarctic *Mesobiotus*, although in 3 out of 4 runs *Meb. philippinicus* Mapalo, Stec, Mirano-Bascos et al., 2016 was was included in this clade resulting in lower node support. Despite a wide range of clockrates, the node age estimates were very similar between runs. The estimated age of the Antarctic node had a 95% highest probable density between 65 and 100 million years before present (range across two clockrates 63–102 mybp) with a most probable estimate of 83 million years before present (range between 82 and 84 mybp). The 18S phylogeny is broadly congruent regarding the genetic spatial groupings and firmly places *Meb. philippinicus* within the non-Antarctic group. In the latter, sequences from Dronning Maud Land belonging to specimens identified as *Meb. hilariae* by Vecchi et al. (2016) cluster with the *Mesobiotus* sp sample of Czechowski et al. (2012). This sequence was found in several individuals and represents the only 18S haplotype from this region (Sør Rondane Mountains, within Dronning Maud Land), although there was some variation in the corresponding COX1 sequences obtained from the same individuals (Czechowski et al., 2012). Although clustered in the same clade, the *Meb. polaris* specimens from Victoria Land appear to be genetically discrete from the Dronning Maud Land *Meb. hilariae* and the Sør Rondane sequence (Fig. 3).

While the analysis of COX1 sequences provided evidence for a single

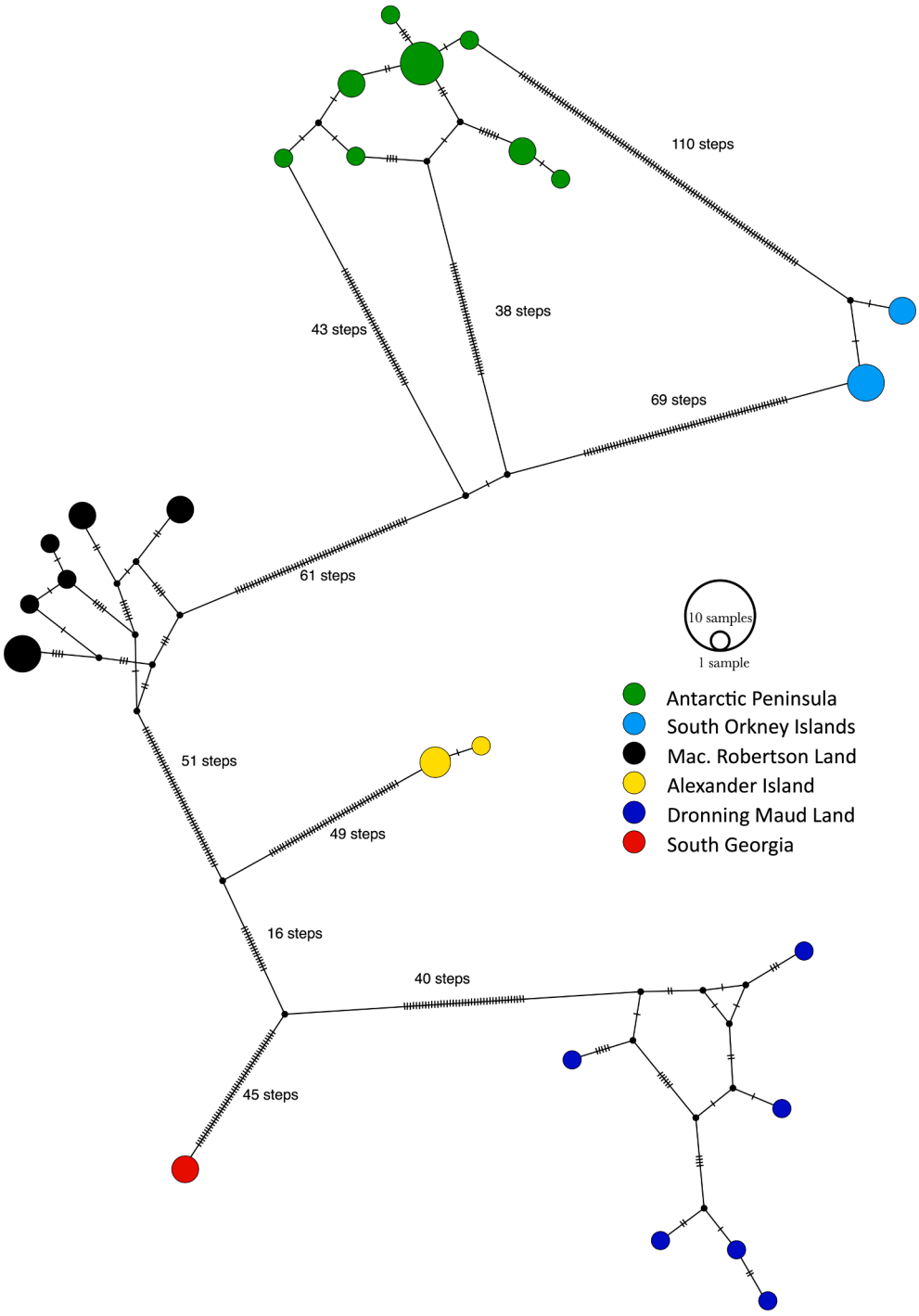

Fig. 2. Haplotype network of COX1 sequences collected for this study showing frequency (proportional to size) and relatedness of Antarctic *Mesobiotus* haplotypes sampled. Haplotypes are represented by circles coloured by geographic region, and size of circle is proportional to haplotype frequency. Solid black circles represent nodes, or ancestral haplotypes, while dashes indicate a base change or missing haplotype.

Antarctic lineage, there was insufficient resolution in the 18S trees, as neither ML or Bayesian analyses provided strong support for bifurcating nodes (pp less than 0.8, bootstrap less than 50 %). However, all non-Antarctic *Mesobiotus* 18S sequences were clustered in a single well supported clade (pp = 1), whereas the non-Antarctic COX1 sequences, generally without strong node support, were excluded from the Antarctic specimens (Figs. 3 and 4).

The only exception to strong spatial genetic partitioning was a single Victoria Land sequence identified as *Meb.* cf *mottai* by Vecchi et al. (2016) that grouped together with the Antarctic Peninsula clade (sequence-specific details in Supplementary Figure 2).

Species delimitation gave conflicting results between techniques ranging from three species (GMYC Single threshold) to ten species (GMYC Multi threshold). Both mPTP strategies found six species that correspond with the six major well supported biogeographical clades produced during the phylogenetic analyses. Graphical results are presented in Fig. 5.

Table 2
Genetic distances between the main geographically explicit COX1 clades. Within group P distances are on the diagonal, between group P distances are below the diagonal, and likelihood adjusted distances are above the diagonal.

	Peninsula	South Orkneys	Mac. Robertson	Alexander Island	Dronning Maud	SouthGeorgia
Antarctic Peninsula	**0.010**	0.392	0.396	0.423	0.452	0.449
South Orkney Islands	0.214	**0.000**	0.479	0.451	0.466	0.480
Mac. Robertson Land	0.219	0.247	**0.020**	0.337	0.440	0.383
Alexander Island	0.225	0.230	0.196	**0.000**	0.394	0.363
Dronning Maud Land	0.233	0.233	0.230	0.216	**0.020**	0.308
South_Georgia	0.231	0.236	0.209	0.205	0.186	**0.000**

Table 3
Summary statistics for the main geographically explicit COX1 clades.

Population Name	N	Prob.	S	HD	π	DT	Fs	R2	Kmax
Antarctic Peninsula	14	0.87	28	0.868	0.0128	−0.3	0.954	0.133	22
South Orkney Islands	6	0.71	3	0.533	0.0025	1.124	2.506	0.267	3
Mac. Robertson Land	11	0.83	25	0.855	0.0191	1.57	3.097	0.226	20
Alexander Island	4	0.6	1	0.5	0.0008	−0.612	0.172	0.433	1
Dronning Maud Land	6	0.71	27	1	0.0198	−0.788	−0.85	0.153	18
South Georgia	2	0.33	0	0	0	NA	NA	NA	NA

N, number of samples.
Prob., probablility of having captured the deepest coalescent event.
S, Number of segregating sites.
HD, Haplotype diversity.
π, Nucleotide divertsity.
DT, Tajima's D.
Fs, Fu's S.
R2, Ramos-Onsins & Rozas' R2.
Kmax, Maximum number of nucleotide differences between any two sequences.

4. Discussion

The genus *Mesobiotus* has been shown to be cosmopolitan, with examples known from every continent and from the Arctic to Antarctica (Kaczmarek et al., 2020). The two groups, "*harmsworthi*" and "*furciger*", have also been suggested to be cosmopolitan (Binda and Rebecchi, 1992), with examples of the "*furciger*" group being proposed from Arctic Norway (Kaczmarek et al., 2018) and Madagascar (Kaczmarek et al., 2020). Binda et al. (2005) made the alternative suggestion that *Meb. furciger sensu stricto* was likely to be Antarctic-specific, redescribing the species and describing several similar but distinct (*furciger*-like) species from the other Southern Hemisphere continents. However, information that has become available subsequently suggests that Binda et al.'s (2005) redescription is confounded by geography. In particular, the material used for the redescription originated from sub-Antarctic South Georgia, not the original type locality in the South Orkney Islands (Murray, 1907). The South Georgia specimens included in the current study do not appear to be comparable to those used in the redescription, as the eggs that were sequenced in this study were morphologically distinct from those described by Binda et al. (2005) and, perhaps, more similar to those described from South Georgia by Dastych (1984) as a likely new species similar to *Marobiotus liviae* Ramazzotti, 1962. A feature that is clear throughout our study is the substantial genetic differences between different geographic regions, again supporting that it is unlikely the redescribed South Georgia samples represent *Meb. furciger sensu stricto*.

The current justification of separating *Mesobiotus* into "*harmsworthi*" and "*furciger*" groups based on egg morphology (simple versus complex or castellate tips on egg processes) is not supported on either phylogenetic or systematic grounds. Kaczmarek et al. (2020, 2018) acknowledge the non-monophyly of the two groups while still assigning new species to one or the other. Our results support the finding of non-monophyly based on the current division of groups within *Mesobiotus*. However, and importantly, our data support the concept of deep divisions within the genus that require further taxonomic attention. Of particular relevance to the aims of this study, it is clear that all Antarctic *Mesobiotus*, regardless of which group they are assigned to, are either monophyletic (COX1 inference) or form two Antarctic lineages independent of all non-Antarctic specimens (18S inference). Furthermore, the non-Antarctic "*furciger*" samples are grouped in the non-Antarctic clade (or clades from COX1 inference) labelled in Fig. 3 as Non-Antarctic *Mesobiotus* species. Interestingly the combined 18S and 28S (large ribosomal subunit) phylogenetic analysis of Vecchi et al. (2016) was very similar to our analysis in that the same three clades, two of which are Antarctic, are identified. Their analysis, similar to our COX1 phylogeny, supported the two Antarctic clades forming a single Antarctic lineage (pp = 0.99) to the exclusion of all non-Antarctic *Mesobiotus*.

Our analyses, with support from that of Vecchi et al. (2016) indicate the existence of two major Antarctic lineages. The first contains *Meb. polaris* from Victoria Land, *Meb. hilariae* and *Mesobiotus* sp. from the Sør Rondane Mountains and *Mesobiotus* sp. from South Georgia. These geographically discrete genetic groups with large genetic distances support treating them as distinct species. It is possible that *Meb.* sp from the Sør Rondane Mountains has already been described but without (as prior to the availability of) genetic data. For example *Meb. krynauwi* was described from Dronning Maud Land in 1995 (Dastych and Harris, 1995). Even within the Sør Rondane Mountains specimens, represented by multiple individuals but a single 18S haplotype, there was substantial COX1 variation detected between collecting sites (Czechowski et al., 2012). *Meb. polaris* has been suggested to be considered *nomina inquirenda* (Kaczmarek et al., 2020) but our results support those of Vecchi et al. (2016) in distinguishing these individuals as a supported monophyletic clade distinct from other *Mesobiotus* species sampled around Antarctica. Vecchi et al. (2016) states that all species of *Mesobiotus* found on continental Antarctica belong to the "*harmsworth"i* group (which may imply that the *"furciger"* group was regarded as a subgroup of "*harmsworthi*") and *Meb. hilariae* in particular was described by them to be of the "*harmsworthi"* group. Although morphology of the eggs

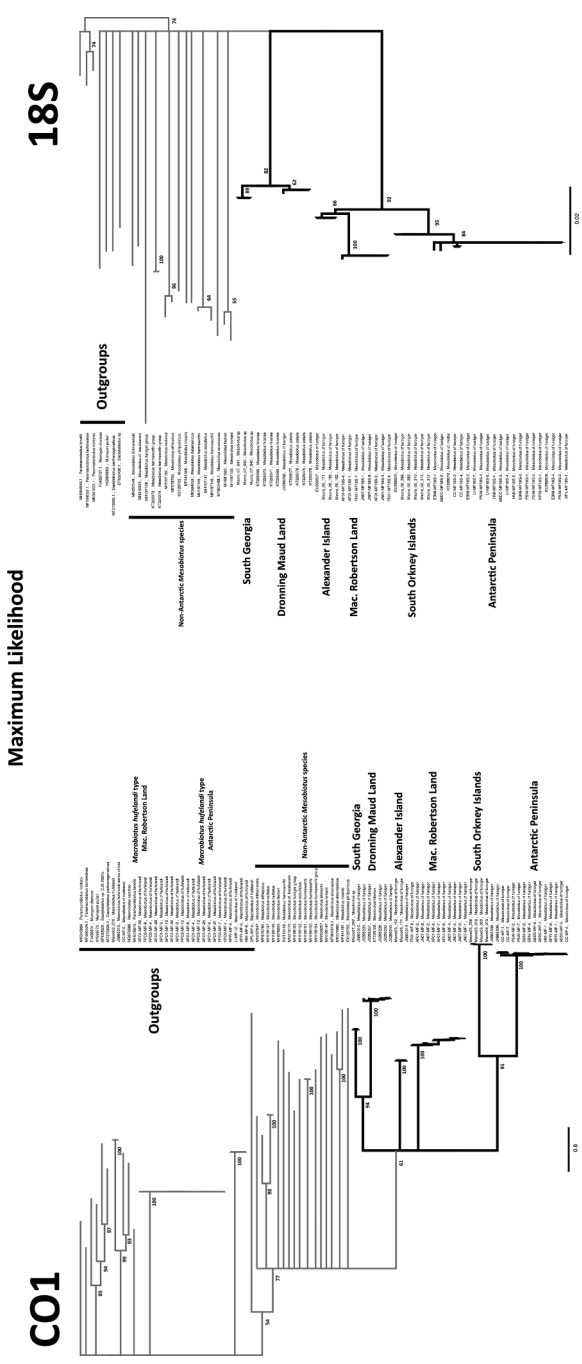

Fig. 3. Maximum Likelihood phylogenies of cytochrome *c* oxidase subunit 1 (COX1) and small sub-unit (18S) from *Mesobiotus* sequences and outgroups. Where bootstrap (node support below the line) was below 50 % nodes were collapsed to the next well supported node (see supplementary Figures 3 and 5 for details). Tips are labelled with GenBank accessions and associated identifications (see Table 1 for details).

associated with the sequences from South Georgia does not match the "*harmsworthi*" (or "*furciger*") groups, it does appear that at least some other lineages in this clade fall within the "*harmsworthi*" group morphotype.

The second Antarctic clade supported by the 18S analysis encompasses the "*furciger*" group morphotypes and is comprised of four biogeographic lineages: three from the Maritime Antarctic (South Orkney Islands, a general Antarctic Peninsula clade that also includes most South Shetland Island samples, and Alexander Island) and one from Mac. Robertson Land. These lineages are mirrored in the COX1 phylogeny. One of these *Meb. furciger* lineages was from samples collected from Signy Island, one of the South Orkney Islands – close to the type locality and possibly representing *Meb. furciger sensu stricto*. This lineage is substantially different from the sequences of all other samples collected throughout the Maritime Antarctic and, even among these Antarctic Peninsula sites. In general the Alexander Island samples were substantially different to all other samples apart from a single specimen from Carlini, King George Island, that differed by a two base deletion. Several of the general Antarctic Peninsula clade samples were from areas elsewhere in the South Shetland Islands very close to the Carlini sample location, and yet were genetically distinct from the latter. For example, Edgell Bay, on Nelson Island, is less than 20 km from Potter Peninsula and Half Moon Island is another of the South Shetland islands less than 75 km distant. Sequences from these two sample locations fall into the general Antarctic Peninsula clade, but are distinct from the Carlini sequence. Binda et al. (2005) described *Meb. aradasi* from King George Island and it is possible that either the Antarctic Peninsula or the Alexander Island lineage represents this species. The single sequence of *Meb*. cf. *mottai* from Victoria Land was clustered within the Antarctic Peninsula sequences, the only example in this study of a clade member not originating from the same general region. It is possible, then, that the Peninsula clade represents *Meb. mottai*. Goodall-Copestake et al. (2012) provides a model of the expectation of haplotype diversity (H_D) and nucleotide diversity (π) within a species (discussed in more detail by Sands et al., 2021). The summary statistics derived from our data show that in three cases the ratio between H_D and π deviated from these expectations suggesting hidden species diversity. These locations were represented by the Antarctic Peninsula clade, the Mac. Robertson Land clade and the Dronning Maud Land clade. This also means that the Victoria Land *Meb.* cf. *mottai*, although grouping together with Antarctic Peninsula samples, may still represent a discrete species.

The *Meb. furciger* like specimens collected from Mac. Roberston Land, East Antarctica, are particularly interesting as they represent a new genetic group and geospatial region and, again, summary statistics suggest the divergences within this region are sufficient to indicate more than one species being present. The Mac. Robertson Land material forms a lineage that groups together with the larger clade that includes the Maritime Antarctic (Antarctic Peninsula, including *Meb*. cf *mottai,* South Orkney Islands and Alexander Island) and is the clade containing vouchers that morphologically are traditionally referred to as "*Mesobiotus furciger*" (Sands et al., 2008a, 2008b). This Antarctic "*furciger*" type clade is certainly widely distributed around Antarctica, and harbours deep genetic divergences, particularly in mitochondrial sequences, separating geographically isolated "sub-groups" – likely a mix of described and undescribed species.

Phylogenetically, the lineage containing the clades that include individuals of *Meb. hilariae*, *Meb. polaris*, and *Meb.* sp. from the Sør Rodane Mountains and South Georgia is distinct from that of *Meb. furciger*, *Meb*. cf. *mottai* and related clades sampled from the Antarctic Peninsula and Mac. Robertson Land. Superficially, the relationship between the two lineages appears to represent a "*harmsworthi*" group lineage and a "*furciger*" group lineage, but only including taxa occurring in Antarctic regions, and not the global diversity of the genus. When placed into context with samples from the rest of the world these two groupings break down, as the "*harmsworthi*" group falls into either two discrete

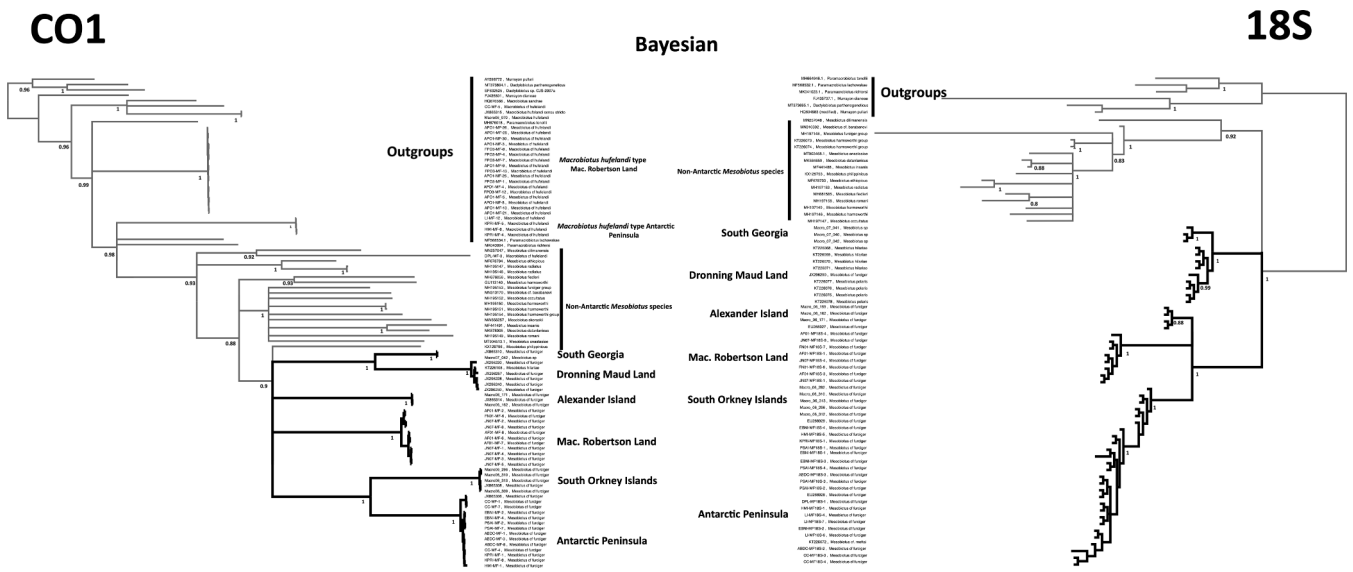

Fig. 4. Bayesian phylogenies of cytochrome *c* oxidase subunit 1 (COX1) and small sub-unit (18S) from *Mesobiotus* sequences and outgroups. Where posterior probabilities (node support below the line) was below 0.8 nodes were collapsed to the next well supported node (see supplementary Figures 4 and 6 for detail). Tips are labelled with GenBank accessions and associated identifications (see Table 1 for details).

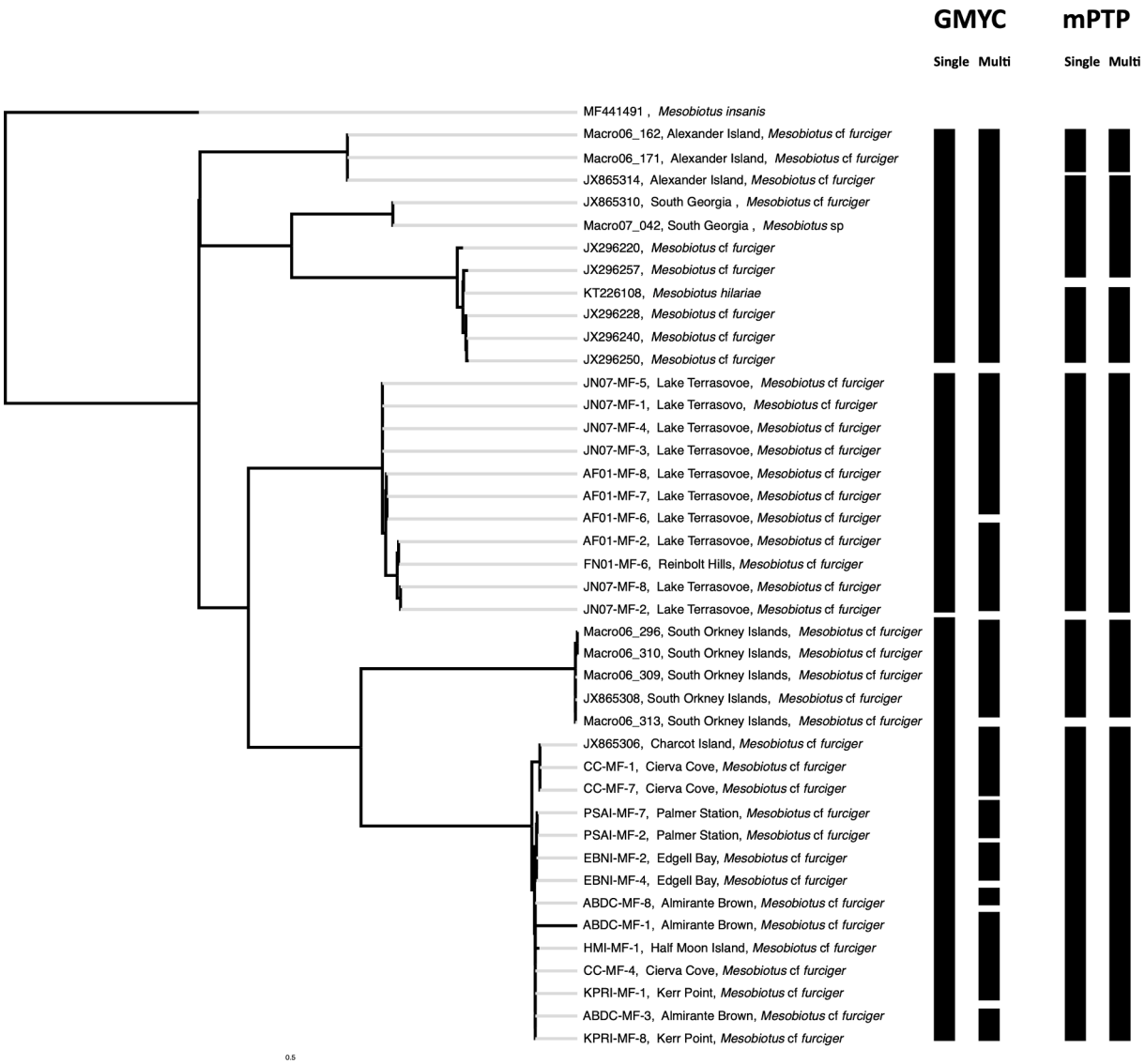

Fig. 5. Species delimitation results shown against a Maximum Likelihood phylogenetic tree of COX1 sequences. Methods for species delimitation used were General Mixed Yule Coalescent (GMYC) using both single threshold and multiple thresholds, and Poisson Tree Process (mPTP) using both single and multiple thresholds.

COX1 clades, interspersed with "*furciger*" from the Arctic and Madagascar, or several unsupported 18S lineages with both groups mixed (Supplementary Fig. 2). Stec et al. (2021) point out that as the groups of "*furciger*" and "*harmsworthi*" are intermixed it is not possible to further sort the genus *Mesobiotus*. We suggest that the current study, along with others mentioned above, have shown that the groups as they stand are of no systematic value and that their use should be abandoned as it has resulted in hiding the true evolutionary relationships and biogeographical structure that have previously been overlooked. Our data and analyses provide strong support for the Antarctic lineage of *Mesobiotus* to be considered as an independently evolving group. A very conservative molecular clock estimate indicates that this lineage is likely to have been independently evolving from the non-Antarctic lineages for over 80 million years, and certainly tens of millions of years, predating the final separation and isolation of the Antarctic continent around 30–40 mya (see discussion in Convey et al., 2009). Within the lineage that forms the current genus *Mesobiotus*, the two deeply divergent clades highlighted by our study could be considered as systematically discrete species groups, possibly appropriate for consideration as separate genera once sufficient supporting evidence is accumulated. Further development of understanding in this field will require the application of integrated taxonomic approaches (Cesari et al., 2009), to facilitate sufficiently detailed and accurate taxonomic descriptions, as well as redescriptions in some cases, in order to reconcile the growing indications of deeply distinct sequence diversity with the legacy of morphological descriptions and the outcomes of new sampling of both known and previously unsurveyed regions.

5. Conclusions

It is clear from this study, with support from previous studies (e.g. Guidetti et al., 2017), that tardigrades have existed on the Antarctic continent since prior to its geographical isolation and glaciation. The collective of *Mesobiotus* species distributed across the continent and its surrounding Islands is likely to be a remnant of a wider fauna that has successfully adapted to the changing conditions and diversified in the isolated habitats in which they have persisted in. There remains a lack of taxonomic clarity at the species level around the Antarctic, and regarding the "group" categories of *Mesobiotus* in general. The successful application of integrated taxonomy across the Antarctic fauna is urgently needed to address species-level taxonomy, while further sampling of *Mesobiotus* from other Gondwanan continents would assist in clarifying the extent and timing of the divergence of the Antarctic fauna from that of the rest of the world. A revision of the use of terminology in the genus is suggested to move away from the two established global "groups" that have been shown here to have no systematic value. Rather we suggest systematic groupings to better capture the lineages that have diversified, particularly those in Antarctica. We conclude that the Antarctic *Mesobiotus* fauna is systematically (and thus should be considered taxonomically) discrete from the non-Antarctic *Mesobiotus*. There are deep divisions within the Antarctic *Mesobiotus* fauna that perhaps should be considered as separate genera housing their own yet to be fully described species groups. Taken together the data and analyses presented here strongly support the growing body of evidence that tardigrades, like other terrestrial invertebrates found across the Antarctic, have a long history of isolated existence and evolutionary divergence on the continent.

CRediT authorship contribution statement

K.A. Short: Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Software. **C.J. Sands:** Data curation, Formal analysis, Investigation, Resources, Software, Supervision. **S.J. McInnes:** Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision. **D. Pisani:** Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision. **M.I. Stevens:** Investigation, Supervision, Formal analysis. **P. Convey:** Funding acquisition, Investigation, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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