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Evolutionary factors affecting the cross-species utility of newly developed microsatellite markers in seabirds

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Abstract

Microsatellite loci are ideal for testing hypotheses relating to genetic segregation at fine spatio-temporal scales. They are also conserved among closely related species, making them potentially useful for clarifying interspecific relationships between recently diverged taxa. However, mutations at primer binding sites may lead to increased nonamplification, or disruptions that may result in decreased polymorphism in nontarget species. Furthermore, high mutation rates and constraints on allele size may also with evolutionary time, promote an increase in convergently evolved allele size classes, biasing measures of interspecific genetic differentiation. Here, we used next-generation sequencing to develop microsatellite markers from a shotgun genome sequence of the sub-Antarctic seabird, the thin-billed prion (*Pachyptila belcheri*), that we tested for cross-species amplification in other *Pachyptila* and related sub-Antarctic species. We found that heterozygosity decreased and the proportion of nonamplifying loci increased with phylogenetic distance from the target species. Surprisingly, we found that species trees estimated from interspecific F_{ST} provided better approximations of mtDNA relationships among the studied species than those estimated using D_C, even though F_{ST} was more affected by null alleles. We observed a significantly nonlinear second order polynomial relationship between microsatellite and mtDNA distances. We propose that the loss of linearity with increasing mtDNA distance stems from an increasing proportion of homoplastic allele size classes that are identical in state, but not identical by descent. Therefore, despite high cross-species amplification success and high polymorphism among the closely related *Pachyptila* species, we caution against the use of microsatellites in phylogenetic inference among distantly related taxa.

Keywords: cross-species transferability, genetic diversity, microsatellite, null alleles, *Pachyptila*, Procellariiformes

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Introduction

Two-thirds of our planet is covered by sea, and albatrosses, petrels and storm-petrels (Aves, Procellariiformes) are, *par excellence*, the seabirds of the open ocean, only coming ashore to breed, usually on remote islands (Brooke 2004). This highly mobile group of seabirds could theoretically maintain high levels of gene flow, but strong philopatry to breeding islands observed in some species (Ovenden *et al.* 1991; Steeves *et al.* 2005; Bicknell *et al.* 2012) may lead to pronounced genetic differentiation between populations. Our understanding of gene flow and genetic structure in petrels has improved considerably in recent years (Smith *et al.* 2007; Lawrence *et al.* 2008; Gangloff *et al.* 2012; Wiley *et al.* 2012; Kerr & Dove 2013), but remains poor for the diverse and often widespread species that breed on sub-Antarctic islands.

Petrels (Procellariidae) of the genus *Pachyptila*, the prions, are ideal monitors of ocean productivity as they feed mainly on zooplankton, which responds rapidly to changing environmental conditions (Hunt *et al.* 1992; Bocher *et al.* 2001; Cherel *et al.* 2002; Quillfeldt *et al.* 2007, 2008). Prions are highly mobile and have a wide distribution in sub-Antarctic waters (Onley & Scofield 2007). Detailed studies on diet, breeding biology and behavioural ecology have been published for several prion species (Strange 1980; Bretagnolle *et al.* 1990; Liddle 1994; Ridoux 1994; Reid *et al.* 1999; Cherel *et al.* 2002; Quillfeldt *et al.* 2003, 2007, 2008; Navarro *et al.* 2013). Much less was known about distributions at sea, particularly during the nonbreeding season. However, recent stable isotope and tracking studies indicate considerable ecological segregation among populations breeding in the Atlantic and Indian sectors of the Southern Ocean (Cherel *et al.* 2002, 2006; Quillfeldt *et al.* 2010, 2013). This spatial and temporal segregation could potentially lead to population differentiation, with consequences for taxonomy and conservation status.

Prions are generally clustered into 2 groups, the species with, or without, palatal lamellae, and hence filtering apparatus (Prince & Morgan 1987). The former (the so-called 'whale birds') includes the Antarctic prion *Pachyptila desolata*, Salvin's prion *P. salvini* and broad-billed prion *P. vittata*, and the latter, the thin-billed prion *P. belcheri*, fairy prion *P. turtur* and fulmar prion *P. crassirostris*. As yet, there are no phylogeographic studies of any prion species, and only scattered genetic information exists (e.g. Ovenden *et al.* 1991). Nor is there agreement regarding the number of prion species or their genetic relationships (see Brooke 2004; Penhallurick & Wink 2004; Rheindt & Austin 2005; Onley & Scofield 2007). Using enzyme electrophoresis, Barrowclough *et al.* (1981) concluded that Antarctic prions were closely related to blue petrels *Halobaena caerulea*, and Viot *et al.* (1993) that Antarctic prions, thin-billed prions and Salvin's prions were very closely related. The low variation at the mitochondrial cytochrome *b* gene also suggests that Antarctic prions, thin-billed prions and blue petrels are closely related species (Nunn & Stanley 1998). Based on the same locus, Penhallurick & Wink (2004) invoked the multidimensional biological species concept to suggest that all prions represent just two species. However, this last study was heavily criticized (Rheindt & Austin 2005). Consequently, taxonomic authorities and field guides still follow the scheme outlined by Bretagnolle *et al.* (1990) which concluded from the combination of morphometrics, breeding biology, genetics and calls that Antarctic, Salvin's, thin-billed and fairy prions were distinct but closely related species. The recent description of distinct thin- and broad-billed morphs, within broad-billed prions that also show strong differences in breeding phenology at Gough Island, raises further questions regarding intra- and interspecific relationships of this group (Ryan *et al.* 2014). More genetic data from the many populations of prions that breed in the sub-Antarctic could potentially shed important light on these open questions.

Microsatellite loci are powerful tools in population and evolutionary genetics that could provide the resolution for detailed analyses of several aspects of prion biology. Given their high mutation rate, they are ideal for testing hypotheses relating to fine-scale spatiotemporal segregation and for the estimation of demographic parameters such as gene flow, effective population size and genetic variability (Bruford & Wayne 1993; Sunnucks 2000). They are also conserved among closely related species (Moore *et al.* 1991) making them potentially useful for clarifying interspecific genetic relationships between recently diverged taxa (e.g. Dawson *et al.* 2010), although their high mutation rate means that loci developed for one species may not always be useful for others. This is because mutations at primer binding sites may lead to increased nonamplification (Moodley *et al.* 2006), or disruptions within tandemly repeated elements may lead to a reduced level of observed polymorphism in the nontarget species (Garza *et al.* 1995; Primmer *et al.* 2005). The taxa of interest must therefore be closely related to maximize utility and offset the costs of microsatellite development. Hence, the testing of newly developed microsatellite loci for cross-species utility has become fairly standard practice (Li *et al.* 2003; Bried *et al.* 2008; Dawson *et al.* 2010; Jan *et al.* 2012; Huang *et al.* 2014); however, few surveys have reported statistical trends in their multispecies data sets.

Here, we used next-generation sequencing technology to develop a set of 26 polymorphic microsatellite markers from a shotgun genome sequence of the

thin-billed prion in order to test for genetic structure among the different populations of this species and to provide an accurate estimation of demographic parameters. We also tested these microsatellite loci for cross-species amplification in other prions, the closely related blue petrel (all Procellariiformes, Procellariidae) and the more distantly related Wilson's storm-petrel *Oceanites oceanicus* (Procellariiformes, Hydrobatidae). While prion species appear to be very closely related, we predict, nevertheless, that average observed microsatellite genetic diversity will decrease, whereas the number of nonamplifying alleles will increase, with increasing phylogenetic distance from the species of origin (thin-billed prion). As any increase in null allele frequency could bias the estimation of essential population parameters in phylogeographic studies (e.g. Astanei *et al.* 2005; Wulff *et al.* 2012; McCormack *et al.* 2013) and perhaps even alter phylogenetic relationships, we corrected our raw data for null alleles using methods developed by Chapuis & Estoup (2007). This allowed for a comparison of the effect of null alleles on levels of interspecific differentiation.

Lastly, high microsatellite mutation rates coupled with mutational limits on allele sizes (Ostrander *et al.* 1993; Bowcock *et al.* 1994) increase the probability of convergent evolution of allele size classes. Given this highly homoplastic scenario, population parameters and evolutionary hypotheses inferred under the assumption that alleles of the same size share a most recent common ancestor (i.e. are identical by descent) could be biased, even in comparisons between sister taxa (Paetkau *et al.* 1997). We expect, therefore, that microsatellite genetic distance between species will be biased to lower values as the evolutionary time separating species increases.

Materials and methods

Molecular methods

Between 2010 and 2012, samples (all from adults) from 77 thin-billed prions, 79 Antarctic prions, 118 broad-billed prions, 18 Salvin's prions, 35 fairy prions, 99 blue petrels and 6 Wilson's storm-petrels were obtained in breeding colonies located on sub-Antarctic island groups (Noir, Diego Ramirez, Falkland/Malvinas, South Georgia, Tristan da Cunha, Gough, Marion, Kerguelen, Macquarie, and Chatham). Genomic DNA was obtained from different sample types: blood in ethanol (Gough and Diego Ramirez), blood in Queens's lysis buffer (Kerguelen and Falkland/Malvinas), blood on FTA classic cards (Whatman International Ltd., Maidstone, UK; South Georgia and Chatham), muscle in ethanol (Macquarie, Gough, Tristan da Cunha and Noir) and feather quills (Marion, Tristan da Cunha and Falkland/Malvinas). DNA was extracted from blood (n = 313), feather quills (n = 101) or muscle tissue (n = 51) using the Qiagen DNeasy® Tissue kit (Qiagen, Germany). DNA quantity and quality was determined by UV spectrophotometry using a NanoDrop 1000 Spectrophotometer, and all samples were standardized to a final concentration of 10 ng/μL.

Microsatellite-containing genomic sequences were isolated by ecogenics GmbH (Switzerland) from a 1:1 pool of two thin-billed prion individuals from Mayes Island in the Kerguelen Archipelago using a modified high-throughput genomic sequencing approach (Abdelkrim *et al.* 2009). Genomic DNA was nebulized to 300–800 bp and ligated into an ssDNA library. These size-selected fragments were then enriched for tandemly repeated element content using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. This enriched shotgun library was then sequenced on a Roche 454 next-generation platform using the GS-FLX titanium reagents. Resulting sequence reads were passed through quality filters and scanned for microsatellite repeats, from the conserved flanking regions of which primer pairs were designed using Primer 3 (Untergasser *et al.* 2012).

After initial testing for amplification and polymorphism, microsatellite loci were visualised in the seven species of petrel through florescent labelling of universal M13 primers, as described in Schuelke (2000). PCR was then performed in a final volume of 10 μL including 1 × Qiagen PCR buffer, 2 mM dNTPs, 2 μM M13-tailed forward primer, 2 μM reverse primer, 2 μM of universal M13 primer 5′-end-labelled, 0.5 U Hotstar Taq (Qiagen) and 10 ng template. The PCR program comprised an initial denaturation step of 95°C for 15 min, the cycling parameters were 30 cycles at 95°C for 30 s, an annealing temperature of 56°C for 45 s, 72°C for 45 s, 8 cycles of 95°C for 30 s, 53°C for 45 s, 72°C for 45 s and a final extension step of 72°C for 30 min. PCR products were visualized on a 1.5% agarose gel to confirm successful amplification and to examine negative controls. Products were run on a AB 3130*xl* genetic analyser along with a ROX size-standard. We repeated all PCRs for individuals that failed to amplify at >4 loci and for those loci with >10% missing data.

We used an 880-bp fragment of the mitochondrial cytochrome *b* gene to estimate the phylogenetic relationships among the seven taxa in our data set, as two of our hypotheses required an independent estimate of interspecific phylogenetic distance. While we do not believe that mtDNA distances are unbiased, we do feel that its slower mutation rate, relative to that of microsatellites, would ensure its linearity among the seven species on our comparison. Furthermore, cytochrome *b* has been

used previously to successfully infer relatedness among a much broader sample of Procellariiformes (Nunn & Stanley 1998), and previous morphological and behavioural analyses (Bretagnolle *et al.* 1990) did not specifically quantify interspecific distances among the species in our study. Generic avian cytochrome *b* primers (e.g. Patterson *et al.* 2011) were problematic for some samples. Therefore, we designed specific primers (CytB_Pri_F: 5′-CTAGCTATACACTACACCGC-3′ and CytB_Pri_R: 5′-CTAGTTGGCCGATGATGATG-3′) for our study group from an alignment of those samples that we successfully sequenced. PCRs were conducted in 20 μL reaction volumes containing 100 ng DNA template, 10 mM of each primer, 10 mM dNTPs (Roth, Karlsruhe), 2 mM MgCl, 5 U *Thermus aquaticus* polymerase (BioLabs Taq DNA polymerase) in a 1x PCR reaction buffer. Thermocycling included initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s and extension at 72°C for 1 min, followed by a final extension step of 5 min at 72°C. Products were purified of excess primers and dNTPs using exonuclease-shrimp alkaline phosphatase (Fermentas Life Sciences following the manufacturer's specifications). PCR products were then sequenced in both directions using Big Dye chemistry (Applied Biosystems) and run on an AB 3130*xl* genetic analyser (Applied Biosystems). Resulting sequences were assembled and aligned in CLC Main Workbench® 6.9.2.

Data analyses

Genotypes were assigned with GENEMARKER 1.85 (SoftGenetics LLC, State College, PA, USA). 20% of the samples were rescored by a separate individual, with a resulting error rate of <5%. The probability of deviation from Hardy–Weinberg equilibrium (HWE) and nonrandom association of loci was calculated for each locus/species combination using GENEPOP (Raymond & Rousset 1995; Tables 1 and 2). Measures of genetic diversity [number of alleles per locus (*A*), observed heterozygosity (H_O) and expected heterozygosity (H_E)] were estimated in CERVUS 3.0.3 (Kalinowski *et al.* 2007) and MSA 4.05 (Dieringer & Schlötterer 2003). The inbreeding coefficient (F_{IS}) and its significance were estimated with GENEPOP (Raymond & Rousset 1995). Null allele frequencies (F null) per locus and species were obtained using FREENA (Chapuis & Estoup 2007).

Phylogenetic distances between species at the cytochrome *b* gene were calculated using the maximum likelihood in MEGA (Tamura *et al.* 2013). We reconstructed the mitochondrial species phylogeny by firstly determining the most suitable substitution model for the cytochrome *b* sequence data using the Akaike information criteria (AIC) in JMODELTEST 2 (Darriba *et al.* 2012), then set the model parameters to the general time reversible (GTR) model with gamma substitution rate heterogeneity estimated from the data using four rate categories.

We examined the cross-species utility of our isolated microsatellite loci by plotting genetic diversity (H_O) and the proportion of missing data (nonamplifying loci after 3x repeat PCRs, with standardized DNA quantity and quality) in each species against phylogenetic (mtDNA, cytochrome *b*) p-distances, calculated in MEGA (Table S1, Supporting Information). We also performed this regression separately for three different phylogenetic groupings: Group 1: all seven species; Group 2: *Halobaena* and *Pachyptila* only; Group 3: *Pachyptila* species only. For a more detailed analysis of these relationships, we used a generalized linear model (GLM, implemented in R, R Development Core Team 2014) to test the effect of phylogenetic distance (as a covariate) and locus (as a factor) on both observed heterozygosity and the proportion of missing data in each of the three species groupings above. A GLM could not be used to test for the effect of phylogenetic distance and locus on null allele frequencies due to a large amount of missing data in Wilson's storm-petrels.

The frequency of null alleles was calculated in our data set using FREENA (Chapuis & Estoup 2007). This method estimates the frequency of null alleles from data sets simulated to contain and not contain null alleles. Then, it uses the expectation–maximization (EM) algorithm of Dempster *et al.* (1977) to adjust homozygote allele frequencies based on true and false homozygote counts, resulting in the estimation of the null allele frequency. Population differentiation indices can then be calculated including null alleles (INA) and also only on the visible allele sizes, thus, excluding null alleles (ENA). To determine the effect of null alleles on cross-species comparisons, we calculated pairwise F_{ST} (Wright 1943) and D_C (genetic distance of Cavalli-Sforza & Edwards 1967) between species using INA and ENA data sets. We reconstructed UPGMA species trees from these triangular F_{ST} and D_C matrices, using MEGA 6.06 (Tamura *et al.* 2013).

We investigated the effect of high mutation rates and constraints on allele size on microsatellite genetic distance by observing the change in the slope of pairwise Mantel regressions performed on the same three phylogenetic data groupings used in Fig. 1. As interspecific microsatellite distance, we used INA and ENA triangular matrices of both F_{ST} and D_C and checked their linearity against the matrix of pairwise maximum-likelihood cytochrome *b* distances calculated previously (Table S1, Supporting Information). All Mantel regressions were calculated in GenAlEx 6.5 (Peakall & Smouse 2012). To formally test the hypothesis that F_{ST} and D_C microsatellite distances were nonlinearly related to mtDNA

Table 1 Locus characteristics and genetic variation at 25 newly isolated microsatellites in the target species, the thin-billed prion *Pachyptila belcheri*

Locus code and name	Motif†	Primer sequences 5′–3′	Size (bp), range	*N*	*A*	H_O	H_E	F_{IS}	*F* (null)
A Pacbel_00386	$(AC)_{14}$	F: GCATGTCTACAAACAAGCACG R: TCACTGGAAACCAGAGTAGGC	120–142	72	11	0.764	0.811	0.058 ns	0.014
D Pacbel_02653	$(AC)_{12}$	F: AGCCATAGCTCAGTACAAGTTC R: TGCAGGCATTTCAGGTTTGG	132–170	77	12	0.325	0.639	0.494***	0.203
E Pacbel_03731	$(AC)_{14}$	F: TAGTGGACTGGTCACAGCAC R: TAGCAGCTGGAGAGCATCAG	122–268	74	48	0.392	0.972	0.599 ns	0.293
F Pacbel_04240	$(AC)_{14}$	F: CCCATTGTCTGGGCAAAGC R: GCATTCTTTGTGGGGATGGG	166–254	47	19	0.511	0.815	0.376***	0.164
G Pacbel_04355	$(AC)_{17}$	F: TACCAGGGACAATCTGGGTG R: GGAAAAATACAGGAGATGCTTGAG	158–212	69	20	0.579	0.931	0.379***	0.181
H Pacbel_04991	$(GT)_{14}$	F: TGTCCATGAGGTCTGGAAGC R: GGTGGAATACAGGGATGCAC	86–106	74	11	0.757	0.877	0.138 ns	0.064
Z Pacbel_07265	$(GT)_{15}$	F: CGTCACTTTAATAGCGCTGGC R: ACCCTGATTTTCCCAGTCCG	148–182	74	15	0.730	0.817	0.108 ns	0.040
I Pacbel_08509	$(TG)_{12}$	F: TCTGGTTTCACAAATACCTACTGC R: CCTAGTTTCGACACAAAGGATGG	156–172	73	9	0.849	0.808	−0.052 ns	0.000
Ñ Pacbel_08867	$(GA)_{13}$	F: TTTGGTCAATTTTCCCTCGC R: ACAGAAAACCAATGTTGTTAATAGG	138–154	74	8	0.689	0.690	0.002 ns	0.000
J Pacbel_08988	$(CT)_{12}$	F: CTGATCGGTTGTGCTCTGTG R: GCGGAAAGATCCTAACAAGCC	184–202	74	10	0.757	0.692	−0.094 ns	0.000
K Pacbel_09021	$(GT)_{12}$	F: ATCTGCGCATGCAGTGATAG R: CACAGCTAGCAGCATTGACC	208–254	76	17	0.829	0.901	0.080 ns	0.034
L Pacbel_09528	$(AC)_{12}$	F: AACTGTTTGCTCCACACCAC R: ATGGCTTGGAAGTCTCCCTG	146–170	75	9	0.600	0.809	0.260**	0.118
M Pacbel_09957	$(GT)_{13}$	F: GCTTTATTTAAGAGCAACAAAAACTTC R: ACAAAGCAAACCTAATCATTCCC	92–110	73	10	0.822	0.830	0.010 ns	0.026
N Pacbel_10033	$(TG)_{12}$	F: CAACGCGCTTTTGGTTTTGC R: GGCCACTCACCACAATACAAG	102–120	77	9	0.688	0.811	0.152 ns	0.058
O Pacbel_10895	$(AC/AT)_8$	F: AGCTTTCTGTCTGGTAGCAC R: TGCTCCTGCCTAAGCTACG	158–196	75	19	0.720	0.890	0.192**	0.092
S Pacbel_12344	$(AC)_{12}$	F: CCAAACCCTGCCCCGATG R: GCCGTGCAGACGTGAATAG	92–116	74	11	0.419	0.805	0.482***	0.211
T Pacbel_15293	$(TG)_{13}$	F: CAAGCTGGTTTTCAATGTGCC R: CTGAAGCATTAGCACCTGCC	254–266	76	7	0.697	0.728	0.042 ns	0.020
Q Pacbel_15327	$(CA)_{13}$	F: TTCTTGTAGCAGTAGGAGACC R: ACCTCATGTGTAAAACCTGCC	146–162	75	8	0.627	0.674	0.071*	0.038
R Pacbel_16671	$(GT)_{13}$	F: TGAAGGTATGCCTGTCCTCC R: TCGCTCCCACACACATGC	126–134	75	5	0.640	0.598	−0.071 ns	0.000
V Pacbel_16989	$(CA)_{12}$	F: TGCTTTTGGACAATGTGGAGG R: TCTGGTACACTTCTCATTTGGAC	100–120	75	10	0.653	0.670	0.025 ns	0.026
W Pacbel_17529	$(AG)_{14}$	F: TGCAAGGTCTTGTGATGAAGC R: AATGCAATTTGTCTGCGGGG	142–164	76	12	0.842	0.821	−0.026 ns	0.000
X Pacbel_17944	$(TG)_{13}$	F: TACAACCGTTCTCCCTGTGG R: GGAGAAGCAGGCAGCAATAC	228–254	75	12	0.880	0.828	−0.063 ns	0.000
U Pacbel_17986	$(GT)_{12}$	F: ATAACCCAGTGTGATGGTGC R: CACAGCTGCTTAGTGCACAG	204–212	75	5	0.507	0.493	−0.028 ns	0.030
Y Pacbel_19907	$(AG)_{12}$	F: TTTCTCCTTAGCTCGGCAGG R: CCATACTTGGTGGCAGTGTG	166–184	74	8	0.622	0.642	0.033 ns	0.000
P Pacbel_20784	$(GT)_{12}$	F: GCAAACGCAAGGCGTACAAG R: ATGGTAGCAAACCTCCTGCC	122–158	76	12	0.500	0.833	0.401***	0.177

†Number of repeats indicated in the subscript. Primer annealing temperature, *Ta* = 56°C. *N*, number of individuals with reliable amplification; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. The probabilities of deviation from Hardy–Weinberg equilibrium (HWE) are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant). *F* (null), null allele frequency estimate.

Table 2 Cross-species genetic variation of microsatellites isolated from 25 thin-billed prions *Pachyptila belcheri* in Antarctic prions *P. desolata* (des), broad-billed prions *P. vittata* (vit), fairy prions *P. turtur* (tur), Salvin's prions *P. salvini* (sal), blue petrels *Halobaena caerulea* (car) and Wilson's storm-petrels *Oceanites oceanicus* (oce)

Locus code	Sp	*N*	*A*	H_O	H_E	F_{IS}	*F* (null)	Sp	*N*	*A*	H_O	H_E	F_{IS}	*F* (null)	Sp	*N*	*A*	H_O	H_E	F_{IS}	F (null)
A	car	97	13	0.835	0.897	0.070*	0.017	des	79	13	0.810	0.862	0.061 ^{ns}	0.011	tur	33	2	0.424	0.403	−0.054 ^{ns}	0.000
	oce	1	2	1	1	−1.000^{nc}	0.000	vit	117	11	0.675	0.828	0.185***	0.074	sal	18	10	0.778	0.856	0.093 ^{ns}	0.033
D	car	95	4	0.042	0.082	0.489***	0.088	des	77	11	0.468	0.754	0.381***	0.167	tur	33	5	0.272	0.525	0.484***	0.167
	oce	6	2	0.167	0.167	0.000^{nc}	0.000	vit	115	9	0.313	0.642	0.513***	0.200	sal	17	8	0.471	0.663	0.297*	0.132
E	car	95	13	0.779	0.795	0.020 ^{ns}	0.022	des	75	51	0.653	0.971	0.329**	0.157	tur	1	1	0.000	0.000	–	0.001
	oce	6	1	0.000	0.000	–	0.001	vit	111	39	0.649	0.951	0.319***	0.157	sal	17	22	0.588	0.973	0.403***	0.184
F	car	83	13	0.313	0.782	0.601***	0.265	des	48	17	0.438	0.927	0.531***	0.252	tur	17	9	0.235	0.818	0.719***	0.311
	oce	2	3	0.500	0.833	0.500 ^{ns}	0.001	vit	65	20	0.477	0.872	0.455***	0.204	sal	13	9	0.615	0.840	0.276*	0.104
G	car	93	10	0.731	0.829	0.118*	0.056	des	76	20	0.684	0.930	0.265***	0.127	tur	14	9	0.429	0.820	0.487***	0.200
	oce	5	6	0.400	0.844	0.556**	0.189	vit	116	23	0.750	0.934	0.198***	0.093	sal	13	9	0.385	0.806	0.533***	0.203
H	car	92	8	0.152	0.259	0.413***	0.116	des	78	10	0.795	0.893	0.111*	0.050	tur	33	4	0.333	0.526	0.370*	0.122
	oce	0	0	–	–	–	–	vit	113	11	0.655	0.826	0.208***	0.094	sal	15	9	0.800	0.880	0.094 ^{ns}	0.043
Z	car	85	28	0.635	0.947	0.331***	0.160	des	72	15	0.694	0.785	0.116 ^{ns}	0.057	tur	21	6	0.619	0.560	−0.109 ^{ns}	0.000
	oce	5	5	0.400	0.844	0.556*	0.203	vit	103	14	0.709	0.777	0.088**	0.033	sal	16	7	0.625	0.756	0.178 ^{ns}	0.048
I	car	95	13	0.832	0.846	0.017 ^{ns}	0.015	des	75	11	0.720	0.785	0.083*	0.027	tur	16	5	0.438	0.688	0.371*	0.156
	oce	6	2	0.167	0.167	0.000^{nc}	0.000	vit	111	12	0.730	0.756	0.034**	0.032	sal	11	6	0.636	0.805	0.218 ^{ns}	0.073
Ñ	car	82	10	0.841	0.795	−0.059 ^{ns}	0.000	des	78	9	0.590	0.664	0.113 ^{ns}	0.041	tur	26	6	0.577	0.728	0.211**	0.060
	oce	6	2	0.500	0.409	−0.250 ^{ns}	0.000	vit	106	5	0.623	0.630	0.012 ^{ns}	0.014	sal	17	6	0.824	0.745	−0.109 ^{ns}	0.000
J	car	89	12	0.348	0.859	0.596***	0.272	des	77	9	0.792	0.697	−0.138 ^{ns}	0.000	tur	33	2	0.030	0.088	0.660*	0.105
	oce	6	2	0.500	0.409	−0.250 ^{ns}	0.000	vit	113	6	0.575	0.635	0.094**	0.040	sal	11	4	0.364	0.688	0.484 ^{ns}	0.190
K	car	95	22	0.821	0.913	0.101*	0.049	des	75	22	0.907	0.893	−0.015 ^{ns}	0.000	tur	10	6	0.600	0.832	0.290 ^{ns}	0.133
	oce	6	2	0.167	0.167	0.000^{nc}	0.000	vit	112	18	0.848	0.911	0.069**	0.019	sal	17	12	0.882	0.895	0.014 ^{ns}	0.000
L	car	95	9	0.589	0.675	0.127*	0.061	des	72	14	0.653	0.814	0.199**	0.079	tur	32	7	0.375	0.609	0.388***	0.151
	oce	1	1	0.000	0.000	–	0.001	vit	114	13	0.500	0.640	0.219***	0.079	sal	11	5	0.636	0.775	0.186 ^{ns}	0.050
M	car	85	2	0.059	0.057	−0.024 ^{ns}	0.000	des	78	9	0.679	0.826	0.179***	0.062	tur	29	5	0.655	0.662	0.011 ^{ns}	0.031
	oce	4	4	0.250	0.821	0.727*	0.278	vit	116	7	0.431	0.674	0.362***	0.152	sal	15	7	0.533	0.766	0.311 ^{ns}	0.098
N	car	95	9	0.568	0.842	0.326***	0.151	des	78	9	0.679	0.825	0.178*	0.075	tur	35	5	0.429	0.737	0.422***	0.170
	oce	5	4	0.600	0.644	0.077 ^{ns}	0.000	vit	117	10	0.752	0.802	0.063 ^{ns}	0.012	sal	14	6	0.571	0.815	0.307 ^{ns}	0.119
O	car	92	36	0.793	0.946	0.162**	0.068	des	78	17	0.487	0.866	0.439***	0.202	tur	10	4	0.100	0.363	0.735**	0.192
	oce	4	5	0.250	0.893	0.750*	0.300	vit	112	21	0.295	0.868	0.662***	0.306	sal	16	11	0.438	0.855	0.496***	0.209
S	car	90	7	0.367	0.729	0.499***	0.207	des	65	11	0.662	0.838	0.212*	0.088	tur	19	5	0.421	0.616	0.322*	0.149
	oce	5	1	0.000	0.000	–	0.001	vit	104	12	0.587	0.839	0.302***	0.133	sal	14	8	0.714	0.796	0.107 ^{ns}	0.005
T	car	96	7	0.708	0.730	0.030 ^{ns}	0.020	des	79	9	0.759	0.774	0.019 ^{ns}	0.005	tur	13	3	0.538	0.495	−0.091 ^{ns}	0.000
	oce	6	1	0.000	0.000	–	0.001	vit	114	6	0.640	0.676	0.053 ^{ns}	0.012	sal	16	6	0.750	0.776	0.035 ^{ns}	0.000
Q	car	89	14	0.798	0.877	0.091 ^{ns}	0.040	des	76	12	0.711	0.756	0.061 ^{ns}	0.000	tur	22	5	0.545	0.636	0.146 ^{ns}	0.053
	oce	1	1	0.000	0.000	–	0.001	vit	113	10	0.726	0.737	0.015 ^{ns}	0.005	sal	17	8	0.647	0.775	0.170 ^{ns}	0.042
R	car	96	5	0.219	0.240	0.091 ^{ns}	0.018	des	78	9	0.372	0.394	0.056 ^{ns}	0.004	tur	21	2	0.095	0.093	−0.026 ^{ns}	0.000
	oce	6	1	0.000	0.000	–	0.001	vit	99	6	0.485	0.514	0.057**	0.046	sal	16	6	0.313	0.512	0.398**	0.126
V	car	95	3	0.158	0.148	−0.070 ^{ns}	0.000	des	75	12	0.720	0.720	0.000 ^{ns}	0.000	tur	33	10	0.636	0.734	0.135 ^{ns}	0.053

Table 2 (Continued)

Locus code	Sp	*N*	*A*	H_O	H_E	F_{IS}	*F* (null)	Sp	*N*	*A*	H_O	H_E	F_{IS}	*F* (null)	Sp	*N*	*A*	H_O	H_E	F_{IS}	F (null)
	oce	6	1	0.000	0.000	–	0.001	vit	109	10	0.780	0.747	−0.045 ns	0.000	sal	14	7	0.643	0.794	0.196 ns	0.087
W	car	89	14	0.888	0.852	−0.042 ns	0.000	des	78	12	0.795	0.827	0.040 ns	0.026	tur	30	10	0.733	0.879	0.168***	0.082
	oce	6	3	0.500	0.439	−0.154 ns	0.000	vit	117	11	0.795	0.850	0.065*	0.026	sal	18	8	1.000	0.835	−0.205 ns	0.000
X	car	94	10	0.617	0.746	0.173***	0.082	des	79	13	0.810	0.866	0.064 ns	0.027	tur	33	8	0.394	0.505	0.222***	0.103
	oce	6	4	0.500	0.455	−0.111 ns	0.000	vit	112	12	0.705	0.804	0.123*	0.050	sal	16	8	0.938	0.857	−0.098 ns	0.000
U	car	97	4	0.082	0.080	−0.025 ns	0.000	des	77	6	0.571	0.500	−0.143 ns	0.000	tur	33	3	0.182	0.224	0.190 ns	0.063
	oce	6	1	0.000	0.000	–	0.001	vit	117	4	0.419	0.469	0.107 ns	0.021	sal	16	5	0.625	0.558	−0.124 ns	0.000
Y	car	94	5	0.511	0.528	0.033 ns	0.006	des	72	5	0.514	0.520	0.013 ns	0.000	tur	17	3	0.176	0.266	0.343 ns	0.093
	oce	0	0	–	–	–	–	vit	104	7	0.692	0.650	−0.066 ns	0.000	sal	11	3	0.636	0.636	0.000 ns	0.040
P	car	90	10	0.244	0.311	0.214**	0.081	des	75	15	0.387	0.854	0.549***	0.250	tur	25	10	0.160	0.839	0.813***	0.364
	oce	6	3	0.500	0.439	−0.154 ns	0.000	vit	117	14	0.504	0.765	0.341***	0.152	sal	14	8	0.429	0.865	0.514**	0.228

N, number of individuals with reliable amplification; *A*, number of alleles. H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient. The probabilities of deviation from Hardy–Weinberg equilibrium (HWE) are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns, not significant; nc, not calculated). *F* (null), null allele frequency estimate.

distance, we fitted the data (INA and ENA for all species) with both linear and polynomial functions and performed model testing within a GLM framework in R using the Akaike information criterion (AIC).

Results

Shotgun 454 sequencing of two pooled thin-billed prion genomes resulted in 22 220 reads after quality filtering, with an average read-length of 177 bp (total of 3.9 Mb). Of these, 517 (2.3%) contained microsatellite repeat elements with tetra- or trinucleotides of at least six repeat units, or dinucleotides of at least 10 repeat units. Suitable primer design was possible in 166 reads. We tested 36 of these primer pairs for cross-species amplification and polymorphism among four other unrelated Mayes Island *P. belcheri* and three individuals from the closely related *P. desolata* from Verte Island, also in the Kerguelen Archipelago. Twenty-six loci were identified as polymorphic in the target species, showing clear amplification profiles and reliable amplification in both species tested. We further tested the reliability of amplification and genotypic disequilibrium in a larger set of 77 thin-billed prions from across the breeding range (Kerguelen, Falkland/Malvinas, and Isla Noir in southern Chile). Among populations of the target species, the number of alleles (*A*) per locus ranged from 5 to 48, the observed heterozygosity (H_O) from 0.325 to 0.880 and the expected heterozygosity (H_E) from 0.493 to 0.972 (Table 1). Cross-species amplification was successful for most primer pairs in Antarctic prions, broad-billed prions, fairy prions, Salvin's prions and blue petrels, whereas only a third of the primer pairs worked successfully in the distantly related Wilson's storm-petrel (Table 2). One of the 26 loci screened (Pacbel_00829) was found to be in significant linkage equilibrium with locus Pacbel_03731 and locus Pacbel_08509, but the latter two loci appeared statistically unlinked. We therefore removed locus Pacbel_00829 from further analyses.

The mean observed heterozygosity decreased in other prion species, blue petrel and Wilson's storm-petrel with increasing mtDNA phylogenetic p-distance (Table S1, Supporting Information) from the thin-billed prion (Group 1 including all species, $f = 0.6-2.7*x$, $R^2 = 0.21$, $P < 0.001$; Group 2 *Pachyptila* and *Halobaena*, $f = 0.6-2.0*x$, $R^2 = 0.07$, $P = 0.001$; Group 3 only *Pachyptila*, $f = 0.7-8.4*x$, $R^2 = 0.25$, $P < 0.001$; Table S1 (Supporting Information), Fig. 1). This tendency was also consistent for most loci in a generalized linear model (GLM), using phylogenetic distance as covariate and locus as factor (Group 1 including all species, effect of distance: $F = 2.189$, d.f. = 1, $P < 0.001$, effect of locus: $F = 3.046$, d.f. = 24, $P < 0.001$; Group 2 *Pachyptila* and

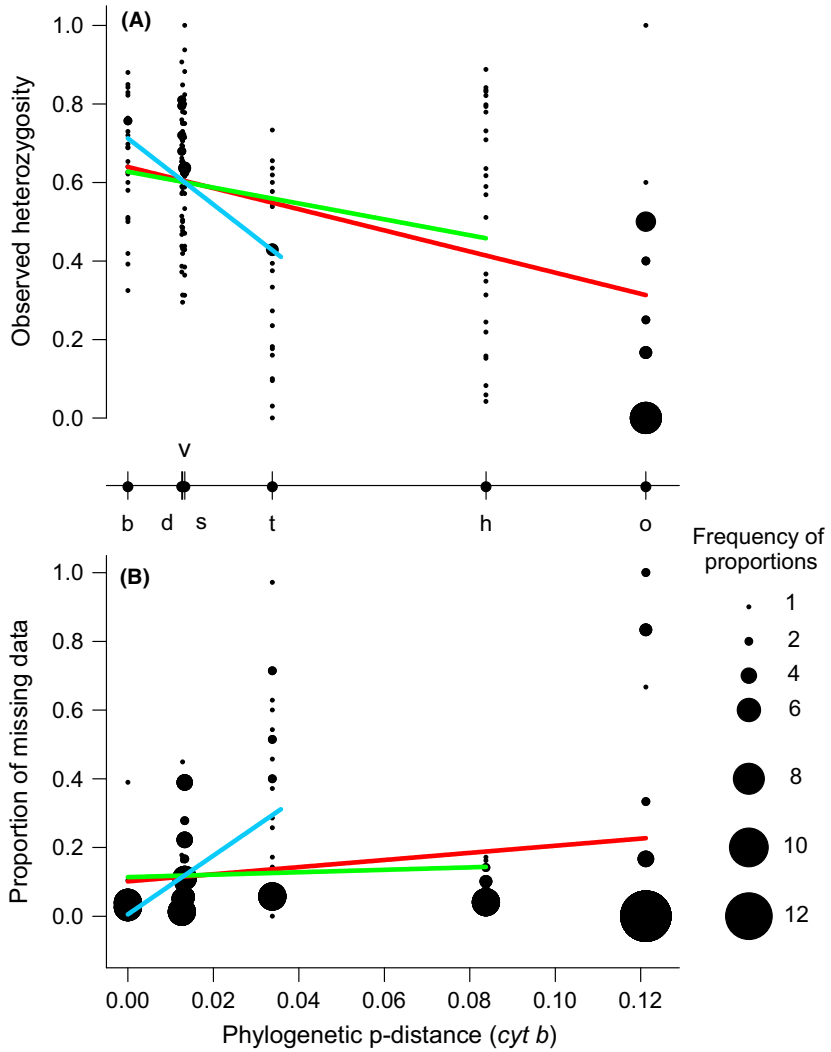

Fig. 1 Observed heterozygosity (A) and proportion of missing data (B) per microsatellite locus (black circles) in seven procellariiform species, against mitochondrial cytochrome *b* phylogenetic distance from the species of origin, the thin-billed prion *Pachyptila belcheri*. Regressions were carried out separately for three different groups of species: Group one (red) includes all species [thin-billed prion *Pachyptila belcheri* (b), Antarctic prion *P. desolata* (d), broad-billed prion *P. vittata* (v), fairy prion *P. turtur* (t), Salvin's prion *P. salvini* (s), blue petrel *Halobaena caerulea* (h) and Wilson's storm-petrel *Oceanites oceanicus*(o)]; Group two (green) includes *Pachyptila* species and *Halobaena caerulea* only; and Group three (blue) includes only the closely related *Pachyptila* species. The size of the circles indicates the frequency of particular proportions among microsatellite loci, as shown in the figure panel. The line between both graphs represents the phylogenetic distance between each species. Regression lines of mean values are shown for each group.

Halobaena, effect of distance: $F = 0.468$, d.f. = 1, $P < 0.001$, effect of locus: $F = 2.843$, d.f. = 24, $P < 0.001$; Group 3 only *Pachyptila*, effect of distance: $F = 1.207$, d.f. = 1, $P < 0.001$, effect of locus: $F = 2.106$, d.f. = 24, $P < 0.001$). The proportion of missing data increased slightly (Group 1, $f = 0.1 + 1.0*x$, $R^2 = 0.04$, $P = 0.006$; Group 2, $f = 0.1 + 0.4*x$, $R^2 = 0.004$, $P = 0.473$; Group 3, $f = 0.01 + 8.6*x$, $R^2 = 0.31$, $P < 0.001$) with phylogenetic distance from thin-billed prion (Fig. 1), and a GLM confirmed this trend for individual loci (Group 1, effect of distance: $F = 0.361$, d.f. = 1, $P = 0.003$, effect of locus: $F = 1.296$, d.f. = 24, $P = 0.134$; Group 2, effect of distance: $F = 0.017$, d.f. = 1, $P = 0.428$, effect of locus: $F = 0.805$, d.f. = 24, $P = 0.191$; Group 3, effect of distance: $F = 1.24$, d.f. = 1, $P < 0.001$, effect of locus: $F = 0.911$, d.f. = 24, $P < 0.001$).

The average frequency of null alleles among the loci and species in our total data set was low (0.076 ± 0.085), although values for some loci/species combinations were quite high (0.364). INA and ENA species trees constructed from pairwise species F_{ST} (Fig. 2A) and D_C (Fig. 2B) values were superimposed onto each other for comparison. F_{ST} values tended to decrease more than D_C when corrected for the presence of null alleles (black relative to grey branches, Fig. 2A/B). This correction did not alter the relationships between taxa for either measure of genetic differentiation. Both trees were compared for topological congruence with the mtDNA phylogeny of the cytochrome *b* gene (Fig. 2C). As with the mtDNA phylogeny, F_{ST} and D_C trees separated the genus *Pachyptila* from out-group genera *Halobaena* and *Oceanites*, and positioned the fairy prions basally within the *Pachyptila* clade. All trees differed regarding the placement of the most derived *Pachyptila* taxa. F_{ST} more closely approximated the mtDNA phylogeny in that *P. desolata* and *P. salvini* were sister taxa, but they differed with respect to the placement of *P. belcheri* and *P. vittata* (Fig. 2).

Mantel regressions (Fig. 3) indicated that a large proportion of DNA sequence variance in the cytochrome *b* data could be significantly explained by the multilocus

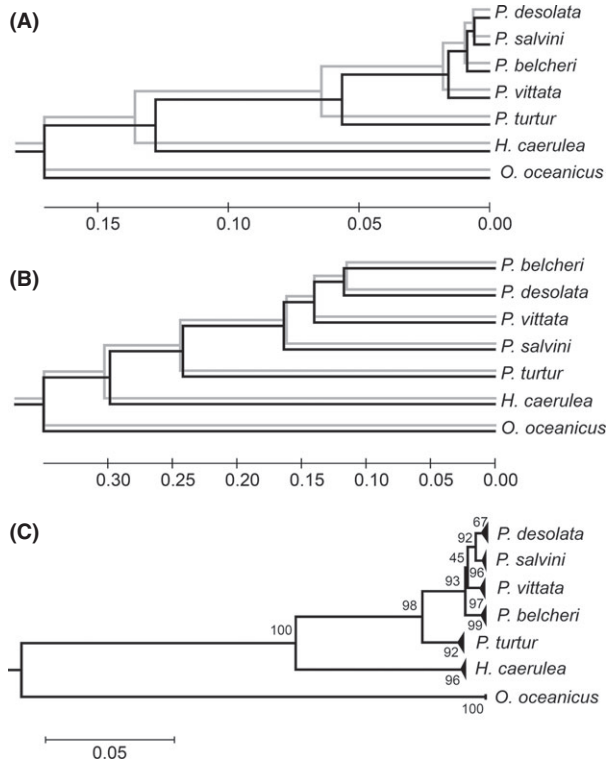

Fig. 2 Nuclear and mitochondrial phylogenetic relationships among thin-billed prions *Pachyptila belcheri*, Antarctic prions *P. desolata*, broad-billed prions *P. vittata*, fairy prions *P. turtur*, Salvin's prions *P. salvini*, blue petrels *Halobaena caerulea* and Wilson's storm-petrels *Oceanites oceanicus*. (A/B) UPGMA species trees reconstructed using interspecies F_{ST} (A) and D_C distances (B) from microsatellite data sets that include null alleles (INA, in grey) and exclude null alleles (ENA, black). (C) Phylogeny of the mitochondrial cytochrome *b* gene reconstructed via maximum likelihood using the GTR substitution model with gamma-distributed rate heterogeneity.

microsatellite distance statistics F_{ST} and D_C ($Rxy > 0.9$ in all cases, see Fig. 3). However, the relationship between microsatellite and mtDNA distance values changed markedly among the three groups of species tested and depended on which species were included. The slopes of the regressions including all species (Group 1) were lowest and increased incrementally as more phylogenetically similar taxa (Group 2, only *Pachyptila* and *Halobaena*; Group 3, only *Pachyptila* species) were grouped together. This effect was more pronounced for D_C, with shallower gradients differentiating F_{ST} regressions. Microsatellite distance statistics calculated including null alleles almost always resulted in a steeper gradient than ENA values, but this difference was smallest in the group that contained all seven species.

We tested the hypothesis that microsatellite distances were nonlinear with evolutionary time by model fitting. We found that linear functions provided a closer fit to F_{ST} distances than to D_C distances, but that second order (quadratic) polynomials provided a significantly better fit than linear functions for both distance statistics (Table 3).

Discussion

Nonamplification and null alleles

We amplified microsatellite loci in 432 individual samples in seven species of petrels. Concordant with expectation, we found that genetic diversity decreased, and the proportion of nonamplifying (missing) data increased with phylogenetic distance from the target species. Although global regressions (Group 1) as well as groups containing *Halobaena* and *Pachyptila* species (Group 2) and *Pachyptila* species (Group 3) were highly significant in most cases, the trend was not observed in all loci, resulting in shallow regression gradients. Nevertheless, this confirmed our expectation that genetic diversity decreases and missing data increases with evolutionary distance from the target species and is compatible with other studies that show increases in nonamplification and decline in polymorphism (e.g. Li *et al.* 2003; Primmer *et al.* 2005; Bried *et al.* 2008; Dawson *et al.* 2010; Jan *et al.* 2012).

The average frequency of null alleles in our data set was low, and therefore, correcting allele frequencies for the presence of null alleles resulted in no change to overall species tree topologies, but decreased F_{ST} branch lengths (Fig. 2). D_C branch lengths, on the other hand, differed much less between corrected and uncorrected data sets, implying that this statistic is more robust to the presence of null alleles.

Utility in analysing interspecific relationships

We found that species trees estimated from interspecific microsatellite data were largely congruent with mtDNA relationships among the studied species, with F_{ST} providing a slightly better approximation than D_C distances. This is a surprising result, as F_{ST} is a fixation index, and as such does not satisfy the triangle inequality as would true distance measures such as D_C. Our data also showed the inherent problem posed by null alleles in the resolution of interspecific branch lengths, which affected F_{ST} more than D_C (Fig. 2A,B). Furthermore, F_{ST} has often been criticized for inaccurately estimating population differentiation when genetic variation is high (Charlesworth 1998; Balloux & Lugon-Moulin 2002; Carreras-Carbonell *et al.* 2006; Jost 2008). Nevertheless, F_{ST} is perhaps the most reported statistic in population and evolutionary genetics. These comparisons with the cytochrome *b* phylogeny, however, do not account for potential biases

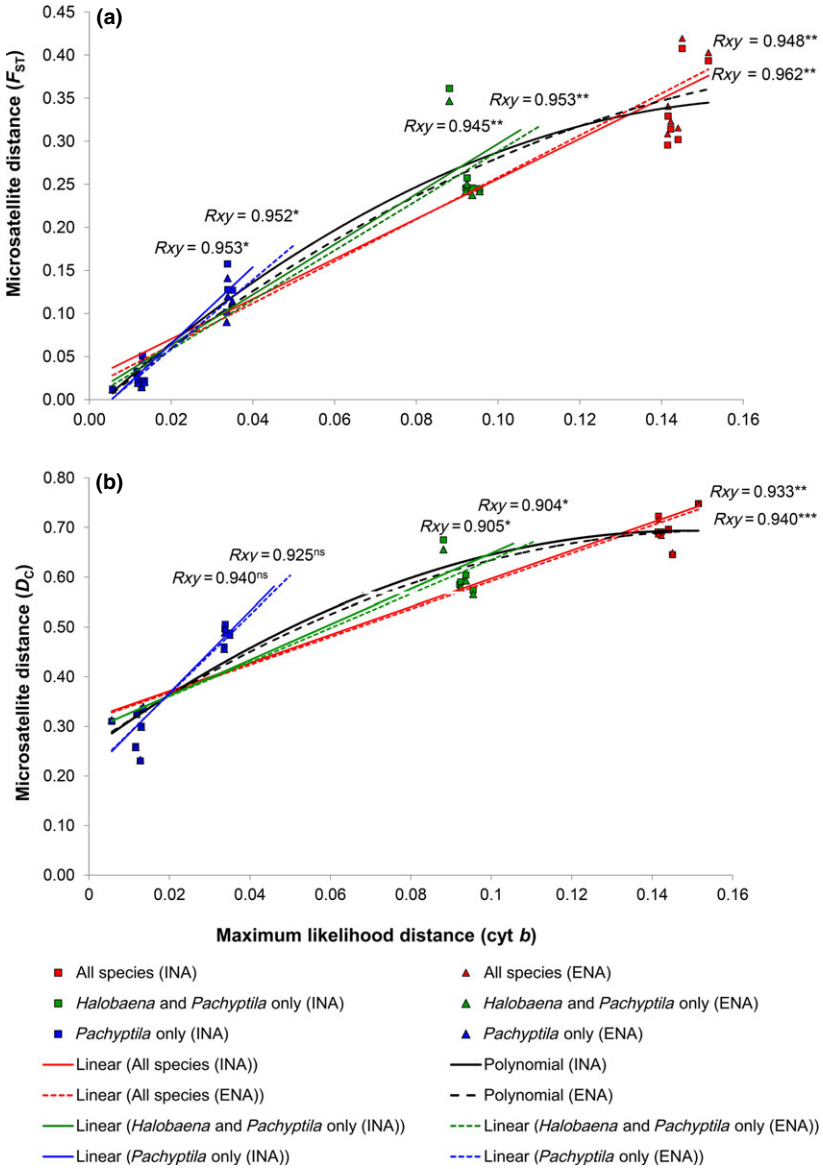

Fig. 3 Mantel regressions of pairwise microsatellite distances F_{ST} (A) and D_C (B) against mitochondrial cytochrome *b* phylogenetic distance calculated for three groups of Procellariiform species. Microsatellite genetic distances tend to lose linearity for groups that include species increasingly distant to the species of origin, *Pachyptila belcheri*. Microsatellite distances are also given including null alleles (INA, squares and solid lines) and excluding null alleles (ENA, triangles and dashed lines) after Chapuis & Estoup (2007). Species are grouped and colour-coded as in Fig. 1. Black lines describe the polynomial function that best fits the INA (solid lines) and ENA (dashed lines) microsatellite distance data.

Table 3 Fitting linear and polynomial functions to variance in microsatellite distance statistics across seven sub-Antarctic seabird species

Statistic	Best fit linear function	AIC linear	Best fit polynomial function	AIC poly.	Delta AIC	*P*
F_{ST} INA	$y = 2.325x + 0.024$	−67.2	$y = -12.599x^2+4.285x-0.015$	−72.5	5.3	0.013
F_{ST} ENA	$y = 2.437x + 0.014$	−72.5	$y = -9.259x^2+3.877x-0.014$	−75.3	2.8	0.045
D_C INA	$y = 2.826x + 0.314$	−53.4	$y = -19.726x^2 + 5.894x + 0.253$	−61.3	7.9	0.004
D_C ENA	$y = 2.802x + 0.312$	−56.3	$y = -17.195x^2 + 5.477x + 0.259$	−62.5	6.3	0.009

in mtDNA itself, nor for differences in tree-building algorithms used for microsatellite (UPGMA) and mtDNA data (maximum likelihood). Therefore, we stress the need for a more thorough reappraisal of the phylogenetic relationships among the prions, using multiple but more slowly evolving nuclear intronic gene sequences.

Linearity of microsatellite genetic distances

The high proportion of explained variation in Mantel regressions of microsatellite and mtDNA genetic distance implies that variation was similarly distributed between the both microsatellite and mitochondrial data sets. However, when regression analyses of the three groups of

varying species diversity imposed a linear relationship between microsatellite and mtDNA distance, the slope of the regression changed considerably, suggesting that the true relationship was nonlinear. Instead, at lower distance values (e.g. among congeners; Group 3), the relationship appears linear, but microsatellite distance gradually reaches a plateau with increasing mtDNA distance (Fig. 3A,B, Groups 1 and 2), implying that the latter statistic is a better estimator of relationships among distantly related taxa. We tested the hypothesis that microsatellite distances are not linear with evolutionary distance by fitting both linear and polynomial functions to the F_{ST} and D_C data and found that in both cases a quadratic function best fitted the data (Table 3).

Interestingly, F_{ST} appeared to remain linear for longer than D_C, especially when corrected for the presence of null alleles (see higher *P* values, Table 3). While this suggests that F_{ST} might be more useful at higher phylogenetic levels, its usefulness is compromised by its higher variance compared to D_C. Because neither microsatellite distance measure maintained linearity in pairwise intergeneric comparisons, we recommend that analyses of genetic differentiation restrict F_{ST} and D_C to studies in which the target species is closely related to the species from which the markers were developed. In either case, ENA correction for null alleles is essential.

Despite high mutation rates of microsatellites, simulations indicate that measures of genetic differentiation will remain linear much longer without constraints in allele size (Nauta & Weissing 1996). Therefore, we propose that the nonlinearity we observed at the intergeneric level is a natural consequence of constrained microsatellite allele size that leads to an increase in the number of convergently evolved allele size classes that, while identical in state, are no longer identical by descent in intergeneric pairwise comparisons. Estoup *et al.* (2002) suggested that at the intraspecific level, the high mutation rates of microsatellites will compensate for the inevitable convergent evolution of some allele classes, while Paetkau *et al.* (1997) detected a loss of linearity among closely related sister taxa (brown bear *Ursus arctos* and polar bear *U. maritimus*). Given that variation in our set of microsatellites remains linear within the genus *Pachyptila*, perhaps because of a slightly lower mutation rate or a slightly larger maximum repeat size, we are confident that population genetic and demographic analyses at this level will not be compromised by constraints in allele size.

Conclusions

We show here that a panel of 25 microsatellite loci developed using next-generation sequencing of a thin-billed prion shotgun library may be applied in studies of molecular ecology among congeners; however, this approach may result in a greater proportion of null alleles and lower amounts of genetic diversity in the non-target species. Genetic diversities therefore may not be directly comparable between species, despite the use of the same conserved microsatellite markers. In addition, the contrasting results from the two measures of differentiation lead us to discourage the use of these microsatellites in phylogenetic reconstruction beyond the genus level, as even at that level this may be associated with high variance.

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P.Q. and Y.M. conceived and designed the study. R.A., Y.C., R.J.C., M. Marin, J.F.M., M.M., J.N., R.A.P., P.Q., P.G.R., C.G.S., and H.W. carried out the extensive fieldwork. M.R.T. isolated the microsatellite sequences. T.L.C., J.F.M., and G.K.M. screened the samples. J.F.M., Y.M., T.L.C., and L.C. carried out the bioinformatic analyses. Y.M., J.F.M., and P.Q. drafted the manuscript. All authors reviewed the final draft of the manuscript.

Data accessibility

DNA sequences: GenBank accessions KP122163-KP122196, KM050769 and KM050770. Shotgun DNA sequence reads, 432 microsatellite genotypes at 25 loci, cytochrome *b* alignment, distance matrices and tree files: Provisional DRYAD entry doi:10.5061/dryad.rc917.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Phylogenetic p-distances and maximum likelihood distances (cytochrome *b*) in Pachyptila species, blue petrels and Wilson's storm-petrels.