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Mitochondrial DNA structuring of Antarctic prions (*Pachyptila desolata*, Procellariidae)

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Abstract

Prions (*Pachyptila*) are small seabirds with a Southern Hemisphere breeding distribution. Antarctic prion (*Pachyptila desolata*) and Salvin's prion (*P. salvini*) are two species that are regularly recorded in New Zealand as beach-wrecks but they are difficult to distinguish morphologically. Salvin's prion is restricted to breeding on the Prince Edward Islands and Crozet Islands in the Indian Ocean but Antarctic prions have a circumpolar breeding distribution on numerous sub-Antarctic and Antarctic islands in the Southern, South Atlantic and Indian Oceans. Our aim was to examine the level of mitochondrial DNA (mtDNA) structuring within Antarctic prion and Salvin's prion colonies, to test whether this technique can determine the provenance of beach-cast birds. The Auckland Islands Antarctic prion population exhibited distinct mtDNA haplotypes from all other populations, supporting the suggestion that these islands may have been an ice-free refugium during the Last Glacial Maximum. All other sampled breeding populations shared haplotypes, limiting the use of these sequences for determining the provenance of beach-cast birds. None of our museum specimens of Salvin's prion collected from breeding colonies produced DNA sequences. This result indicates that the method by which these specimens, which were collected in the 1960s and 70s, were preserved, or subsequent treatments, has resulted in the loss of their DNA.

Keywords

cytochrome *b*, cytochrome oxidase I, phylogeography, population structure, Procellariiformes, seabird

Introduction

Prions (*Pachyptila*) are small seabirds with a Southern Hemisphere breeding distribution. Eight extant species of prions are currently recognised (Checklist Committee (OSNZ) (2022); Shepherd et al. 2022) and they are closely related and estimated to have diverged in the last 6 million years (Masello et al. 2019). The species are similar in appearance and behaviour, making their identification challenging, especially at sea (Harper 1980). Prions mainly differ in the structure and size of their bills (Warham 1990), which reflects differences in prey selection and feeding strategies.

New Zealand is the centre of prion diversity, with five species breeding within the region (Checklist Committee (OSNZ) (2022); Shepherd et al. 2022), mostly on remote, predator-free islands. Prions are also regularly found beach-cast on the New Zealand mainland, particularly during mass mortality events (wrecks) when many thousands of individuals may die, often following stormy weather (Harper 1980; Powlesland 1989; Warham 1996). Such beach-cast birds are particularly difficult to identify because immature birds of larger-billed species can look like adults of smaller-billed species and shrinkage in bill dimensions can occur as dead specimens dry out (Harper 1980).

In this paper we focus on Antarctic prion (*Pachyptila desolata* (Gmelin, 1789)) and Salvin's prion (*P. salvini* (Mathews, 1912)), two species that are commonly confused morphologically (Harper 1980) and that have been regularly recorded in New Zealand wrecks (Harper 1980; Powlesland 1989). Salvin's prion appears to have arisen through hybridisation between broad-billed prion (*P. vittata* (G. Forster, 1777)) and Antarctic prion and has a bill intermediate in size between these two species (Massello et al. 2019). The breeding distribution of Salvin's prion is restricted to the Prince Edward Islands

and Crozet Islands in the Indian Ocean (Fig. 1) (Marchant and Higgins 1990). In contrast, Antarctic prions have a much wider circumpolar distribution, breeding on numerous sub-Antarctic and Antarctic islands in the Southern, South Atlantic and Indian Oceans (Fig. 1) (Marchant and Higgins 1990). Morphological variation is present within Antarctic prions that is geographically based. Historically, the taxonomy of Antarctic prions has been unstable with various authors recognising up to six subspecies (e.g. Mathews 1912, 1934; Falla 1940; Tickell 1962; Bretagnolle 1990) or no subspecies (Harper 1980). Most recent treatments consider the species monotypic (Marchant and Higgins 1990; Dickinson and Remsen 2013; Checklist Committee (OSNZ) (2022)).

A number of population genetic studies on prions have recently been published and these have shown that some species exhibit little differentiation between populations whilst others exhibit considerable structuring. The fairy and fulmar prion clade (*P. turtur*, *P. crassirostris* and *P. pyramidalis*) demonstrated a high level of genetic structuring with both genomic SNPs and mitochondrial DNA (mtDNA) sequences, resulting in further taxa being recognised (Shepherd et al. 2022). In contrast, populations of broad-billed prion from the Atlantic Ocean and the New Zealand region could not be distinguished with either mtDNA sequences (cytochrome *b* and cytochrome oxidase I (COI)) or 18 microsatellite loci (Masello et al. 2021). Quillfeldt et al. (2017) examined cytochrome *b* sequences plus genotypes from 25 microsatellite loci of Indian and Atlantic Ocean populations of Antarctic prion and Thin-billed prion *P. belcheri* (Mathews, 1912) and found no population structuring within either species. Masello et al. (2021) subsequently genotyped the same 25 microsatellite loci from Macquarie Island Antarctic prions and found them indistinguishable from Indian and Atlantic Ocean birds. There are no published mtDNA sequences (or microsatellite data) from New Zealand Antarctic prions. For Salvin's prions there are published COI and cytochrome *b* sequences published from Marion Island but only a single sequenced individual from the Crozet Islands (Masello et al. 2021), which hold the largest populations of this species.

Our aim for this study was to determine the level of mtDNA genetic structuring within Antarctic prion and Salvin's prion colonies. In particular, is the morphological

Figure 1. Distribution map of Antarctic (*Pachyptila desolata*) and Salvin's (*Pachyptila salvini*) prion breeding colonies. The symbol '?' indicates a likely but unconfirmed breeding colony and '†' denotes a colony thought to be extinct. Populations included in this study are underlined.

variation observed in Antarctic prions supported by genetic differences? Determining the level of connectivity between populations of these species is important for tracking threats on their populations and to assess whether DNA sequencing can be used to determine the provenance of beach-wrecked birds.

Materials and methods

Both modern samples and historical skins were included in this study in order to cover the geographic spread of Antarctic and Salvin's prions (Fig. 1, Table 1, Suppl. material 1), when combined with published sequences. Thirty-six Antarctic prion and six Salvin's prion specimens were selected for sampling. Footpad or skin tissue was sampled from study skins from the Museum of New Zealand Te Papa Tongarewa collection using a new sterile scalpel blade for each specimen. These study skins dated from between 1912 and 1992 and most were collected from breeding colonies (Table 1). Additionally, blood samples of Antarctic prions from the Auckland Islands were collected under a permit from the Department of Conservation (permit number 97330-FLO). Sampling methods followed approved ethical procedures as required under this permit.

We targeted the mitochondrial cytochrome oxidase (COI) and cytochrome *b* loci because these markers have previously been used successfully to differentiate prion populations (Shepherd et al. 2022) and primers are available, including short internal primers for amplifying the degraded DNA typically found in museum specimens. Also, cytochrome *b* sequences for Antarctic prions from South Georgia and the Kerguelen Islands are available on Genbank for comparison.

DNA extraction, PCR amplification and sequencing of COI and cytochrome *b* loci followed Shepherd et al. (2022), except that cytochrome *b* from the historical specimens was sequenced by amplification of five short overlapping fragments. These fragments were amplified and sequenced with the internal primers provided in Masello et al. (2021).

Sequences were edited in Sequencer 5.4.6 (Gene Codes Corporation) and, because they contained no insertion/deletion events (indels), were aligned manually to sequences available in GenBank (Table 1). Thin-billed and Salvin's prion sequences were used as outgroups (Masello et al. 2019).

The relationships between the mtDNA sequences were examined by constructing median-joining networks (Bandelt et al. 1999) in PopART (Leigh and Bryant 2005) and phylogenetic trees using maximum likelihood (ML) and Bayesian Inference (BI). Separate networks were produced for each locus because only cytochrome *b* was available for some individuals on GenBank. For the phylogenetic analyses only the samples with both loci sequenced were included and the two loci were concatenated.

ML analyses were performed with the PhyML v3.0 (Guindon et al. 2010) web server (http://www.atgcmontpellier.fr/phyml/). Heuristic searches were performed with SPR branch-swapping and 10 random addition sequence replicates, with the best fit model of sequence evolution (HKY85) determined by Smart Model Selection (Lefort et al. 2017) and the Akaike Information Criterion. Branch support was assessed with 1000 bootstrap (BS) pseudoreplicates.

MrBayes v3.2.7 (Ronquist et al. 2012) was used to perform BI with the dataset partitioned by locus and substitution model parameters unlinked between the loci. Two concurrent analyses, each with four Markov chains of fifty million generations were run, sampling every 1000 generations and with nst = 6, rates = invgamma and default parameters. The first 20% of samples were discarded as ''burn-in", after this point the standard deviation of split frequencies was below 0.01. Tracer v.1.71 (Rambaut et al. 2018) also confirmed that stationarity had been reached.

Population differentiation between colonies was estimated by calculating the global fixation index (F_{ST}) and two parameters of population subdivision (G_{ST} and N_{ST}) from the cytochrome *b* data (this locus had the least missing data) in SPADS v1.0 (Dellicour and Mardulyn 2014). Statistical significance of these values was assessed by 1000 random permutations. N_{ST} considers the relationships between haplotypes, whereas G_{ST} is calculated using only haplotype frequency data. G_{ST} and N_{ST} were compared with a permutation test with 10 000 permutations.

The geographic structure of the cytochrome *b* variation in Antarctic prions was examined by spatial analysis of molecular variance (SAMOVA, Dupanloup et al. 2002), implemented in SPADS 1.0. SAMOVA partitions groups of populations by maximising the proportion of the total genetic variance due to differences between groups of populations (F_{CT}). The number of groups (K) was set to vary between 2 and 5, with SAMOVA run with 10 000 iterations and 10 repetitions. For calculating the population differentiation measures and running the SAMOVA only birds from known breeding colonies were included and the single specimen from the South Sandwich Islands was grouped with the larger population sample from nearby South Georgia (they shared a haplotype).

Results

Twenty-three of the thirty-six Antarctic prions amplified for at least one locus, but none of the Salvin's prions produced any PCR products (Table 1, Suppl. material 1). Newly-generated sequences have been deposited in GenBank (Accession numbers provided in Table 1).

The COI and cytochrome *b* alignments of Antarctic prion sequences were 702 bp and 812 bp in length, respectively. Neither locus contained internal stop codons when translated. For the COI locus we recovered 8 haplotypes, defined by 9 variable sites and for cytochrome *b* there were 22 haplotypes, defined by 21 variable sites.

Table 1. Specimens included in this study that produced DNA sequences. Newly-generated sequences are shown in bold. * denotes samples not collected from breeding colonies. ł denotes a sample from an extinct colony.

Voucher/ identifier	Location	Collection date	Collector	Type of sample	COI	Cytochrome *b*
Pachyptila desolata						
NMNZ OR.018142	**Auckland Is.**	**16/12/1973**	**FC Kinsky**	**footpad**	**OR751463**	**OR753539**
NMNZ OR.018143	**Auckland Is.**	**16/12/1973**	**FC Kinsky**	**footpad**	**OR751464**	**OR753540**
NMNZ OR.017551	**Auckland Is.**	**2/02/1973**	**BD Bell**	**footpad**	**OR751472**	**OR753544**
NMNZ OR.017552	**Auckland Is.**	**2/02/1973**	**BD Bell**	**footpad**	**OR751473**	**OR753545**
NMNZ OR.013037	**Auckland Is.**	**9/10/1942**	**CA Fleming**	**footpad**	**OR751465**	**OR753546**
NMNZ OR.017550	**Auckland Is.**	**2/02/1973**	**BD Bell**	**footpad**	**OR751471**	**OR753543**
AP1	**Auckland Is.**	**22/01/2018**	**C Miskelly, A Tennyson**	**blood**	**OR751474**	**OR753550**
AP6	**Auckland Is.**	**29/01/2018**	**C Miskelly, A Tennyson**	**blood**	**OR751476**	**OR753549**
AP7	**Auckland Is.**	**29/01/2018**	**C Miskelly, A Tennyson**	**blood**	**OR751480**	**OR753547**
AP9	**Auckland Is.**	**29/01/2018**	**C Miskelly, A Tennyson**	**blood**	**OR751475**	**OR753548**
NMNZ OR.013345	***Campbell I.**	**21/02/1968**	**G Surrey**	**skin from leg**	**OR751459**	**OR753536**
NMNZ OR.021931	***Campbell I.**	**1943**	**JH Sorenson**	**footpad**	**OR751460**	**OR753537**
NMNZ OR.019432	**ł Cape Denison**	**1913**	**HH Hamilton**	**footpad**	**OR751461**	**OR753538**
NMNZ OR.014446	***Ross Sea, Antarctica**	**2/02/1967**	**P Harper**	**footpad**	**OR751477**	**-**
NMNZ OR.012753	***At Sea, Antarctica (63'S, 40'W)**	**12/02/1966**	**PC Harper**	**footpad**	**OR751469**	**-**
NMNZ OR.012757	***At Sea, Antarctica (55.45'S, 42.52'W)**	**10/03/1966**	**PC Harper**	**footpad**	**OR751470**	**OR753554**
NMNZ OR.024779	**Heard I.**	**12/02/1992**	**P Scofield**	**footpad**	**OR751468**	**OR753541**
NMNZ OR.024776	**Heard I.**	**12/02/1992**	**P Scofield**	**footpad**	**OR751467**	**OR753542**
NMNZ OR.022311	**Kerguelen Is.**	**6/02/1981**	**JA Bartle**	**footpad**	**OR751466**	**OR753553**
NMNZ OR.023067	**Kerguelen Is.**	**28/01/1985**	**H Weimerskirch**	**footpad**	**OR751462**	**-**
Kerguelen2	**Kerguelen Is.**	**10/01/2016**	**C Miskelly**	**blood**	**OR751478**	**OR753551**
Kerguelen3	**Kerguelen Is.**	**10/01/2016**	**C Miskelly**	**blood**	**OR751479**	**OR753552**
104dVE	Kerguelen Is.				-	KX139130
101dVE	Kerguelen Is.				-	MF421887
102dVE	Kerguelen Is.				-	MF421888
103dVE	Kerguelen Is.				-	MF421889
105dVE	Kerguelen Is.				-	MF421890
89dVE	Kerguelen Is.				-	MF421891
92dVE	Kerguelen Is.				-	MF421892
93dVE	Kerguelen Is.				-	MF421893
94dVE	Kerguelen Is.				-	MF421894
95dVE	Kerguelen Is.				-	MF421895
97dVE	Kerguelen Is.				-	MF421896
98dVE	Kerguelen Is.				-	MF421897
99dVE	Kerguelen Is.				-	MF421898
PetraFA06	Kerguelen Is.				KX092013	-
NMNZ OR.012579	**South Sandwich Is.**	**6/03/1966**	**PC Harper**	**footpad**	**OR751458**	**OR753555**
346dBI	South Georgia				-	MF421870
347dBI	South Georgia				-	MF421871
348dBI	South Georgia				-	MF421872
51dBI	South Georgia				-	MF421873
353dBI	South Georgia				-	MF421874
55dBI	South Georgia				-	MF421875
356dBI	South Georgia				-	MF421876
357dBI	South Georgia				-	MF421877
358dBI	South Georgia				-	MF421878
359dBI	South Georgia				-	MF421879
360dB	South Georgia				-	MF421880
361dBI	South Georgia				-	MF421881
362dBI	South Georgia				-	MF421882
363dBI	South Georgia				-	MF421883
364dBI	South Georgia				-	MF421884
365dBI	South Georgia				-	MF421885
Pachyptila salvini						
SP4MA/401sMA	Marion I.				KX092041	KX139069
Pachyptila belcheri						
NMNZ OR.030193	NZ wreck				OM212715	OM240595

The relationships between Antarctic prion haplotypes at the COI and cytochrome *b* loci are shown in the median-joining networks (Fig. 2A, B). The outgroups (thin-billed prion and the Salvin's prion) both joined the COI network by connecting to different haplotypes from the Auckland Islands. In the cytochrome *b* network both outgroups connected to the same haplotype that was sequenced in Antarctic prions from the Auckland Islands and one of the Campbell Island specimens.

For both COI and cytochrome *b*, no haplotypes were shared between the Auckland Islands and elsewhere, except for one of the two specimens from Campbell Island. However, the genetic differences were small, with only a single substitution separating the most closely-related haplotypes from the Auckland Islands and South Georgia, Kerguelen and Antarctica. There was no clustering by location for the remaining sequences (Kerguelen, Heard, South Georgia, South Sandwich and Antarctica).

The ML and BI phylogenies had similar topologies and the ML phylogeny is presented in Fig. 3 with the support values from both analyses. The deeper relationships within the phylogeny were largely unresolved. However, the sequences from Kerguelen, Heard, South Sandwich, Antarctica and one from Campbell Island formed a well-supported clade (1.00 PP/85 BS). Within the Auckland Islands two clades received support in the BI analysis (1.00 PP/61 BS and 0.95 PP/65 BS) and the second Campbell Island sequence was sister to one of these clades (1.00 PP/61 BS).

Measures of population differentiation between Antarctic prion colonies were moderate. $G_{ST}$was 0.287 ($P < 0.05$), whereas N_{ST} was 0.447 ($P < 0.001$). N_{ST} was significantly higher than G_{ST} ($P < 0.01$), which suggests a phylogeographic component to the structuring with haplotypes in close geographic proximity more likely to also have a close genetic relationship. The global F_{ST} was also moderate (0.321; $P < 0.0001$).

The SAMOVA (Suppl. material 2) indicated a lack of higher-level structuring in Antarctic prions. For all values of K, including $K = 2$, single populations were partitioned into exclusive groups rather than groups of populations. At $K = 2$ the Auckland Islands was split from the remaining populations.

Discussion

Genetic structuring in Antarctic prions

The mtDNA variation in Antarctic prions was not distributed randomly, as indicated by our G_{ST} N_{ST} and F_{ST} calculations. There was a significant phylogeographic component to this structuring, as revealed by the significantly higher N_{ST} than G_{ST}, indicating that population structuring is influenced by genealogical relationships. However, the SAMOVA indicated no higher-level structuring; instead the main division in the

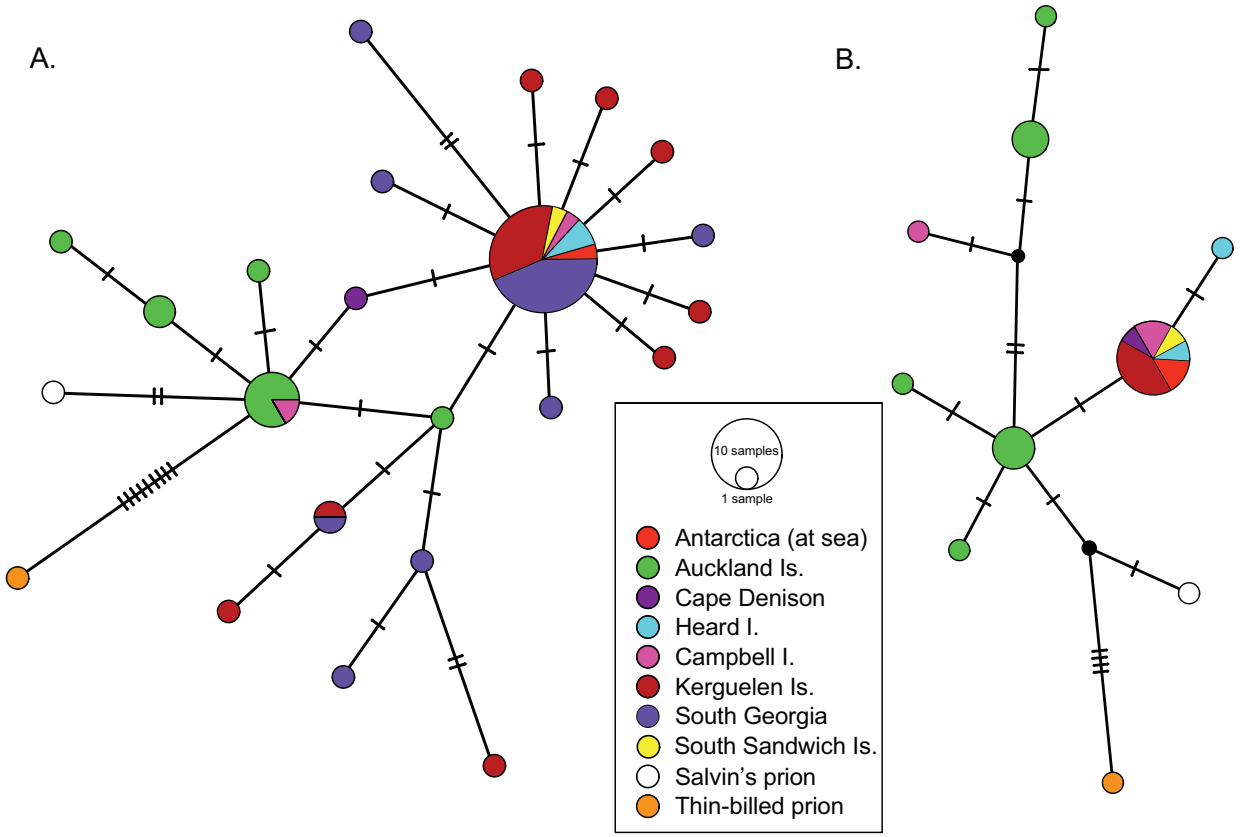

Figure 2. Median-joining networks of (**A**) cytochrome *b* and (**B**) COI for Antarctic prions. Sampling locations are colour-coded and the size of each circle is proportional to frequency. Mutational steps between haplotypes are shown as hatch marks.

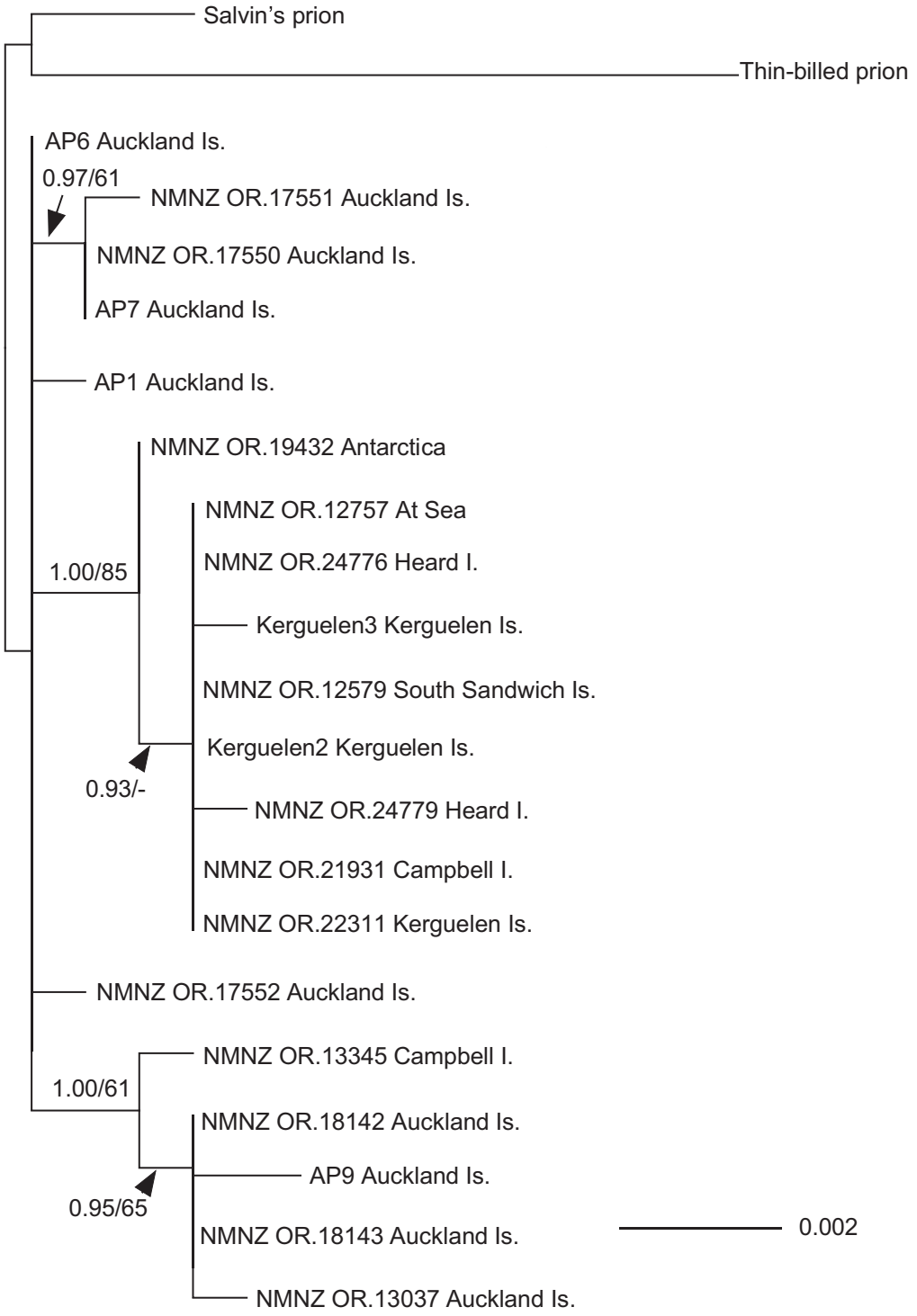

Figure 3. Maximum likelihood phylogeny of concatenated COI and cytochrome *b* sequences for Antarctic prions. Support values for nodes are as follows: Bayesian posterior probability (PP)/maximum likelihood bootstrap (BS). Only PP values over 0.9 and BS values over 60% are shown.

data was between Antarctic prions from the Auckland Islands and the remaining breeding populations. There was a lack of differentiation between the Indian and Atlantic populations, consistent with Quillfeldt et al. (2017) who found South Georgia (Atlantic Ocean) and Kerguelen birds (Indian Ocean) indistinguishable.

The two specimens from Campbell Island exhibited different haplotypes. One specimen (NMNZ OR.013345) shared a cytochrome *b* haplotype with Auckland Island Antarctic prions and had a unique COI haplotype most closely related to haplotypes detected from Auckland Island Antarctic prions. This specimen was caught at Beeman Camp and was most likely attracted by the lights. No detailed collection locality data is available for the other Campbell Island specimen (NMNZ OR.021931). It shared both its COI and cytochrome *b* haplotype with Indian Ocean birds. Antarctic prions have not been confirmed as breeding on Campbell Island (Jamieson et al. 2016; Checklist

Committee (OSNZ) (2022)) so it is likely that the two sampled birds originate from different breeding colonies.

Three specimens obtained at sea from near Antarctica (NMNZ OR.012753, NMNZ OR.012757; NMNZ OR.014446) also shared haplotypes found in Indian Ocean and South Atlantic Antarctic prions but the specimen from the now extinct breeding colony on Antarctica at Cape Denison (NMNZ OR.019432; Checklist Committee (OSNZ) 2022) had a unique cytochrome *b* haplotype but shared a COI haplotype with the Indian Ocean birds.

Taxonomy of Antarctic prions

Antarctic prions from the Auckland Islands have previously been recognised as a distinct taxon (*Pachyptila desolata alter* (Mathews)) based limited bill measurements that Mathews (1912) used to define a number of now synonymised subspecies. Although this population exhibited distinct mtDNA haplotypes, there is currently not enough evidence to reinstate this name, with detailed morphological analysis and nuclear DNA markers required prior to accepting taxonomic differentiation within this species. Nuclear microsatellite DNA data are available for some populations of Antarctic prion (Quillfeldt et al. 2017; Masello et al. 2021) but not from the Auckland Islands population. Furthermore, obtaining mtDNA sequence from Antarctic prions from Macquarie Island is crucial to determine their relationship to Auckland Islands birds (our attempts to sequence specimens from Macquarie Island failed; Suppl. material 1). Macquarie Island and the Auckland Islands are geographically close but studies of other seabirds, such as wandering albatross (*Diomedea exulans* Linnaeus, 1758; Alderman et al. 2005), have indicated that geographic proximity does not necessarily lead to close genetic relationships in mobile seabirds (see *Phylogeography* section).

Phylogeography of Antarctic prions

The distribution of mtDNA haplotypes in Antarctic prions is similar to that observed in several other seabird taxa from the Southern Ocean, including wandering albatross (*Diomedea exulans*; Alderman et al. 2005), white-chinned petrel (*Procellaria aequinoctialis* Linnaeus, 1758; Techow et al. 2009) and fairy prion (*Pachyptila turtur*; Shepherd et al. 2022). Such patterns of genetic differentiation have been suggested to correlate with the location of ice-free refugia providing suitable breeding habitat during the Last Glacial Maximum (LGM) (Munro and Burg 2017; Lombal et al. 2020). The general pattern is that more northerly islands were more likely to remain unglaciated (Munro and Burg 2017). The Auckland Islands, Crozet Islands and Macquarie Island were less impacted by glaciation than more southerly islands (Munro and Burg 2017) and may have acted as refugia for Antarctic prions from which other sites were subsequently colonised following the end of the LGM. In contrast, the level of glaciation on South Georgia, South Sandwich Islands, Kerguelen Islands and Heard Island (Munro and Burg 2017) means that the Antarctic prion populations on these islands likely have a more recent origin.

DNA for identifying the provenance of wrecked Antarctic prions

The different species of prion can be distinguished with mtDNA sequences (Masello et al. 2019; Masello et al. 2021; Shepherd et al. 2022), making this a useful technique for identifying the species of beach-wrecked prions. However, the level of haplotype sharing between populations that we detected in Antarctic prions suggests that these sequences will have limited use for identifying the population of origin of wrecked birds of this species. The only Antarctic prion breeding population with distinct haplotypes was from the Auckland Islands, with all other breeding populations that were sampled sharing haplotypes. Furthermore, no mtDNA sequences are available from Macquarie Island birds, which may share haplotypes with the geographically-close Auckland Islands, or the more distant Indian and Atlantic populations, which are at a similar latitude. Alternatively, Macquarie Island Antarctic prions may exhibit their own unique haplotypes. Obtaining sequences from this population is particularly pertinent because Harper (1980) hypothesised that most of the Antarctic prions that beach-wreck in New Zealand originate from Macquarie Island and the Auckland Islands, based on the dates that the birds fledge and the time of year they typically wreck.

Salvin's prions and DNA preservation in museum specimens

None of the six museum specimens of Salvin's prion from the Crozet Islands produced DNA sequences. Interestingly, six specimens of fairy prion collected by the same collectors from the same location during a similar time period (1969–1974) also failed to amplify (Shepherd et al. 2022). This suggests that the preservation method or treatment of the specimens collected during these expeditions to the Crozet Islands is not conducive to DNA preservation and that alternative specimens should be prioritised for future genetic studies. This result reinforces the importance of reporting negative results in ancient DNA studies because avoiding specimens that are likely to fail can save time and money, as well as limit destructive sampling to specimens.

Author Contributions

LS - Writing - Investigation, Formal analysis, Writing - Original draft. AT - Conceptualisation, Investigation, Writing - Review and Editing. CM - Conceptualisation, Investigation, Writing - Review and Editing

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Supplementary material 1

Prion specimens that failed to yield any amplifiable DNA

Authors: Lara D. Shepherd, Alan J. D. Tennyson, Colin M. Miskelly
Data type: docx
Explanation note: Prion specimens sampled from Te Papa's collection that failed to yield any amplifiable DNA.

Link: https://doi.org/10.3897/tuhinga.35.115090.suppl1

Supplementary material 2

Fixation indices for Antarctic prion colonies

Authors: Lara D. Shepherd, Alan J. D. Tennyson, Colin M. Miskelly
Data type: docx
Explanation note: Fixation indices (FCT) and population groupings from a spatial analyses of molecular variance (SAMOVA) of breeding colonies of Antarctic prions.

Link: https://doi.org/10.3897/tuhinga.35.115090.suppl2