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Overlooked Species Diversity and Distribution in the Antarctic Mite Genus *Stereotydeus*

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Abstract: In the harsh Antarctic terrestrial ecosystems, invertebrates are currently confined to sparse and restricted ice free areas, where they have survived on multi-million-year timescales in refugia. The limited dispersal abilities of these invertebrate species, their specific habitat requirements, and the presence of geographical barriers can drastically reduce gene flow between populations, resulting in high genetic differentiation. On continental Antarctica, mites are one of the most diverse invertebrate groups. Recently, two new species of the free living prostigmatid mite genus *Stereotydeus* Berlese, 1901 were discovered, bringing the number of Antarctic and sub-Antarctic species of this genus up to 15, of which 7 occur along the coast of Victoria Land and in the Transantarctic Mountains. To examine the biodiversity of *Stereotydeus* spp., the present study combines phylogenetic, morphological and population genetic data of specimens collected from nine localities in Victoria Land. Genetically distinct intraspecific groups are spatially isolated in northern Victoria Land, while, for other species, the genetic haplogroups more often occur sympatrically in southern Victoria Land. We provide a new distribution map for the *Stereotydeus* species of Victoria Land, which will assist future decisions in matters of the protection and conservation of the unique Antarctic terrestrial fauna.

Keywords: Victoria Land; molecular phylogeny; *cox1*; *28S*; biogeography; terrestrial invertebrates; acari; *Stereotydeus* spp.

Citation: Brunetti, C.; Siepel, H.; Convey, P.; Fanciulli, P.P.; Nardi, F.; Carapelli, A. Overlooked Species Diversity and Distribution in the Antarctic Mite Genus *Stereotydeus*. *Diversity* **2021**, *13*, 506. https://doi.org/10.3390/d13100506

Academic Editor: Michael Wink

Received: 24 August 2021
Accepted: 13 October 2021
Published: 19 October 2021

1. Introduction

Due to Antarctica's isolation and extreme environmental conditions, the continent's terrestrial biota has limited species level diversity and many higher taxonomic groups are completely missing or very poorly represented [1,2]. As a result of the climatic factors and the typically low availability of organic nutrients in soils, lichens and mosses are the only macroscopic flora present on the continent [1,3–6]. Similarly, the Antarctic terrestrial fauna consists of a small number of microarthropod species (mites and springtails) as well as other microscopic invertebrates (nematodes, tardigrades and rotifers), making the continental region amongst the simplest ecosystems on Earth [2,7].

The challenging environmental conditions, isolation and the patchy distribution of ice free areas have been recognized as the main factors affecting and defining populations of the Antarctic terrestrial invertebrate fauna, both physiologically and genetically [8]. As a consequence, in order to survive the harsh Antarctic conditions, these terrestrial animals have evolved impressive biochemical and physiological adaptations, to tolerate prolonged periods of freezing and dry conditions, amongst other severe stresses [9–12]. Behavioral strategies also play a role. For instance, continental Antarctic springtails (Collembola) and

mites (Acari) are often found concentrated under rocks, where the environment tends to be moister, rich in organic carbon and with low salinity [13], and where microbial diversity is also present, stabilizing mineral soils and allowing colonization by both micro-invertebrates and flora [2]. Although temperature plays an important role in regulating microarthropod life cycles, the major factor regulating their survival and growth remains the availability of liquid water [5,14]. An additional challenge for microarthropod survival derives from the bottleneck caused by their dispersal abilities, especially over longer distances.

Studies have suggested that rafting on the surface of melt water streams is a possible route for dispersal [15–18], as is the use of animal vectors (zoochory; e.g., on bird plumage or in nesting materials) [19–23] and, also, human mediated transport [22]. A further mechanism is dispersal by wind (anemochory). However, although the latter is known to be an effective dispersal strategy in, for instance, some oribatid mites [24], it may not be effective for Antarctic microarthropods, at least over longer distances/timescales, due to the risk of desiccation and the lack of an anhydrobiotic dispersal stage [7,25,26]. In order to understand the dispersal, over short and long distances, of microarthropods in Victoria Land, molecular studies have been conducted on different springtail species [22,27–31]. These have identified that the presence of glacial barriers strongly influences species distributions, and that these have likely limited gene flow between restricted and isolated refugia during various glacial maxima [22,28,32]. Analogous biogeographical patterns have been reported for the prostigmatid mite *Stereotydeus mollis* by Womersley and Strandtmann, 1963, in Victoria Land [33–36], although with higher genetic divergence, possibly due to higher activity levels and shorter generation time [33,37] and/or to a longer evolutionary history than for the springtails. As the evolution of these microarthropods in Antarctica has taken place over many millions of years, they represent suitable subjects to test speciation hypotheses and identify evolutionary trends and patterns of Antarctic fauna [33,38,39].

Free living mites are one of the most abundant and widespread microarthropod groups in Antarctica [40] and, among these, the best represented groups are the suborders Prostigmata and Oribatida and the order Mesostigmata. Within the Prostigmata, one of the most diverse families is the Penthalodidae, which includes the cosmopolitan genus *Stereotydeus* Berlese, 1860 [7]. However, while many studies have been conducted on the morphological and, more recently, genetic characteristics of springtails [27–29,31,35,41,42] present in Victoria Land, very few particularly genetic studies have investigated the biodiversity of Antarctic mites generally, and specifically *Stereotydeus*. Indeed, after early morphological studies in the 1960s [43–45], few studies on the physiology and ecology of the genus have been conducted [11–13,40,46,47], these are particularly focusing on *S. mollis*. Very recently, two new *Stereotydeus* species (*S. ineffabilis* and *S. nunatakis*) have been described from an area of Victoria Land [48], bringing the number of known Antarctic and sub-Antarctic members of the genus to 15 [48]. Focusing on Victoria Land, five species (*S. delicatus* Strandtmann, 1967, *S. punctatus* Strandtmann, 1967, *S. belli* Trouessart, 1902, *S. ineffabilis* Brunetti and Siepel, 2021 and *S. nunatakis* Brunetti, 2021) are currently known from North Victoria Land and two (*S. mollis* and *S. shoupi* Strandtmann, 1967) from South Victoria Land and the central Transantarctic Mountains [36]. Given the harsh field conditions and the small size and cryptic characters of members of this genus, the precise taxonomic determination of specimens in situ is challenging. In the laboratory, the combination of genetic and morphological approaches provides a powerful tool for detecting different levels of diversity. During the last two decades, the development of barcoding techniques using the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene in combination with different nuclear markers has helped to discriminate cryptic species and determine the origin of morphological variation in multiple taxa [31,49,50]. However, over the period since this technology has become available, only three genetic studies have been conducted on Antarctic representatives of the genus, focusing exclusively on *S. mollis* in Southern Victoria Land [33,34,36] and giving a tantalizing hint of the high level of diversity hidden within and between different populations of this single species.

At the same time, given the recent discovery of the two new *Stereotydeus* species in Northern Victoria Land in a study that also reviewed the morphological characters relevant to the identification of Antarctic *Stereotydeus* species [48], the question of a possible overlap between these new taxa with the species already known from the area (*S. belli*, *S. punctatus* and *S. delicatus*) and with *S. mollis* from Southern Victoria Land has to be addressed. In addition to that, the current lack of genetic knowledge of a species morphologically described more than fifty years ago needs addressing, not only for the systematic understanding of the genus, but also to contribute to the future development and implementation of sustainable conservation planning in Antarctica. Although Antarctica is often assumed to be a pristine continent, it is increasingly clear that Antarctic ecosystems and biodiversity are facing the same threats as in the rest of the world, particularly from climate change, pollution, biological invasions and an increase in direct human impacts and activities [51–53]. In this context, the poor existing knowledge of species diversity and their dispersal ability are considered limiting factors to their effective management and conservation [31,54,55].

In the current study, we investigated, using a combined taxonomic approach, the distribution, phylogenetic relationships and the population genetics of the genus representatives of the *Stereotydeus* present in Victoria Land, with the support of morphological characteristics fundamental for species identification. In the Antarctic Conservation Biogeographic Regions system (ACBRs [52,56,57]), Victoria Land is divided into Northern and Southern Victoria Land. Nevertheless, the area between Mount Melbourne and the Drygalski Ice Tongue has been singled out for its unusual biogeographic connections and possible role in the promotion of the genetic differentiation of terrestrial taxa in numerous studies targeting Collembola [27,58,59]. As such, this region, named "Central" for convenience, has been separated from the northern ACBR in our analyses. Furthermore, we provide more than 150 new sequences for the mitochondrial barcode region *cox1*, and the nuclear *28S*, of five different *Stereotydeus* species from Victoria Land.

2. Materials and Methods

2.1. Sample Collection

Stereotydeus specimens were collected from nine different localities in Victoria Land (Figure 1; Table 1) during the 2017–2018 and 2018–2019 austral summer expeditions of the Italian National Antarctic Research Program (PNRA: PNRA16_00234), and were immediately preserved in >99.5% ethanol and stored at −80 °C. A total of 159 individuals were used for the molecular analyses. Of these, the whole body of 137 specimens was used for the genetic analyses (Table 1; see Section 2.2). The remaining 22 individuals (see Section 2.3) were used in the morphological investigation, with only 2–4 legs used for the DNA extraction.

Table 1. Coordinates and altitudes of sampling localities and ID codes for the different populations sampled; the numbers of individuals (*n.*) extracted and used for the molecular analyses and the species found at each locality, are given.

ID	Locality	Victoria Land	Lat (S)	Long (E)	Altitude	*n.*	Species
CHA	*Cape Hallett (Adelie Cove)*	North	72°26′25″	169°56′32″	140 m	10	*S. belli*
CCI	*Crater Cirque*	North	72°37′52″	169°22′22″	200 m	14	*S. belli; S. punctatus*
CJO	*Cape Jones*	North	73°16′38″	169°12′54″	310 m	17	*S. belli*
KAY	*Kay Island*	North	74°04′14″	165°18′60″	140 m	10	*S. belli*
CIC	*Campo Icaro*	Central *	74°42′45″	164°06′21″	70 m	35	*S. ineffabilis; S. delicatus*
VEG	*Vegetation Island*	Central *	74°47′00″	163°37′00″	120 m	10	*S. delicatus*
INE	*Inexpressible Island*	Central *	74°53′39″	163°43′44″	30 m	10	*S. ineffabilis; S. delicatus*
PRI	*Prior Island*	South	75°41′31″	162°52′34″	130 m	17	*S. ineffabilis; S. nunatakis*
SNU	*Starr Nunatak*	South	75°53′57″	162°35′08″	60 m	14	*S. ineffabilis; S. nunatakis*

* CIC, VEG and INE have been considered as "Central" to facilitate the division of the sampling area based on geography, although they all formally lie within the defined ACBR North Victoria Land [52,57].

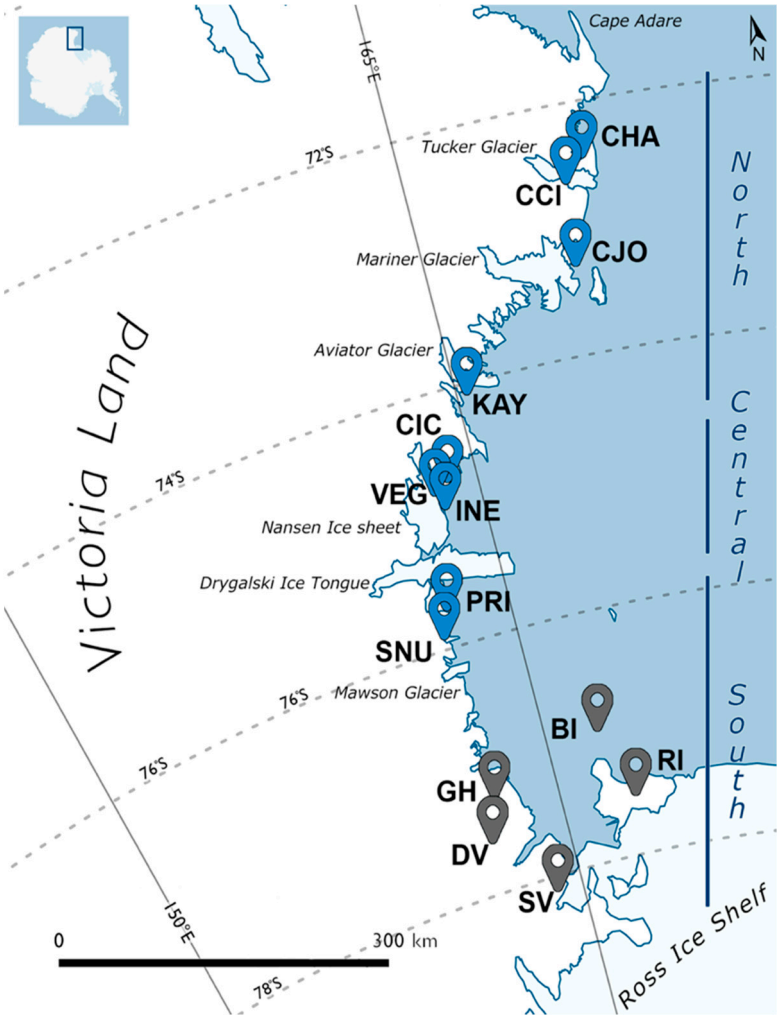

Figure 1. Map of sampling localities for the *Stereotydeus* spp. samples analyzed in this study (blue; see Table 1 for locality abbreviations) and in published studies of *S. mollis* [33,34,36] (dark grey): DV = McMurdo Dry Valleys (Taylor, Wright and Victoria Valleys and vicinity), SV = southern Dry Valleys (Garwood, Marshall and Miers Valleys and vicinity), BI = Beaufort Island; RI = Ross Island and GH = Granite Harbour (coastlines from ADD Simple Basemap, NPI/Quantarctica 3 [60]).

2.2. Molecular Dataset

Total genomic DNA was extracted from 137 whole individuals from the nine collection sites (Table 1) and the outgroup specimen, the winter grain mite *Penthaleus major* (Acari: Penthaleidae; Accession number *cox1*: MZ350753; Accession number *28S*: MZ442288; Table S1 in the Supplementary Materials) using the Wizard® SV genomic DNA Purification System (Promega, Madison, WI, USA) and eluting in 50 µL ddH_2O.

Region II of mtDNA cytochrome *c* oxidase subunit I (*cox1*) was amplified using the mite specific primers COI-2F (5′-TTYGAYCCIDYIGGRGGAGGAGATCC-3′) and COI-2R (5′-GGRTARTCWGARTAWCGNCGWGGTAT-3′) [61]. A preliminary amplification of the 28S gene was performed on a restricted pool of five *Stereotydeus* individuals from each of six localities (CHA, CCI, CJO, CIC, INE and SNU) and including all the species, with the primer pair D1a (5′-CCCSCGTAAYTTAAGCATAT-3′) and D5b1 (5′-ACACACTCCTTAGCGGA-3′) [62]. A new specific primer pair (Ste-28S-F (5′-GGACGTGAAACCGCTTGTA-3′) and Ste-28S-R (5′-TCTGACGATCGATTTGCAC-3′)) was designed in conserved regions (750 bp) and used to amplify all the remaining *Stereotydeus* specimens and the outgroup. PCRs were performed in 25 µL reaction volume containing: 2.5 µL of genomic DNA, 0.5 mM of each primer, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 2.5 mM of $MgCl_2$, 5 µL of

Green GoTaq Flexi buffer and 0.625 U of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). The amplifications were performed in a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA) thermal cycler. The initial denaturation step was set at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 45 °C (for *cox1*) or 50 °C (for the *28S*) for 1 min, and 72 °C for 90 s, and a final extension step at 72 °C for 7 min. PCR products were then purified using the kit Wizard® SV Gel and PCR Clean-up (Promega, Madison, WI, USA) and sequenced on both strands (with the same primers used for PCRs) with a DNA Analyzer ABI 3730, at the core facility of the Bio-Fab Research Lab (Rome, Italy). The sequences were assembled and manually corrected using the MacVector™ software (MacVector, Inc., USA; version 16.0.8-[63]).

In addition to the new samples extracted for this study, all 56 publicly available *cox1* sequences for the genus *Stereotydeus* were downloaded from GenBank (Table S2 in Supplementary Materials) and included in the analyses. These included 50 of *S. mollis*, 2 of *S. shoupi*, 1 of *S. belli*, 1 of *S. villosus* and 2 of *Stereotydeus* sp. together with a second outgroup, another eupodid mite *Eriorhynchus* sp. (Acari: Eriorhynchidae; see Table S2).

The two haplotypes, DQ305366 (S2-[34]) and DQ305388 (B-[33]), were excluded from this analysis because they are homonyms of DQ305362 and DQ305389, respectively. An error in naming them may have occurred when deposited in GenBank, therefore, following the analyses of Demetras et al. [36], the latter two were used in our analyses. Although we included all the remaining deposited haplotypes, some incongruences are noted in three other sequences: (i) for DQ305362 (S2-[34]), coordinates are missing because the precise sampling site in Wright Valley is not clear (W3 and/or W5); (ii) for DQ305382 (S20–V11 from Victoria Valley [34]), coordinates were not included in the original article [34]; (iii) DQ305367 (S6-[34]) was used in Demetras et al. [36] but is missing in the original article of McGaughran et al. [34], therefore, the coordinates are not shown (see Table S2). For the specimens from Demetras et al. [36], only the generic location of southern Dry Valleys (i.e., Garwood, Marshall and Miers Valleys, Shangri La and vicinity, according to Collins et al. [64]) was given, but not the exact coordinates, so they are not shown in this study.

2.3. Combined Morphological Analysis

In parallel to this study, morphological analyses have been performed on numerous specimens (between 20–50 for each sampled species, data not published). The morphological comparisons clearly defined the boundaries between all the *Stereotydeus* species occurring on Victoria Land, as recently published in Brunetti et al. [48], where not only the new species of *S. ineffabilis* and *S. nunatakis* are described, but also all the characters so far used to describe and distinguish the Antarctic *Stereotydeus* species are reviewed (see [48] Tables A1–A7), and the keys to identification are provided. Unfortunately, the lack of specimens of *S. mollis, S. shoupi* and *S. villosus* from accessible localities prevented us from improving the original descriptions with the new characters studied in these species and, therefore, were not available for combined morphological analyses.

In addition, after a quick molecular screening, we decided to deeply investigate the morphological aspects of *S. delicatus* and *S. ineffabilis* in relation to their genetic differentiation. We focused our attention on Campo Icaro, Inexpressible Island, Prior Island and Starr Nunatak, due to the presence at those localities of the new species described (*S. ineffabilis* and *S. nunatakis*). We also questioned the exact correspondence of previously published sequences to specific *Stereotydeus* taxa. In this respect, the combination of morphological and molecular analyses performed on the same specimens, collected in the central and southern sites of our sampling area, and the recent taxonomic description of new species of the genus (i.e., *S. ineffabilis*), challenged the attribution of some haplotypes to *S. mollis*.

Due to the small size of the specimens and, consequently, of the characteristics useful for an accurate taxonomic determination, 22 adult individuals (13 *S. ineffabilis* from four localities and 9 *S. delicatus* from Campo Icaro; Table 2) were selected for the joint morphological/molecular investigation and also used in all the molecular analyses. Only adult specimens were considered in the morphological comparison because, at the nymphal

stages, most of the characteristics useful for the positive identification of *Stereotydeus* species are not yet developed (e.g., small size, sex structures not developed, division of the femora absent or incomplete, reduced number of aggenital and genital setae, and reduced number of rhagidial organs; see [48]).

From each specimen, 2–4 legs were removed (to perform the genetic analyses) while the remainder of the body was incubated on a slide with few drops of lactic acid (20%) at 37–45 °C for 30 min to clear the samples, which were then observed under a Leica DM RBE microscope for morphological analysis. The morphological characters considered for identification of *S. delicatus* and *S. ineffabilis* were: (a) the length (µm); (b) the division of the femora (presence/absence); (c) the position of the anal pore; (d) the number of aggenital and (e) the number of genital setae; (f) the length of the 4th segment of the pedipalp compared to the 3rd segment; (g) the shape of the epirostrum; and (h) the disposition of the rhagidial organs on tarsi I and II.

Table 2. New specimens extracted for the haplotypic and morphological analyses. Sampling localities with their ID codes, date of collection and the slide codes, and the sex and species of the new *Stereotydeus* individuals are given.

Locality	ID	Date	Slide	Sex	Species
Campo Icaro	CIC	28 January 2019	CI1	M	*S. delicatus*
			CI3	F	*S. ineffabilis*
			CI5	F	*S. delicatus*
			CI7	M	*S. delicatus*
		24 December 2017	CI9	M	*S. delicatus*
			CI10	M	
			CI11	F	
			CI12	F	
			CI13	F	
			CI14	M	
Inexpressible Island	INE	21 January 2019	I1	F	*S. ineffabilis*
			I2	M	
			I3	F	
			I4	M	
			I5	F	
Prior Island	PRI	11 January 2019	P1	M	*S. ineffabilis*
			P2	M	
			P3	F	
			P5	M	
Starr Nunatak	SNU	11 January 2018	S1	M	*S. ineffabilis*
			S2	M	
			S5	F	

2.4. Phylogenetic Analyses

For both mitochondrial and nuclear markers, 159 sequences were obtained and the datasets were separately aligned using the online tool Clustal Omega [65]; and manually corrected and trimmed (147 bp and 54 bp were trimmed for the *cox1* and *28S* respectively) using the MacVector™ software (MacVector, Inc., Cary, NC, USA; version 16.0.8-[63]). The resulting *cox1* dataset was then aligned with the two outgroups, while the *28S* dataset was aligned only with the *P. major* outgroup, due to the lack of the ribosomal DNA sequence in Genbank for *Eriorhynchus* sp. The outgroups were selected from mite families related to ingroups in order to reduce the phylogenetic distance with the Antarctic *Stereotydeus* spp. In detail, the species *P. major* (from a closely related family to that of ingroups) was selected as outgroup both for combined and single locus analyses. In addition, the *cox1* sequence of *Eryorinchus* sp. was also included as outgroup because it has been widely used in previous studies on Antarctic *Stereotydeus* spp.

The *cox1* dataset was concatenated to the *28S* alignment to generate a multilocus dataset through FaBox [66], with the online tool Fasta alignment joiner (available at https://users-birc.au.dk/palle/php/fabox/alignment_joiner.php; accessed on 18 September 2020).

The multilocus alignment was then run on the Gblocks server 0.91b ([67]; available at http://molevol.cmima.csic.es/castresana/Gblocks_server.html; accessed on 18 September 2020) under strict settings and the hypervariable regions of the *28S* alignment were discarded. After the run, 1034 positions, out of the 1171 of the initial dataset (88%), were kept. Ultimately, the four single- and the multilocus alignments used for the phylogenetic and population genetics analyses were: (i) *cox1* with outgroup; (ii) *cox1* all haplotypes; (iii) combined *cox1-28S*; and (iv) combined *cox1-28S* with associated morphological information (Table 3).

Table 3. List of the datasets (single and multilocus), number of new sequences obtained and used in each dataset (*n.*), markers, reference sequences (Ref.) and outgroups used for the analyses and models of nucleotide evolution that best fitted, divided according to the partition applied and to the respective tree search optimization criteria.

		n.	Single/Multi Locus	*cox1*	*28S*	Ref.	Outgroups	Best Model			
								1st	2nd	3rd	Non-Cod
i	*cox1 with outgroups*	159	single	x	-	*S. shoupi* (2) *S. villosus* *Stereotydeus* sp. (2) *S. belli*	*Eriorhynchus* sp. *P. major*	K81UF+I+G	GTR+I	F81+I	-
ii	*cox1 all haplotypes*	159	single	x	-	*S. shoupi* (2) *S. villosus* *Stereotydeus* sp. (2) *S. belli* *S. mollis* (50)	*Eriorhynchus* sp. *P. major*	K81UF+G	GTR+I+G	F81+I	-
iii	*combined cox1-28S*	159	multi	x	x	-	*P. major*	K81UF+I+G	TRN+I	F81+I	GTR+G
iv	*combined cox1-28S with morphologica information*	99	multi	x	x	-	*P. major*	HKY+I+G	TIM+G	F81+G	TVM+G

To identify the haplotypes and their frequencies within populations, all the alignments were run with the online software DNA-Collapser [66]. The sequences of the resulting haplotypes were used to calculate the genetic distances between the haplotypes using the software R 3.6.1 [68] with the "ape 5.3" package [69]. The best evolutionary models were selected before the tree search (Table 3), partitioning the datasets with the software PartitionFinder 2.1.1 [70] based on Akaike's information criterion (AIC) and a greedy strategy: 1st, 2nd and 3rd codon positions for the *cox1* protein-encoding gene and one single partition were considered for the *28S* (Table 3). Bayesian analysis was performed with MrBayes 3.2.7 software [71], applying four chains (three hot and one cold) for 10^6 generations, with a sampling frequency of one tree every 1000 iterations and with 25% of the tree topologies discarded (burn in step) from the final result. For better visualization, the resulting phylogenetic trees were then zoomed and expanded and the node labels (posterior probabilities) were added with FigTree 1.4.4 software [72]. The new *Stereotydeus* mitochondrial and nuclear sequences were deposited in GenBank (*cox1* Accession numbers: MZ350724-MZ350752; *28S* Accession numbers: MZ442270-MZ442287; Table S1 in Supplementary Materials).

2.5. Population Structure Analyses

The population genetics study was performed using the *cox1* dataset without the outgroups applied for the phylogenetic analysis. *S. mollis* sequences were not included in the analysis. This was due to: (i) the incongruences found in the Genbank sequences (see Section 2.2. and Table S2 in Supplementary Materials), (ii) the fact that no morphological investigations were performed on these individuals, and (iii) because new *S. mollis* specimens were not available for a morphological analysis during this study. Haplotype frequencies

were obtained using the online tool DNA collapser [66]. The network clade analysis was performed on TCS 1.21 [73] using a connection limit of 98% and visualized with the online tool tcsBU ([74]; available at https://cibio.up.pt/software/tcsBU/; accessed on 28 November 2020) to estimate the haplotype networks for each species. To investigate the genetic characteristics of populations and to test for the presence of population structure, Arlequin version 3.11 [75] was used for each species separately. The haplotype (h) and nucleotide (π) diversity indices [76], as well as the mean number of pairwise differences (θ) and segregating sites (θS), were computed at the population level. Analysis of molecular variance (AMOVA; [77]) was used to measure the extent to which genetic variance could be assigned to the hierarchical structure of population organization (testing them with the structure according to the populations: "Cape Hallett", "Crater Cirque", "Cape Jones" and "Kay Island" for *S. belli*; "Campo Icaro", "Vegetation Island" and "Inexpressible Island" for *S. delicatus*; "Campo Icaro", "Inexpressible Island", "Prior Island" and "Starr Nunatak" for *S. ineffabilis* and "Prior Island" and "Starr Nunatak" for *S. nunatakis*), with the statistical significance of variance components tested with 16,000 permutations. Pairwise differences between haplotypes (Φ_{ST} values) were calculated using simple distances and these were used to look for significant relationships between population genetic distance (Φ_{ST}).

3. Results

Using the *cox1* haplotypes of the 50 *S. mollis* specimens already available on GenBank as templates, 495 bp of a uniform and unambiguous alignment from 159 sequenced individuals were used for all genetic analyses. For *28S*, 1034 positions of the 159 sequenced individuals, together with the outgroup *P. major*, were used for phylogenetic analyses.

For each *Stereotydeus* species, between 2–14 *cox1* and 1–9 *28S* haplotypes were found (Table 4) while, for each locality, between 1–11 *cox1* and 1–4 *28S* haplotypes were found. Most *28S* haplotypes were unique at the species level, with the only exception being RX1 from CIC, shared by both *S. delicatus* and *S. ineffabilis*. In addition, for the combined set of *cox1* and *28S,* from 3–16 and from 2–9 haplotypes were found for the *Stereotydeus* species and the localities, respectively. The number of *Stereotydeus* species identified per site ranged from 1–2 (Table 5).

Thirty-six unique haplotypes for *cox1*, ranging in divergence from 0.2 to 2.5% and 18 unique haplotypes for *28S* (from 0.2 to 9.0%), were identified. The compiled matrix of percentage genetic distances (Table 6) showed a gradient of arbitrarily estimated comparisons corresponding to intraspecific distances (0% to 8.48%), intermediate values between intra- and interspecific distances (8.49% to 10.7%), and interspecific distances (10.8% to 16.8%).

Table 4. Number of specimens analyzed per species and number of haplotypes detected within the species for the mitochondrial and nuclear markers and the combined set of the *cox1* and *28S* (combined).

Species	Specimens	Haplotypes		
		cox1	*28S*	Combined
S. belli	39	10	9	14
S. punctatus	12	4	1	4
S. ineffabilis	59	14	3	16
S. delicatus	39	6	2	10
S. nunatakis	10	2	2	3

Table 5. Sampling locality codes (ID), number of sequenced individuals per area (*n*.), number of species per area (N.) and their names, and list of all haplotypes for each species. Haplotype code: the first letter indicates the marker (M: mitochondrial; R: nuclear ribosomal DNA) and the genus (S: *Stereotydeus*) in the combined haplotypes; the second letter is the initial of the species name (B/b = *belli*; P/p = *punctatus*; D/d = *delicatus*; I/i = *ineffabilis*; N/n = *nunatakis*; RX identifies the haplotype only present in the Campo Icaro (CIC) area and found in both *S. delicatus* and *S. ineffabilis*) followed by the progressive number of the haplotype.

ID	*n*.	N.	Species	Haplotypes		
				cox1	***28S***	**Combined**
CHA	10	1	*S. belli*	MB1(10)	RB1(9), RB2(1)	Sb1(9), Sb2(1)
CCI	14	2	*S. belli* *S. punctatus*	MB10(2) MP1(3), MP2(7), MP3(1), MP4(1)	RB8(1), RB9(1) RP1(12)	Sb12(1), Sb13(1) Sp1(7), Sp2(3), Sp3(1), Sp4(1)
CJO	17	1	*S. belli*	MB2(2), MB3(3), MB4(3), MB5(8), MB6(1)	RB3(16), RB4(1)	Sb3(8), Sb4(2), Sb5(3), Sb6(3), Sb7(1)
KAY	10	1	*S. belli*	MB7(1), MB8(3), MB9(6)	RB5(7), RB6(1), RB7(1), RB8(1)	Sb8(1), Sb9(2), Sb10(5), Sb11(1), Sb14(1)
CIC	45	2	*S. delicatus* *S. ineffabilis*	MD1(18), MD2(1), MD3(2), MD4(1), MD5(6) MI1(6), MI2(1), MI3(1), MI5(4), MI12(4), MI13(1)	RD1(12), RD2(2), RX1(14) RX1(17)	Sd1(1), Sd2(1), Sd3(9), Sd4(2), Sd5(8), Sd6(1), Sd9(1), Sd10(4), Sd11(1) Si1(1), Si2(4), Si5(4), Si11(1), Si12(6), Si13(1)
VEG	10	1	*S. delicatus*	MD5(9), MD6(1)	RD1(10)	Sd8(1), Sd10(9)
INE	15	2	*S. delicatus* *S. ineffabilis*	MD6(1) MI6(3), MI7(3), MI8(6), MI12(1), MI14(1)	RD2(1) RI1(14)	Sd7(1) Si3(1), Si6(3), Si7(3), Si8(6), Si14(1)
PRI	21	2	*S. ineffabilis* *S. nunatakis*	MI4(15), MI9(1), MI10(1), MI11(2) MN1(2)	RI3(19) RN1(2)	Si4(2), Si9(1), Si10(1), Si16(15) Sn1(2)
SNU	17	2	*S. ineffabilis* *S. nunatakis*	MI4(5), MI9(3), MI11(1) MN1(7), MN2(1)	RI2(1), RI3(8) RN1(7), RN2(1)	Si4(1), Si9(3), Si15(1), Si16(4) Sn1(6), Sn2(1), Sn3(1)

Table 6. Matrix of percentage genetic distances between *Stereotydeus cox1* sequences. Cells were colored in a gradient from green (0%-low divergence) to red (16.8%-high divergence) to facilitate interpretation (see Table 5 for the haplotype labels).

	MI4	MI1	MI2	MI3	MI14	MI5	MI9	MI10	MI6	MI8	MI7	MD6	MD1	MD4	MD3	MD2	MD5	MP3	MP4	MP2	MP1	MB10	MB9	MB8	MB7	MB1, *S. belli*	MB6	MB5	MB3	MB4	MB2	MN1	MN2	MI12	MI13	MI11	*Stereotydeus* sp.a	*Stereotydeus* sp.b	*S. villosus*	*S. shoupi*	*S. shoupi* 1
MI4	0																																								
MI1	0.4	0																																							
MI2	0.61	0.2	0																																						
MI3	0.61	0.2	0.4	0																																					
MI14	0.61	0.2	0.4	0.4	0																																				
MI5	2.42	2.42	2.63	2.63	2.22	0																																			
MI9	2.42	2.83	3.03	3.03	2.63	1.41	0																																		
MI10	2.42	2.83	3.03	3.03	2.63	1.82	0.4	0																																	
MI6	2.63	2.63	2.83	2.83	2.42	0.2	1.62	2.02	0																																
MI8	2.63	2.63	2.83	2.83	2.42	1.01	1.62	2.02	0.81	0																															
MI7	2.83	2.83	3.03	3.03	2.63	1.21	1.82	2.22	1.01	0.2	0																														
MD6	1.82	1.82	2.02	2.02	1.62	1.41	2.42	2.83	1.62	1.62	1.82	0																													
MD1	2.22	2.22	2.42	2.02	2.02	1.62	2.42	2.83	1.82	1.82	2.02	1.41	0																												
MD4	2.42	2.42	2.63	2.22	2.22	1.82	2.63	3.03	2.02	2.02	2.22	1.62	0.2	0																											
MD3	2.42	2.42	2.63	2.22	2.22	1.82	2.63	3.03	1.62	1.62	1.82	1.62	0.2	0.4	0																										
MD2	2.63	2.63	2.83	2.42	2.42	2.02	2.83	3.23	2.02	2.02	2.22	1.82	0.4	0.61	0.4	0																									
MD5	8.28	8.69	8.89	8.89	8.89	7.88	8.48	8.69	7.88	8.28	8.08	8.48	8.48	8.69	8.48	8.69	0																								
MP3	6.46	6.87	6.87	7.07	7.07	8.28	7.88	8.28	8.48	8.28	8.08	7.68	7.68	7.88	7.88	8.08	5.86	0																							
MP4	6.67	7.07	7.07	7.27	7.27	8.08	7.68	8.08	7.88	7.68	7.47	7.47	7.88	8.08	7.68	8.08	5.66	1.21	0																						
MP2	6.67	7.07	7.07	7.27	7.27	8.08	7.68	8.08	8.28	8.08	7.88	7.47	7.88	8.08	8.08	8.28	5.66	0.2	1.01	0																					
MP1	6.87	7.27	7.27	7.47	7.47	8.28	7.88	8.28	8.48	8.28	8.08	7.68	8.08	8.28	8.28	8.48	5.45	0.4	1.21	0.2	0																				
MB10	9.49	9.9	9.9	10.1	10.1	9.9	9.7	9.9	9.9	9.7	9.9	9.7	9.9	10.1	9.9	10.1	8.89	6.46	6.26	6.26	6.46	0																			
MB9	9.29	9.7	9.7	9.9	9.9	9.7	9.49	9.7	9.7	9.49	9.7	9.49	9.7	9.9	9.7	9.9	9.09	6.67	6.46	6.46	6.67	0.2	0																		
MB8	9.49	9.9	9.9	10.1	10.1	9.9	9.7	9.9	9.9	9.7	9.9	9.7	9.9	10.1	9.9	10.1	8.48	6.06	5.86	5.86	6.06	0.4	0.61	0																	
MB7	10.7	11.1	11.1	11.3	11.3	11.1	10.9	11.1	11.1	10.9	11.1	10.9	11.1	11.3	11.1	11.1		7.07	6.87	6.87	6.87	4.04	4.24	3.64	0																
MB1, *S. belli*	10.5	10.9	10.9	11.1	11.1	10.7	11.3	11.5	10.5	10.7	10.5	10.7	11.1	11.3	10.9	11.3	9.09	8.08	8.08	7.88	8.08	5.05	5.25	5.05	5.45	0															
MB6	10.3	10.7	10.7	10.9	10.9	10.7	10.5	10.7	10.5	10.3	10.5	10.5	10.7	10.9	10.5	10.9	10.3	8.28	7.68	8.08	8.28	3.43	3.64	3.43	4.24	5.45	0														
MB5	10.7	11.1	11.1	10.9	11.3	11.1	10.9	11.1	10.9	10.7	10.9	10.9	10.7	10.9	10.5	10.5	10.5	8.48	7.68	8.28	8.48	3.84	4.04	3.84	4.65	5.45	1.01	0													
MB3	10.7	11.1	11.1	10.9	11.3	11.1	10.9	11.1	10.9	10.7	10.9	10.9	10.7	10.9	10.5	10.9	10.1	8.08	7.27	7.88	8.08	3.64	3.84	3.23	4.04	5.66	0.81	0.61	0												
MB4	10.7	11.1	11.1	10.9	11.3	11.1	10.9	11.1	10.9	10.7	10.9	10.9	10.7	10.9	10.5	10.9	10.1	8.08	7.27	7.88	8.08	3.84	4.04	3.43	4.24	5.45	1.01	0.4	0.2	0											
MB2	11.1	11.5	11.5	11.3	11.7	11.1	11.3	11.5	10.9	11.1	11.3	11.3	11.1	11.3	10.9	11.3	10.1	8.48	7.68	8.28	8.48	3.84	4.04	3.43	4.24	5.45	1.41	1.21	0.61	0.81	0										
MN1	11.3	11.5	11.5	11.7	11.3	12.7	12.3	11.9	12.9	12.9	13.1	11.7	12.3	12.5	12.5	12.7	13.7	9.49	9.9	9.29	9.49	11.92	11.9	11.9	12.1	11.72	12.3	12.5	12.7	12.5	12.9	0									
MN2	11.5	11.7	11.7	11.9	11.5	12.9	12.5	12.1	13.1	13.1	13.3	11.9	12.5	12.7	12.7	12.9	13.5	9.7	10.1	9.49	9.29	12.12	12.1	12.1	12.1	11.92	12.5	12.7	12.9	12.7	13.1	0.2	0								
MI12	13.3	13.7	13.7	13.5	13.9	14.1	13.7	14.1	14.1	14.1	14.3	13.7	13.3	13.1	13.3	13.5	13.5	12.5	12.3	12.5	12.7	12.93	12.7	12.7	13.3	13.94	14.6	14.1	13.9	13.7	14.1	14.6	14.8	0							
MI13	13.9	14.1	14.1	14.3	14.3	14.8	13.9	14.1	14.6	14.1	13.9	14.3	14.3	14.1	14.1	14.6	11.9	11.7	11.3	11.9	12.1	12.12	12.3	11.9	11.7	13.13	12.3	12.7	12.3	12.5	12.9	16.6	16.8	13.1	0						
MI11	14.3	14.8	15	14.6	15	14.8	15	15.2	14.8	15	15.2	14.8	14.8	14.6	14.8	14.6	12.9	12.1	11.5	11.9	11.9	12.12	12.3	12.5	13.1	13.94	13.5	12.9	13.5	13.3	13.5	12.9	12.9	15.8	14.1	0					
***Stereotydeus* sp.a**	12.3	12.7	12.7	12.9	12.9	13.7	13.3	13.3	13.7	13.5	13.3	13.9	13.1	13.3	13.1	13.1	12.1	9.49	9.7	9.7	9.7	11.11	11.1	10.7	10.9	12.32	11.9	11.7	11.7	11.5	11.9	13.7	13.7	13.3	15.4	14.1	0				
***Stereotydeus* sp.b**	12.3	12.7	12.7	12.9	12.9	13.7	13.3	13.3	13.7	13.5	13.3	13.9	13.1	13.3	13.1	13.1	12.5	9.49	9.7	9.7	9.7	11.52	11.5	11.1	10.9	12.73	11.9	11.7	11.7	11.5	11.9	13.7	13.7	13.9	15.6	13.9	1.21	0			
S. villosus	12.9	12.9	12.9	13.1	13.1	13.1	12.7	13.1	12.9	12.7	12.9	13.1	12.9	13.1	12.7	13.1	13.9	10.5	10.1	10.5	10.7	11.31	11.3	10.9	11.3	12.12	11.1	11.3	11.1	10.9	11.5	14.3	14.6	15.6	15.8	16.4	11.11	10.71	0		
S. shoupi	14.3	14.8	14.8	14.6	15	14.6	14.3	14.6	14.3	14.3	14.1	15	14.6	14.8	14.3	14.8	14.6	12.5	12.1	12.3	12.5	12.93	13.1	12.5	12.1	13.13	13.3	12.9	12.7	12.5	12.5	15.2	15.4	16	15	15.8	12.53	11.92	13.54	0	
***S. shoupi* 1**	14.6	15	15	14.8	15.2	14.8	14.6	14.8	14.6	14.6	14.3	15.2	14.8	15	14.6	15	14.8	12.7	12.3	12.5	12.7	12.73	12.9	12.7	12.3	13.33	13.1	12.7	12.9	12.7	12.7	15	15.2	16.6	15	15.2	12.73	12.12	13.74	0.61	0

Green frame—*S. ineffabilis*; orange frame—*S. delicatus*; red frame—*S. punctatus*; purple frame—*S. belli*; blue frame—*S. nunatakis*; light blue frame—*S. ineffabilis*.

3.1. Haplotype Network Analyses

The total number of nucleotide substitutions (absolute changes) ranged from 1 (*S. nunatakis* in SNU) to 117(*S. ineffabilis* in CIC) within all the populations of the five different taxa. Four subnetworks were found for *S. belli*, with two single haplotypes not connected with any other haplotype: MB1 and MB7, from CHA and KAY, respectively. Within the species and within the clusters, the number of nucleotide substitutions ranged from a minimum of nine, recorded in CJO, to a maximum of 21, in KAY (mean 7.50 ± 25.48) (Figure 2). For *S. punctatus*, one single network was observed where all haplotypes were connected with each other within an upper range of seven nucleotide changes (Figure 2). Three clusters were found for *S. delicatus*, with two single haplotypes not connected with any other haplotype: MD5 (VEG and CIC) and MD6 (VEG and INE). The number of nucleotide substitutions ranged from 42, in VEG, to 46, in CIC, in this species and within the populations (mean 29.33 ± 9.95) (Figure 2). Six networks were found for *S. ineffabilis*, with three single haplotypes not connected with any other haplotype: MI11 (SNU and PRI), MI12 (CIC and INE) and MI13 (CIC). These haplotypes are also placed together in a different position in the phylogenetic trees, with the respect to the other conspecific haplotypes (see Section 3.2). The differences within both species and populations ranged from 79, in SNU, to 117, in CIC (mean 89 ± 18.67) (Figure 2). For *S. nunatakis*, only two haplotypes were observed, differing by a single substitution (mean 0.50 ± 0.71) (Figure 2).

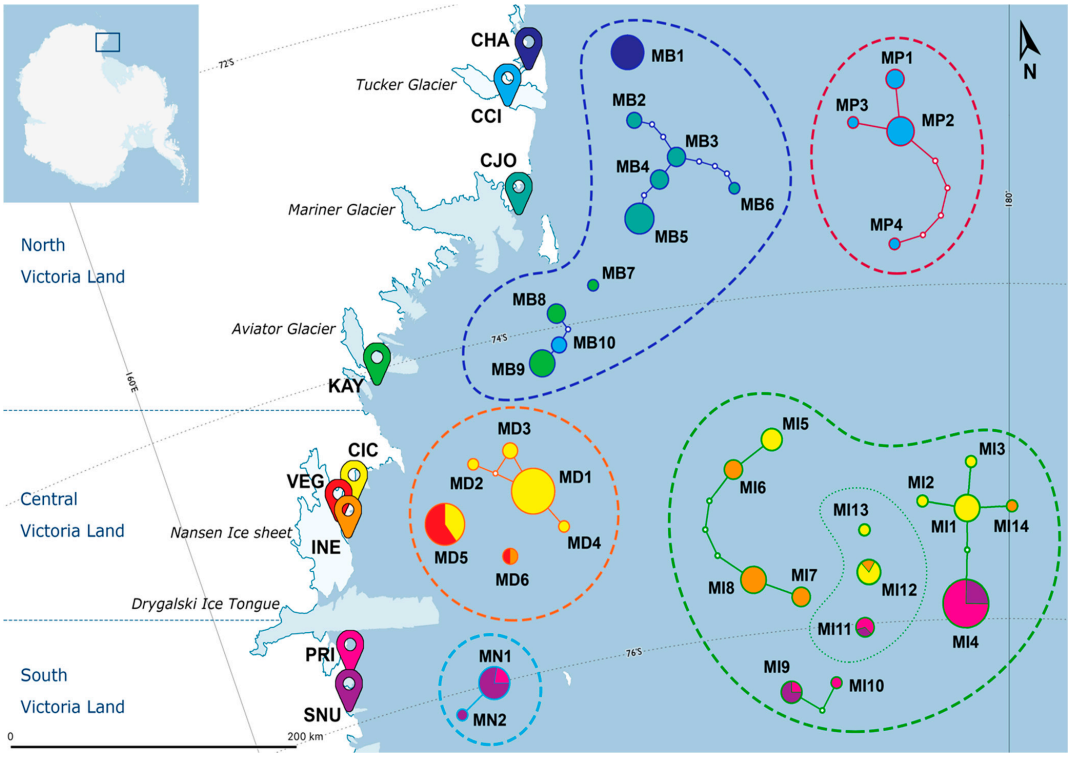

Figure 2. Haplotype networks of *cox1* for the five *Stereotydeus* species in Victoria Land (from 72 °S to 76 °S). Collection sites are indicated by the pie chart colors; the species are identified by the outlines of the networks together with the haplotype ID and the dashed lines around the clusters (coastlines from ADD Simple Basemap, NPI/Quantarctica 3 [60]).

3.2. Phylogenetic Analyses

- *cox1* with outgroups

For this single locus analysis, a total of 165 *Stereotydeus* sequences and two outgroups (*Eriorhynchus* sp. and *P. major*) were used. Before the addition of the outgroups, two unrooted analyses were also performed (Table S3 and Figure S1 in Supplementary Materials). One monophyletic group was formed by the haplotypes of *S. belli* (MB1-10) and includes 29 specimens from Northern Victoria Land (CHA, CJO and two from CCI), all those of the

KAY population (Central Victoria Land) and also the single sequence of *S. belli* (specimen from Cape Hallett) (Figures 2 and 3). Another monophyletic group included all 12 *S. punctatus* sequences (MP1-4) belonging to the CCI population. One paraphyletic group included the *S. delicatus* specimens (MD1-6) and the S. *ineffabilis* specimens (MI1-10, 14), with individuals from Southern Victoria Land (CIC, VEG, INE for *S. delicatus* and CIC, INE, PRI and SNU for *S. ineffabilis*) (Figures 2 and 3). Three haplotypes of *S. ineffabilis* were not included in the latter group, but they were clustered together, although with low statistical support (Figures 2 and 3). These three sequences, together with the branch that carries the two *S. nunatakis* haplotypes, did not cluster with the remaining ingroup, due to the insertion of three sequences of other species, although with medium statistical support (pp = 0.74 and 0.87) (Figure 3).

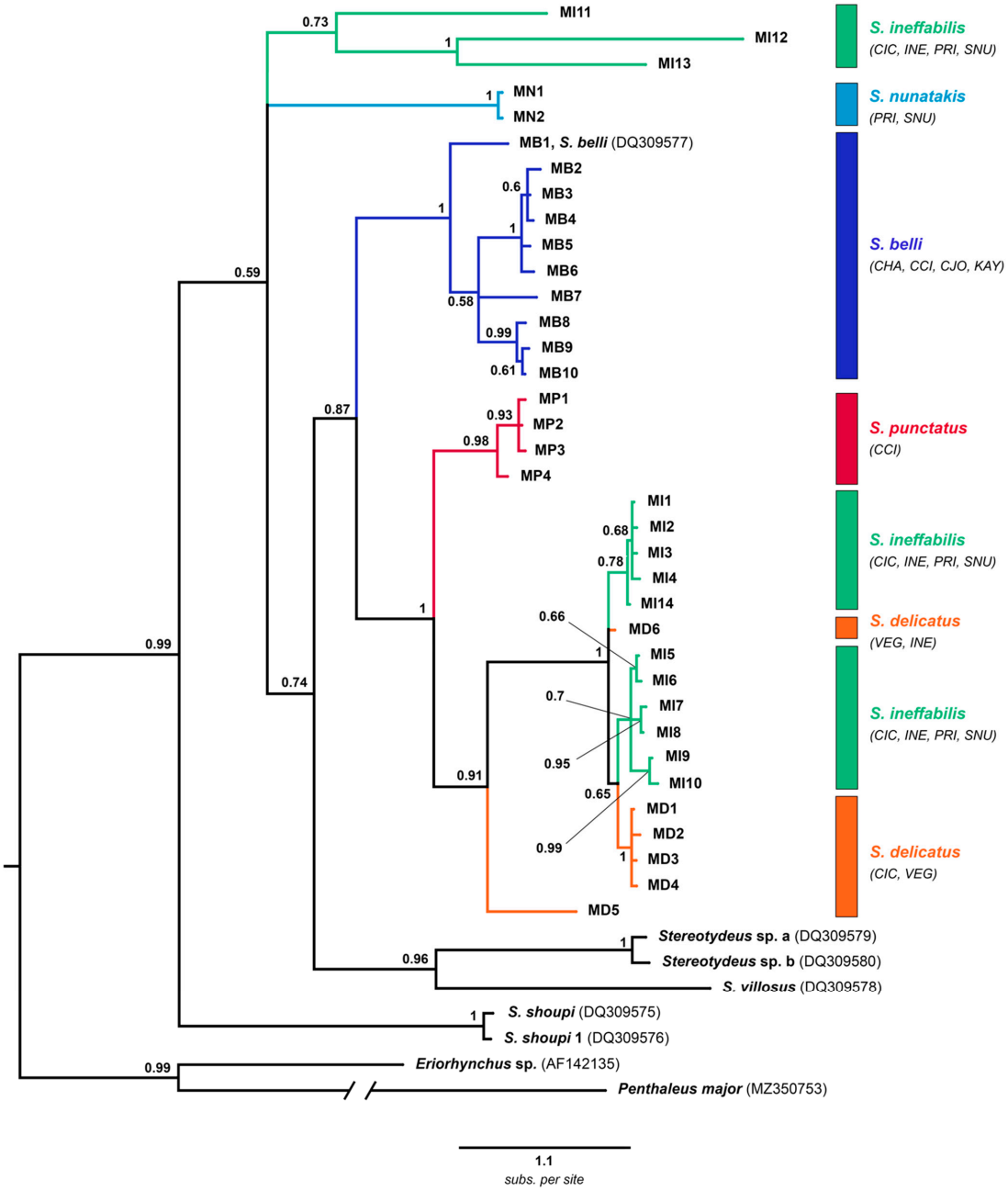

Figure 3. The phylogenetic tree of the *cox1* dataset of *Stereotydeus* specimens from Victoria Land (locality IDs are shown in brackets). Posterior probabilities are shown at the nodes. Labels indicate the ID of the haplotypes (detailed description of haplotypes in Table 5). Accession numbers for reference sequences and *Eriorynchus* sp. [33,34,36] and *P. major* outgroups are also shown.

- *cox1* all haplotypes

Fifty previously published *S. mollis* reference sequences were included for this analysis (Table S2). Despite the *S. ineffabilis* haplotypes being spread throughout the entire phylogenetic tree and not all nodes being statistically well supported, the two monophyletic groups of *S. belli* and *S. punctatus* were still distinct from the remaining species with good support at nodes (pp = 0.95 and 1, respectively). *S. delicatus* was, once again, recovered as a paraphyletic group: one cluster of four haplotypes (MD1-4) and two separated branches (MD5 and MD6), although with low support at nodes. The cluster of two haplotypes for *S. nunatakis*, together with MI11 and two *S. mollis* haplotypes (Sm49 and Sm50), was collapsed with the other three sequences at the base of the main cluster. Six (MI1, 4, 6, 9, 11, 12) out of the fourteen *S. ineffabilis* haplotypes were identical to previously published sequences (L, K, J, Sm44, R, O, respectively) originally assigned to *S. mollis* before the identification and description of *S. ineffabilis* as a new species [48] (Table 7). After the morphological identification of the specimens related to these haplotypes (Table 7), these sequences are now considered as *S. ineffabilis*. In addition, when sequences initially assigned to *S. mollis* clustered together with the *S. ineffabilis* haplotypes and were statistically well supported (pp > 0.85), we tentatively considered them as belonging to *S. ineffabilis* (e.g., Sm50, P; Figure 4).

Table 7. *S. ineffabilis* specimens used for the haplotypic and morphological analyses (Slide) with *cox1* haplotypes (*cox1*) identical to previously published sequences of *S. mollis* (haplo.). Sampling localities with their ID codes where the specimens were found and accession numbers (Acc. num.) of the *S. mollis* haplotypes are also provided.

	Slide	ID	*cox1*	Haplo.	Acc. Num.
S. ineffabilis	CI3	CIC	MI1	L	DQ305390
	P1, 2, 5; S5	PRI, SNU	MI4	K	DQ305385
	I2, 4	INE	MI6	J	DQ305397
	P3; S1	PRI, SNU	MI9	Sm44	HM537086
	S2	SNU	MI11	R	DQ309574
	I3	INE	MI12	O	DQ309572

- Combined *cox1-28S*

Following the phylogenetic analyses of the combined dataset of *cox1* and *28S* sequences (1034 bp), four phylogroups were detected: three monophyletic groups (*S. belli*, *S. punctatus* and *S. nunatakis*, although with variable support, pp = 0.55–1) and one paraphyletic clade (statistically low support, pp = 0.66, including *S. ineffabilis* and *S. delicatus* as mutually para/polyphyletic groups). The combination of the two datasets generated 14 unique haplotypes for *S. belli* from northern Victoria Land (CHA, CJO and CCI) and central Victoria Land (KAY), 4 unique haplotypes for *S. punctatus* from northern Victoria Land (CCI), 3 unique haplotypes for *S. nunatakis* from southern Victoria Land (PRI and SNU), 11 unique haplotypes for *S. delicatus* from southern Victoria Land (CIC, VEG and INE) and 16 unique haplotypes for *S. ineffabilis* from southern Victoria Land (CIC, INE, PRI and SNU) (Figure 5).

- Combined *cox1-28S* with morphology

In order to further clarify the paraphyletic relationships, a table of some morphological characteristics was linked to the combined *cox1-28S* tree, restricted to *S. ineffabilis* and *S. delicatus* sequences. All the nodes clustering the deepest branches together were statistically well supported, with the exception of that separating the Si 4 haplotype from the main cluster (pp = 0.64) (Figure 6).

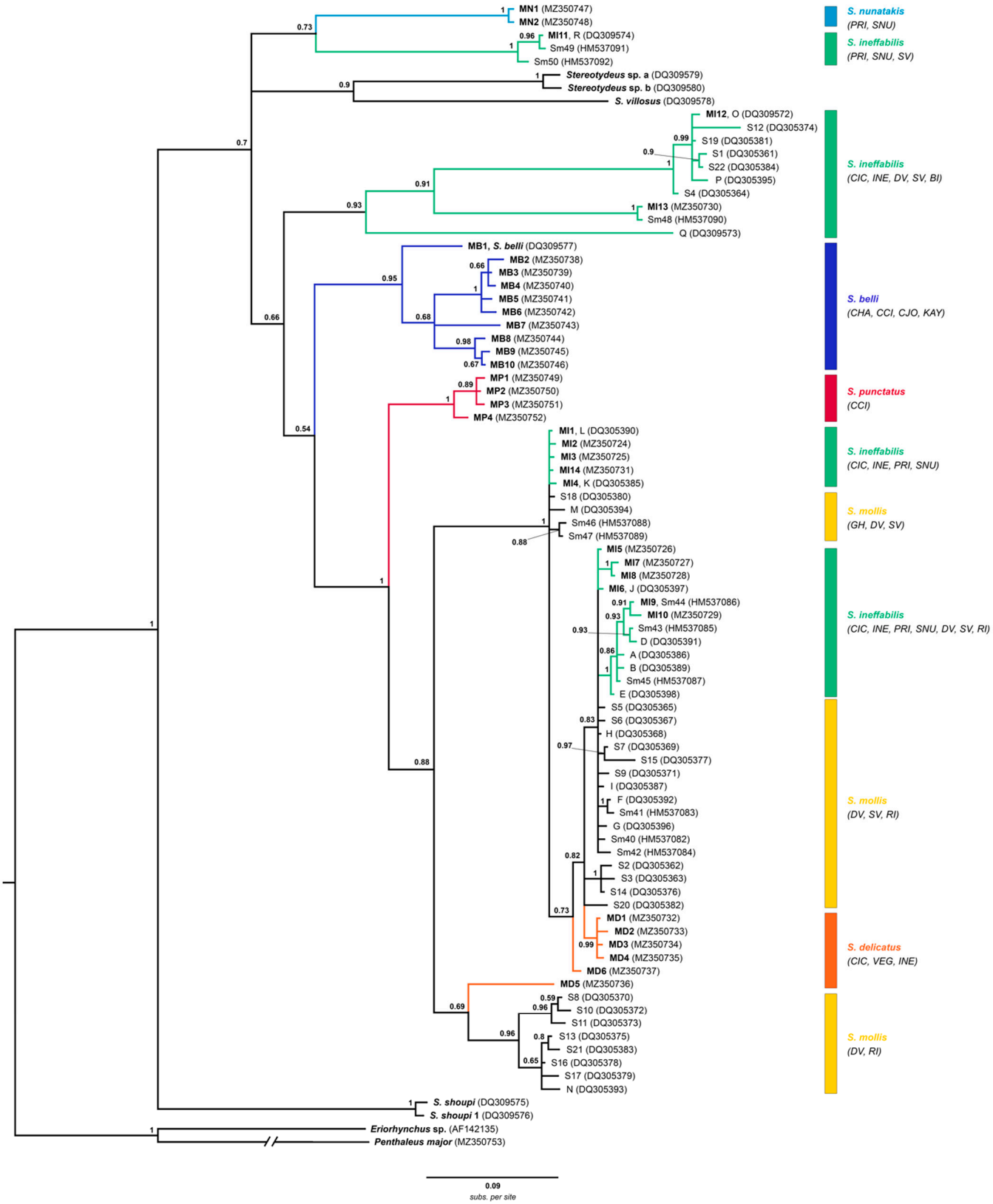

Figure 4. Phylogenetic tree of *cox1* haplotypes of *Stereotydeus* specimens from Victoria Land and the McMurdo and southern Dry Valleys [33,34,36]. Posterior probabilities are shown at the nodes. New haplotypes (in bold): labels indicate the ID of the haplotype; accession numbers are shown in brackets.

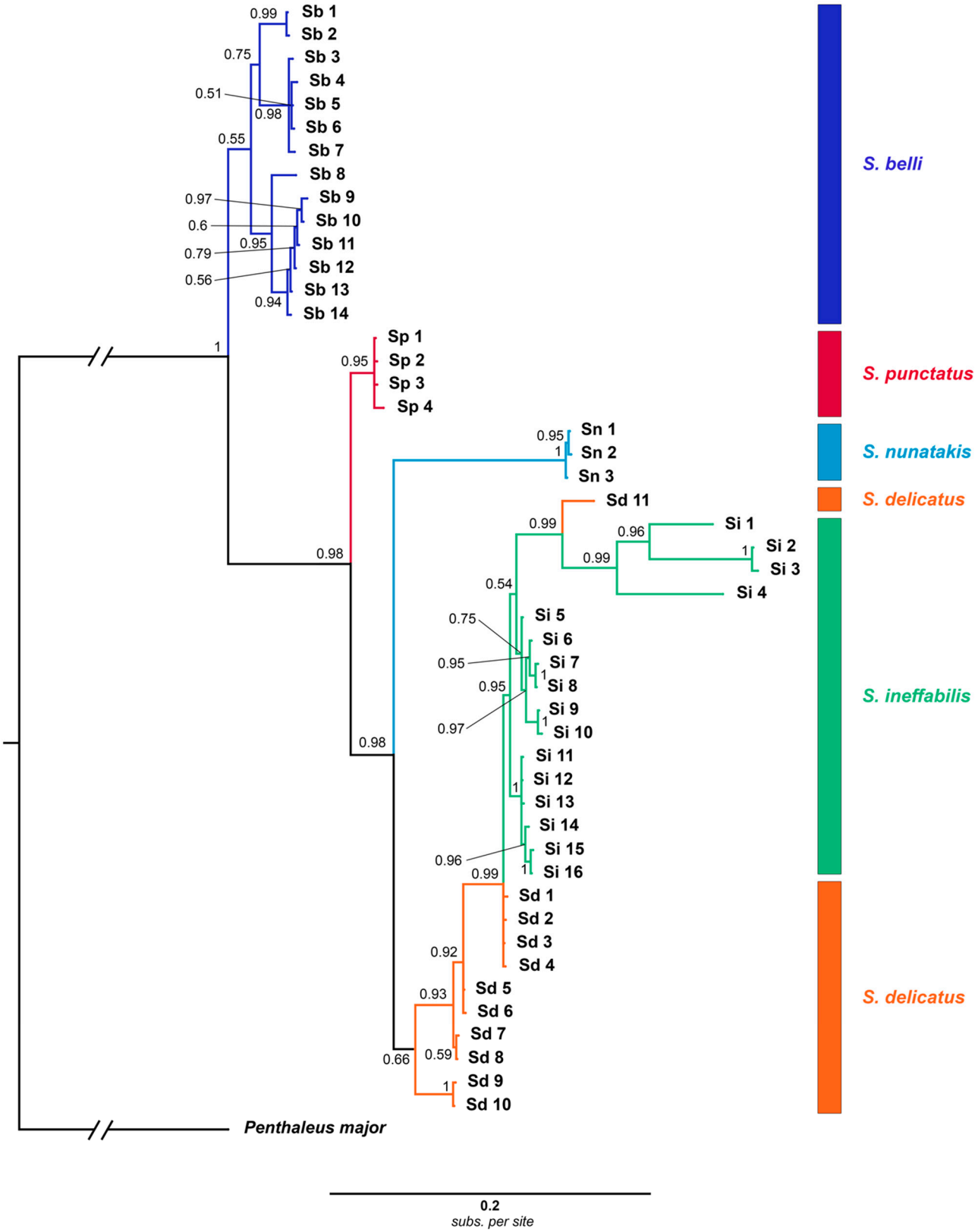

Figure 5. Phylogenetic tree of the combined mitochondrial and nuclear sequences of *Stereotydeus* specimens from Victoria Land. Posterior probabilities are shown at the nodes; labels indicate the ID of the haplotypes. For detailed description of haplotypes, see Table 5.

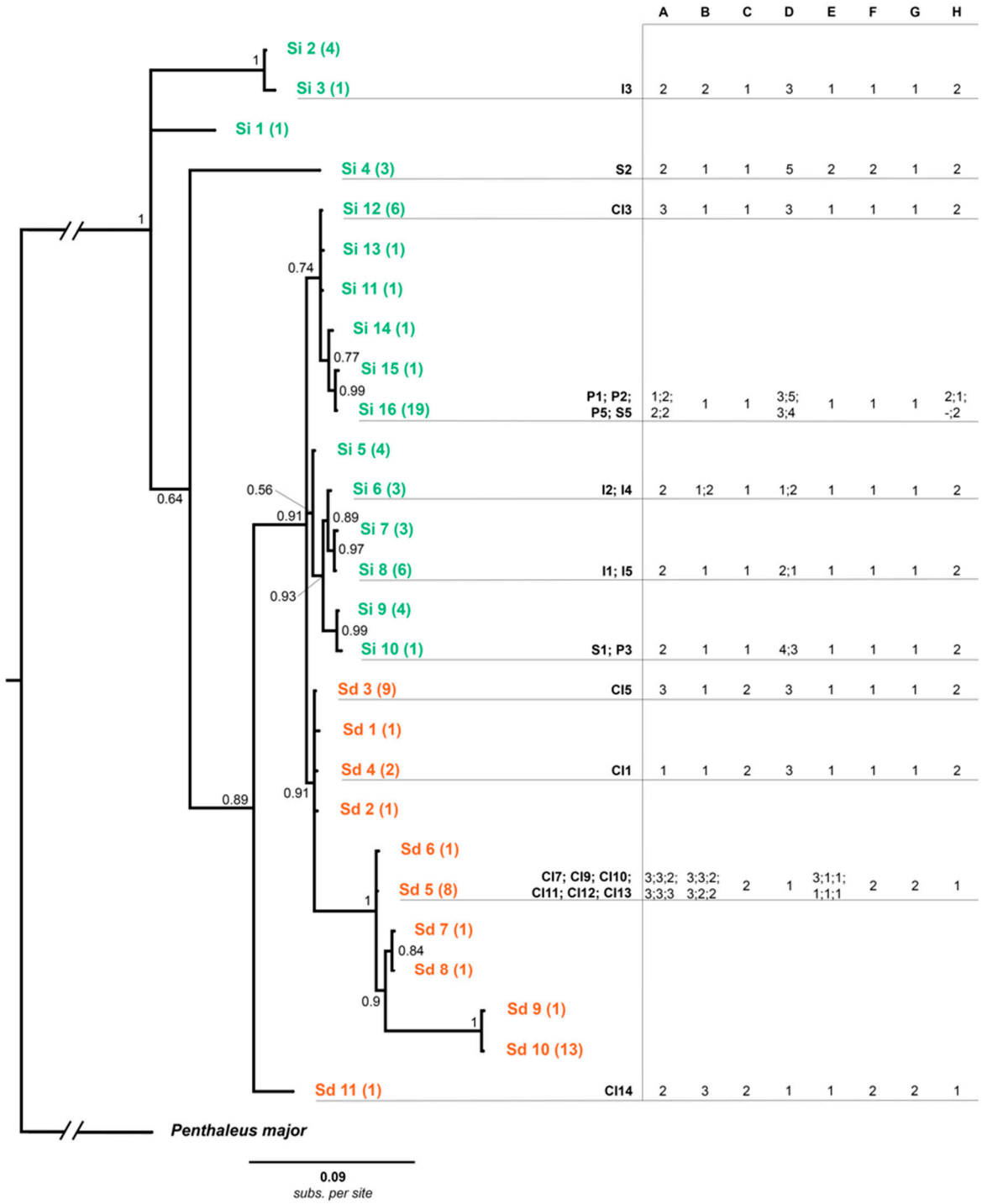

Figure 6. Phylogenetic tree of the combined mitochondrial and nuclear sequences of *S. delicatus* (orange labels) and *S. ineffabilis* (green labels) and table with codes of the morphological characters (see Table 8). Posterior probabilities are shown at the nodes of the phylogenetic tree. For the specimen ID (black, bold), see Table 2.

The *S. delicatus* specimens had a mean length of 451.83 µm (± 27.39 µm), ranging from CI1 (389.99 µm) to CI7 (481.55 µm). The femora were divided in CI7, CI9, CI11 (Sd 5) and CI14 (Sd 11), undivided in CI1 (Sd 4) and CI5 (Sd 3) and with partial division in CI10, CI12 and CI13 (Sd 5). The position of the anal pore was always apical. In all the specimens observed with haplotype Sd 5 and CI14 (Sd 11), there were four pairs of aggenital setae, while CI1 and CI5 had five pairs. Six pairs of genital setae were present in all the specimens, with the exception of CI7, which had seven pairs. The length of the 4th segment of the pedipalp was longer than the 3rd in all the specimens with haplotypes Sd 5 and CI14 (Sd 11),

while in CI1 and CI5 the two segments were comparable in length. The trilobe shaped epirostrum was weakly developed in CI1 and CI5, while, in the remaining specimens, it was evident and strongly developed. The three rhagidial organs on tarsi I and II showed an axis of symmetry in all specimens, with the exception, again, of CI1 and CI5.

Table 8. Morphological characters considered for the identification of *S. delicatus* and *S. ineffabilis*. Every row of a character is represented by a number (1–5) used to link them to the *combined morphology* phylogenetic tree (Figure 6). A. Length (µm); B. Femora; C. Position of the anal pore; D. Aggenital setae; E. Genital setae; F. Length of the 4th segment of pedipalp compared to the 3rd; G. Epirostrum; H. Disposition of the rhagidial organs on tarsi I and II.

Code	A	B	C	D	E	F	G	H
1	<400	undivided	ventral	4/4	6/6	IV = III	weak	symmetry
2	401–450	barely divided	apical	4/5	6/7	IV > III	evident	no symmetry
3	451–489	divided		5/5	7/7			
4	>490			5/6				
5				6/6				

The *S. ineffabilis* specimens had a mean length of 427.62 µm (± 18.61 µm), ranging from P1 (386.62 µm) to CI3 (460.44 µm). The femora were undivided except for individuals I3 (Si 3) and I4 (Si 6), where the division was only partial. The anal pore was always ventral in the terminal portion (see [48] Figures 1b and 5b). The number of aggenital setae was variable: two specimens (I2, I5) had four pairs, five (I3, CI3, P1, P3 and P5) had five pairs, two (S2, P2) had six pairs, while four had an intermediate number (I1 and I4 had 9 setae; S1 and S5 had 11 setae). Six pairs of genital setae were present in all specimens with the exception of S2 (Si 4), which presented an asymmetry with 13 setae. The length of the two terminal segments of the pedipalps was comparable in all the specimens examined except in S2 (Si 4), where the 4th segment was longer than the 3rd. The trilobed shape of the epirostrum was weakly developed in all specimens. The three rhagidial organs on tarsi I and II showed an axis of symmetry only in P2 (Si 16). P5 legs I and II were missing, so it was not possible to determine the positions of the rhagidial organs (for the morphological features see [48], Figures 1–5).

Although character C seems the only listed character that sharply sorts out the two species, when few exceptions of specimens are not considered, the list of characters increases (see [48] for the keys and the synoptic Tables A1–7 of the Antarctic *Stereotydeus* species).

3.3. Population Structure Analyses

Haplotype diversity (*h*) for *cox1* in *S. belli* ranged from 0 to 0.743 (mean 0.336). Within populations, CJO had the highest haplotype diversity and CHA and CCI the lowest. Nucleotide diversity (π) was low for all four populations, with the highest value being in the KAY population (0.010) (Table 9). The values of mean nucleotide pairwise differences $\theta(\pi)$ and mean number of segregating sites $\theta(S)$ ranged from 0 to 5.200 (mean 1.194 ± 2.486) and from 0 to 7.423 (mean 2.521 ± 3.501), respectively. The KAY population had the highest values of both $\theta(\pi)$ and $\theta(S)$, while CHA and CCI had the lowest. For *S. delicatus*, *h* ranged from 0 to 0.553 (mean 0.384), with the highest values in CIC (0.553) and the lowest in INE. Measures of π showed a similar pattern to haplotype diversity, with the highest values found in CIC (0.030). The highest values of $\theta(\pi)$ and $\theta(S)$ were recorded in CIC (14.966) and in VEG, respectively, while the INE population had the lowest values for both parameters. In *S. ineffabilis* populations, *h* ranged from 0.380 to 0.801 (mean 0.647). Within the populations, CIC, again, had the highest haplotype diversity, while PRI had the lowest. Reflecting the *h* measures, π had the highest value in CIC (0.071), with the lowest recorded in INE (0.026). The values of $\theta(\pi)$ and $\theta(S)$ ranged from 13.121 to 35.375 (mean 21.460 ± 9.829) and from 22.317 to 29.579 (mean 26.202 ± 3.497), respectively. The CIC population had the highest values of both $\theta(\pi)$ and $\theta(S)$, while INE and PRI had the lowest. These parameters were also calculated for the two *S. nunatakis* populations. However,

because, in PRI, only one haplotype was detected, all parameters for this population were 0, while in SNU the values were 0.250 for h and $\theta(\pi)$ and 0.001 and 0.386 for π and $\theta(S)$, respectively.

Table 9. Population genetic parameters for *cox1* in *S. belli*, *S. delicatus*, *S. ineffabilis* and *S. nunatakis* sampled across Victoria Land (Area). n, number of individuals; N_H, number of haplotypes within the populations and their frequencies; h, haplotype diversity; π, nucleotide diversity; $\theta(\pi)$, mean number of pairwise differences; θ(S), mean number of segregating sites; haplotypes shared between populations are indicated in italics (see Table 5 for details).

			Stereotydeus belli			
Area	**n.**	**N_H**	**h ± σ**	**π ± σ**	**θ(π) ± σ**	**θ(S) ± σ**
CHA	10	MB1(10)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
CCI	2	MB10(2)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
CJO	17	MB2(2), MB3(3), MB4(3), MB5(8), MB6(1)	0.743 ± 0.086	0.005 ± 0.003	2.559 ± 1.616	2.662 ± 1.247
KAY	10	MB7(1), MB8(3), MB9(6)	0.600 ± 0.130	0.010 ± 0.006	5.200 ± 3.108	7.423 ± 3.330
			Stereotydeus delicatus			
Area	**n**	**N_H**	**h ± σ**	**π ± σ**	**θ(π) ± σ**	**θ(S) ± σ**
CIC	28	MD1(18), MD2(1), MD3(2), MD4(1), *MD5(6)*	0.553 ± 0.093	0.030 ± 0.015	14.966 ± 7.682	11.307 ± 3.860
VEG	10	*MD5(9)*, *MD6(1)*	0.200 ± 0.154	0.017 ± 0.010	8.400 ± 4.807	14.846 ± 6.322
INE	1	*MD6(1)*	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
			Stereotydeus ineffabilis			
Area	**n**	**N_H**	**h ± σ**	**π ± σ**	**θ(π) ± σ**	**θ(S) ± σ**
CIC	17	MI1(6), MI2(1), MI3(1), MI5(4), *MI12(4)*, MI13(1)	0.801 ± 0.060	0.071 ± 0.037	35.375 ± 18.143	29.579 ± 10.687
INE	14	MI6(3), MI7(3), MI8(6), *MI12(1)*, MI14(1)	0.769 ± 0.083	0.026 ± 0.014	13.121 ± 7.058	24.213 ± 9.242
PRI	19	*MI4(15)*, *MI9(1)*, MI10(1), *MI11(2)*	0.380 ± 0.134	0.033 ± 0.017	16.316 ± 8.502	22.317 ± 7.940
SNU	9	*MI4(5)*, *MI9(3)*, *MI11(1)*	0.639 ± 0.126	0.042 ± 0.023	21.028 ± 11.648	28.699 ± 12.242
			Stereotydeus nunatakis			
Area	**n**	**N_H**	**h ± σ**	**π ± σ**	**θ(π) ± σ**	**θ(S) ± σ**
PRI	2	*MN1(2)*	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
SNU	8	*MN1(7)*, MN2(1)	0.250 ± 0.180	0.001 ± 0.001	0.250 ± 0.355	0.386 ± 0.386

As in [27,35,54], to establish the best combination for the population structure, AMOVA screenings were run for three species testing different combinations of population clusters: 10 runs were performed for *S. belli* (four populations: CHA, CCI, CJO and KAY), 3 for *S. delicatus* (three populations: CIC, VEG and INE) and 9 for *S. ineffabilis* (four populations: CIC, INE, SNU and PRI). As *S. nunatakis* was found only in two populations (PRI, SNU), the AMOVA was not calculated. For *S. belli*, the best resulting asset was (CHA vs. CCI+KAY vs. CJO), for *S. delicatus* (VEG vs. CIC+INE) and for *S. ineffabilis* (CIC vs. INE vs. SNU+PRI).

When group structure was assigned to populations for each species, the AMOVA analysis revealed more variation among groups and within populations (for *S. ineffabilis*) than among populations within groups (Table 10). In particular, for *S. belli* and *S. delicatus*, the Φ_{CT} values were similar (10.48068 and 9.51162, respectively). while for *S. ineffabilis* the value was only 2.94891. In contrast, Φ_{ST} values were higher in *S. ineffabilis* (10.89525) than in *S. belli* and *S. delicatus* (1.25345 and 6.66210, respectively).

Table 10. Percentage of variation (%) of molecular variance (AMOVA) of different levels of hierarchical population structure for *Stereotydeus* spp. for the mtDNA cytochrome *c* oxidase subunit *I* (*cox1*). The test was carried out with structure enforced according to geographical regions (see Section 3.3. for details).

Species		**Among Groups** Φ_{CT}	**Among Populations within Groups** Φ_{SC}	**Within Populations** Φ_{ST}
S. belli	Variance component	10.48068	0.05397	1.25345
	p	(0.16735 ± 0.00273)	(0.45057 ± 0.00422)	(0.00000 ± 0.00000)
	%	88.91	0.46	10.63
S. delicatus	Variance component	9.51162	0.28149	6.66210
	p	(0.33383 ± 0.00347)	(0.24403 ± 0.00340)	(0.0006 ± 0.00006)
	%	57.80	1.71	40.49
S. ineffabilis	Variance component	2.94891	−0.55777	10.89525
	p	(0.16135 ± 0.00259)	(0.62355 ± 0.00382)	(0.00056 ± 0.00018)
	%	22.19	−4.20	82.00

4. Discussion

This study provides over 150 new sequences representing all species of the mite genus *Stereotydeus* from Victoria Land. Combined with the morphological assessments that we provided, this information sheds light on an understudied taxon and provides a good starting point for further taxonomic studies of the species of the genus (Figure 7).

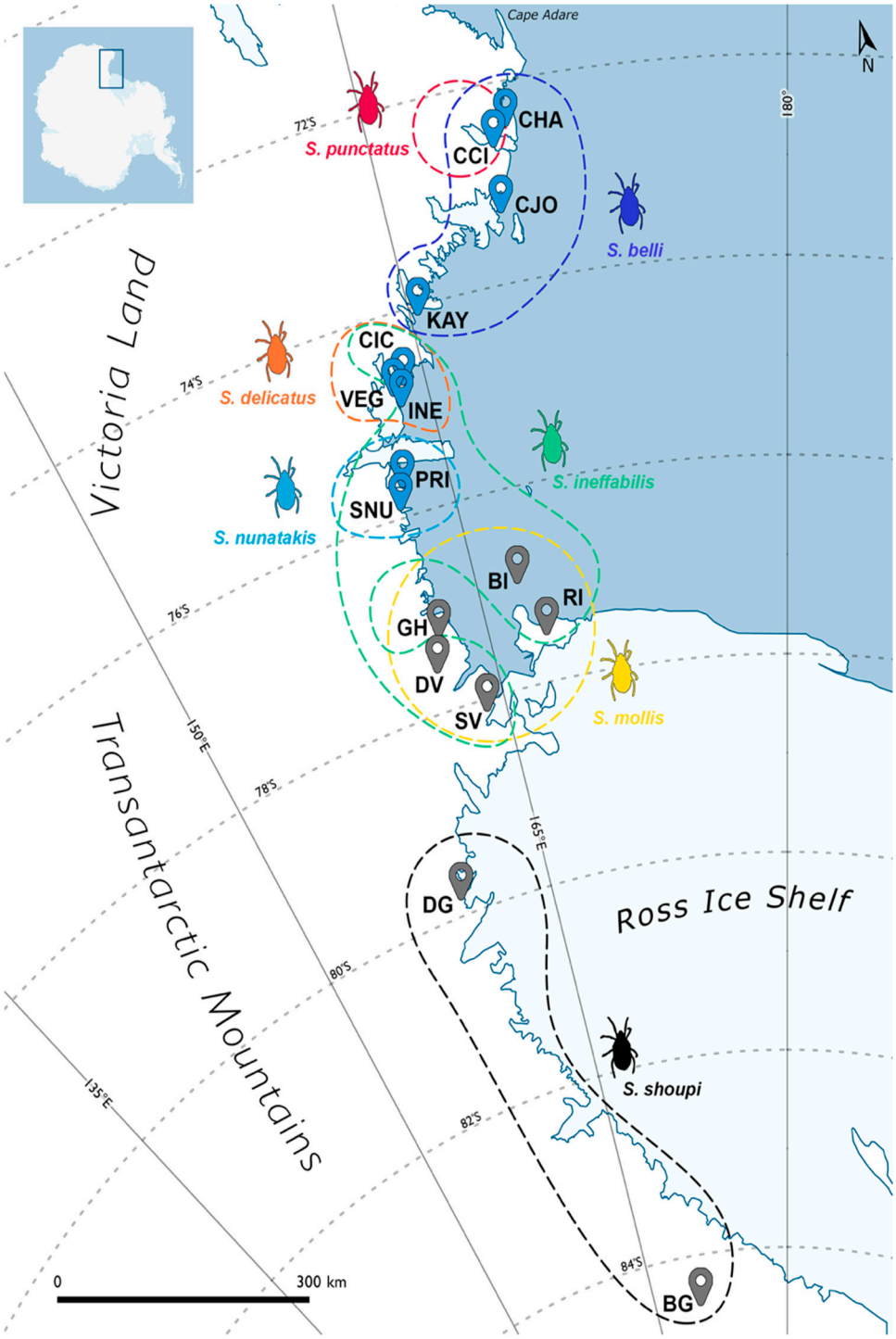

Figure 7. Updated map of the distribution of the *Stereotydeus* spp. of Victoria Land, based on the combination of new morphological and molecular data obtained this study and [48] (blue) and previous molecular data from [33,34,36] (grey). (coastlines from ADD Simple Basemap, NPI/Quantarctica 3 [60]).

4.1. North Victoria Land Taxa

Based on the analyses performed in this study, we found a latitudinal pattern in the distribution of *Stereotydeus* species in the Victoria Land coastal region. The presence of *S. belli* characterizes all populations from Cape Hallett (CHA) to Kay Island (KAY), while *S. punctatus* has, so far, been detected only in Crater Cirque (CCI). This is the first genetic study to be conducted on the latter species and, although only comprising a limited number of samples (12 individuals analyzed resulting in 4 *cox1* and 1 *28S* unique haplotypes), the presence of genetic variability is already evident. In addition, this is an easy species to identify morphologically due to the peculiar dorsal position of the anal opening that does not occur in any other *Stereotydeus* species. Early records of this taxon were reported by Strandtmann [44] and Gressitt and Shoup [40], also from Cape Adare and Cape Hallett.

For *S. belli* it is possible that historical events, such as habitat fragmentation due to glacial events, divergence in isolation and subsequent range expansion [32–34,78–80], are responsible for the patchy distribution of these populations and their genetic isolation. All the *S. belli* populations were clearly distinct, with KAY and CCI clustered together and separated from both CHA and CJO. This fragmented and apparently disjointed distribution is consistent with reports from other terrestrial invertebrate species in north Victoria Land (e.g., [31,64]). The same studies have reported that invertebrate populations in the region of the Tucker Glacier are genetically more closely related to populations in central–south Victoria Land, compared to others in relatively closer geographical proximity. Recent studies of springtail species endemic to Victoria Land, including *Cryptopygus cisantarcticus* [64] and *Friesea gretae* and *F. propria* [31] (*F. grisea* in Collins et al. [64]), highlighted the important role of the Tucker Glacier as an insurmountable barrier leading to high levels of genetic divergence between populations from either side of the glacier, plausibly representing distinct species. Combining the inferences made in the current study with previous springtail studies specific to northern Victoria Land [31,64], a comparably important role may be played by Crater Cirque, where *S. belli* and *S. punctatus* occur in sympatry.

4.2. Central-South Victoria Land Taxa

This study presents the first record in the central part of Victoria Land of *S. delicatus*, which was originally discovered and described by Strandtmann [44] (although with only one individual from each location) from Cape Adare and Edisto Inlet; thus, our new data considerably expand the known distribution of this species southwards. Our sampling area is located in a part of Terra Nova Bay that is affected by powerful winds, while the Hells Gate moraine creates an abrupt interruption between Inexpressible Island (INE) and the peninsula of the Northern Foothills where Campo Icaro (CIC) is located. The distribution of the haplotypes found in this area suggests a possible role played by Vegetation Island (VEG) in acting as a bridge to connect CIC and INE. It is plausible that gene connectivity bypassed the inhospitable Hells Gate channel by using VEG as a midpoint between CIC and INE, although further intermediate steps may have been available at different points in the past.

Considering the phylogenetic relationship of *S. delicatus* with the other *Stereotydeus* taxa reviewed in this study, the link with the newly described species *S. ineffabilis* is immediately evident. Even though the species are morphologically distinct (Figure 6, Table 8; see also [44,48] for species descriptions), individuals of the two species from the CIC locality share the same unique haplotype (RX1) for the nuclear marker *28S*, although the combined analyses of the latter with the mitochondrial marker *cox1* and the morphological characteristics provided a good resolution of the boundary between the two species. A possible explanation for these results is that these taxa have "recently" undergone a speciation process and, because of the different resolutions of the two markers, it is possible that the large ribosomal subunit may not yet have accumulated sufficient mutations to enable distinguishing between the two sympatric species. A "slow" nucleotide substitution rate in *28S* is not unusual and has recently also been recorded in *Friesea* lineages from Victoria

Land [31]. Specifically in relation to understanding the geographic distribution and genetic diversity of *S. delicatus*, it is now crucial to expand the sampling and study effort to include the north Victoria Land locations of Cape Adare and Edisto Inlet, where the species was first recorded and described by Strandtmann [44].

While *S. delicatus* shows a well defined pattern of distribution, that of *S. ineffabilis* appears to be more complex. As for *S. delicatus*, the presence of the Hells Gate moraine isolates the populations north of the Drygalski Ice Tongue but, observing the haplotype networks, it is possible that, in the past, the two areas were linked, with the populations starting to differentiate only when the connection was broken. It is notable that the two *S. ineffabilis* populations south of the Drygalski Ice Tongue show a genetic connection to the population north of the glacier, although also showing some differentiation. As the Drygalski Ice Tongue is considered the geographical barrier that sharply delimits the faunas of north and south Victoria Land, our data provide a first indication of geneflow between north and south Victoria Land, and the first record of a terrestrial microarthropod species shared between the two regions.

In comparing the genetic diversity present in north and south Victoria Land, this study included also *Stereotydeus* spp. *cox1* haplotypes reported in previous studies [33,34,36] in the phylogenetic analyses performed. A striking outcome of these analyses is the strong link that emerged between *S. mollis* and *S. ineffabilis* sequences. The great genetic variability of the *cox1* marker alone proved ineffective in drawing a clear distinction between the two taxa. In order to stabilize the phylogenetic signal of the mitochondrial marker, it will be crucial to include one or more nuclear markers in future studies, as well as combining genetic and morphological approaches. In the absence of nuclear DNA sequence data from the *Stereotydeus* specimens, several morphological characteristics (e.g., the smaller size of the adults, the asymmetry in the tarsal rhagidial organs, the position of the solenidia on the tibiae and the genua, the number of the aggenital setae; see [48] for more details) were useful in identifying boundaries between *S. mollis* and *S. ineffabilis*. A high level of genetic diversity of recent origin (see branching pattern on Figures 4 and 5) is generally interpreted as an indication of recent demographic expansion. However, the present distributions of the *S. ineffabilis*, *S. delicatus* and *S. mollis* phylogroups may best be interpreted as being the result of alternative and temporally disjunct colonization events and speciation processes that occurred several times and started from different glacial refugia over a time interval of more than 10 Myr.

Together with *S. ineffabilis*, *S. nunatakis* was also present in the Prior Island (PRI) and Starr Nunatak (SNU) sampling locations [48]. Although the number of samples for genetic and morphological analyses was low, some variability and divergence was apparent. Based on the combined mitochondrial and nuclear phylogenetic analysis and the computation of genetic distances, *S. nunatakis* appears to be more closely related to *S. punctatus*, from north Victoria Land, than to the other species from south Victoria Land, *S. ineffabilis*, *S. mollis* and *S. shoupi*.

4.3. Speciation in Action

The patterns of diversity observed today in many Antarctic species can be traced back to historic events, such as habitat fragmentation, divergence in isolation and subsequent range expansion, that influenced the distribution of species particularly at local scales [32–34,81]. The resulting patterns of genetic variation can be used to infer ecological factors (e.g., effective population size, dispersal capacity), as well as those affecting speciation processes. Allopatric speciation in populations that are geographically separated appears to be characteristic for populations of many terrestrial invertebrate species native to Victoria Land, and is considered the result of the different fragmentation and isolation events of ancestral and widespread lineages [19,20,27]. As for these other invertebrates, we suggest that, due to their limited dispersal abilities and the presence of physiological barriers such as low tolerance to desiccation and abiotic barriers, our resulting populations also started to differentiate independently. However, especially for the southern Victoria Land species of *Stereotydeus*, the scenario appears to be more complex, due to the

presence of four geographically and genetically closely related species. In recent decades, the suggestion that speciation might also occur in populations that are not geographically isolated (i.e., sympatric speciation [82–84]) has become increasingly accepted. It is possible, for example, that, when limited resources are available to members of sympatric populations, interactions through both direct (i.e., interference) and indirect (i.e., exploitation) competition could lead groups of individuals, especially those belonging to populations of large size, to adopt different behaviors, select different habitats, establish temporal shifts of activity patterns or avoid mating or generating hybrids with low fitness. Thus, ecologically based barriers to gene flow evolve between populations resulting in an "ecological selection" [83,85,86]. This selection can occur under different geographic conditions [83], so it cannot be excluded that this process may also have contributed to the current patterns of variability and distribution of *Stereotydeus* species in Antarctica.

Although the biogeographical patterns of springtails and mites in coastal Victoria Land share some similarities [28,33,35], their intra- and interspecific genetic distances are not entirely comparable. Interspecific genetic distances calculated between species of Acari are generally greater than those observed in comparisons between Collembola (e.g., [33,34]), and it is not possible to exclude this being influenced by the different survival strategies and/or life histories of free living mites [12]. It is possible that all aspects of the life history strategy of Antarctic terrestrial invertebrates (e.g., including generation time, life cycles, physiology and metabolism), in combination with environmental conditions, could be major factors influencing evolutionary rates (nucleotide substitutions). However, it is also not clear, in general, how rates of evolution differ across species or, if they do, what factors drive these differences. The factors responsible for the high levels of divergence shown by mites have previously been suggested to include the smaller size of the animals, their shorter generation time and higher activity levels [37] and their greater recolonization/dispersal abilities [33] in comparison with springtail taxa. However, these hypotheses have not been explicitly tested. Prostigmatid mites lack an impermeable cuticle, and behavioral strategies, such as microhabitat selection, along with physiological acclimatization [12] are likely to play a fundamental role in the isolation of populations and their survival. As suggested by Demetras et al. [36], some behavioral differences may have a role in increasing genetic divergence, as has also been noted for some Antarctic springtail species [87]. Thus, through combining morphological, genetic and ecological studies of terrestrial fauna, we can better understand the evolutionary origins, dispersal history and current distribution of Antarctic invertebrates.

Due to the close phylogenetic relationships between the central and southern species (*S. ineffabilis*, *S. delicatus* and *S. mollis*), in the future it will be fundamental to carry out and implement new combined taxonomical studies and enlarge the number of specimens in the analyses. The inclusion of a more recent revision of the original materials used for the first description of *S. delicatus* and *S. mollis* will help to identify additional morphological characters, if any, necessary to distinguish these species with respect to *S. ineffabilis*. In fact, when the amount of divergence at inter- and intraspecific level is overlapping, morphology is important to identify species boundaries. In addition, the genetic differentiation of species of "recent" origin may be less variable with respect to more ancient ones. Thus, the combination of new morphological analyses and a deeper genetic screening through the incorporation of more nuclear markers and/or genome comparisons will be the starting point to better define some of the phylogenetic relationships of all the Victoria Land *Stereotydeus* species.

In summary, the contemporary distributions of species of *Stereotydeus* occurring in Victoria Land follow defined latitudinal patterns, including two major features. These are characterized by, first, a more genetically defined cluster in the north Victoria Land populations of *S. belli* and *S. punctatus* and, second, a more complex, in terms of species composition, cluster including populations in south Victoria Land.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13100506/s1, Figure S1: Unrooted phylogenetic trees of *Stereotydeus* specimens with pos-

terior probabilities shown at nodes, Table S1: Accession numbers of the *cox1* and *28S* sequences of *Stereotydeus* species and *Penthaleus major* deposited on GenBank and included in the analyses, Table S2: Accession numbers of the *cox1* sequences of *Stereotydeus* species and one Eriorhynchidae mite downloaded from GenBank and included in the analyses, Table S3: List of the datasets, number of new sequences obtained and used in each dataset, markers, reference sequences and outgroups used for the analyses and models of nucleotide evolution that best fitted, divided according to the partition applied and to the respective tree search optimization criteria.

Author Contributions: Conceptualization, C.B. and A.C.; methodology, C.B., A.C. and F.N.; validation, A.C., F.N., H.S. and P.P.F.; formal analysis, C.B.; investigation, C.B.; resources, A.C., P.P.F., F.N. and H.S.; data curation, A.C., F.N. and C.B.; writing—original draft preparation, C.B.; writing—review and editing, C.B., A.C., F.N., P.C., H.S. and P.P.F.; visualization, C.B.; supervision, A.C., H.S., P.C. and F.N.; project administration, C.B. and A.C.; funding acquisition, A.C., F.N. and P.P.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Program of Research in Antarctica (PNRA16_00234) to A.C. Partial support was also provided by the University of Siena. P.C. is supported by NERC core funding to the British Antarctic Surveys "Biodiversity, Ecosystems and Adaptation" Team. The paper also contributes to the SCAR "State of the Antarctic Ecosystem" (AntEco) international program.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data are contained within the article. The sequences are available on NCBI under the Accession Numbers MZ350724-MZ350753 (*cox1*) and MZ442270-MZ442288 (*28S*) (see also Table S1 in Supplementary Materials).

Acknowledgments: We would like to thank Hatef Servatibeiragh, Bernardo Stockl Junior, Veronica Cannucci, Chiara Leo and Claudio Cucini for their help with the lab work. We wish to thank also the four anonymous reviewers for their helpful comments.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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