A TEST OF MECHANISMS OF POPULATION DIFFERENTIATION IN GANNETS (*MORUS* SPP.) USING COMPARATIVE PHYLOGEOGRAPHY AND MORPHOMETRICS

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ABSTRACT

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The relative importance of gene flow, genetic drift, and natural selection in driving genetic divergence of local populations, as well as the factors that disrupt gene flow in natural populations, are uncertain. Comparative analyses enable us to test explicit hypotheses regarding the roles of these factors. Gannets (*Morus* spp.) include three morphologically and ecologically similar seabird species: the Cape Gannet *M. capensis*, which breeds in southern Africa; the Australasian Gannet *M. serrator*, which breeds in southeastern Australia and New Zealand; and the Northern Gannet *M. bassanus*, which breeds in the North Atlantic. We assessed the extent of differentiation in mitochondrial control region sequences and morphometrics among colonies of each species to test mechanisms of divergence. No evidence was found for introgression of mitochondrial DNA between Cape and Australasian gannets, despite the existence of mixed-species pairs, consistent with their taxonomic status as biological species. Significant population differentiation, both in control region sequences and in morphology, was found within both Australasian and Northern gannets. Analyses based on coalescent theory indicated that little to no female-mediated gene flow occurs either between Australasian Gannets in Australia versus New Zealand, or between Northern Gannets in Canada versus Europe. Divergence of these regional populations, as well as Cape versus Australasian gannets, appears to pre-date the Pleistocene glaciations and corresponds to differences in non-breeding distributions. Northern and Australasian gannets may each represent at least two management units for conservation, but additional data are needed on nuclear DNA variation in individuals sampled from more colonies to confirm this result.

Key words: coalescence, gene flow, hybridization, management unit, Pleistocene glaciation, seabird, speciation

INTRODUCTION

Understanding the mechanisms by which populations diverge genetically and adapt to the local environment is important not only for understanding ecology and evolution, but also for predicting the potential for populations to adapt to anthropogenic change (Waldvogel *et al*. 2019) and for defining management units (Mortiz 1994, Barbosa *et al*. 2018). The extent to which conspecific populations diverge genetically and adapt to local environmental conditions is determined by complex interactions between gene flow (which tends to inhibit local adaptation), genetic drift (which can inhibit local adaptation but promote differentiation in neutral genetic variation), and natural selection (which can either promote or inhibit population differentiation, depending on the nature of selection; e.g., Wright 1951, 1978, Olson-Manning *et al*. 2012, Tigano & Friesen 2016). The outcomes of interactions among these forces are far from predictable. For example, many species of seabirds have high gene flow and little geographic variation in neutral genetic variation, yet exhibit local variation in morphology, sometimes even on small geographical scales, e.g., Common Murres *Uria aalge* (Bedard 1985, Morris-Pocock *et al*. 2008); Little Auks *Alle alle* (Wojczulanis-Jakubas *et al*. 2015); Northern Fulmars *Fulmarus glacialis* (van Franeker & J. Wattel 1982, Kerr & Dove 2013); and Band-rumped Storm Petrels *Hydrobates castro* (Smith & Friesen 2007). In contrast, some local populations of seabirds are morphologically and ecologically similar yet genetically distinct, even representing cryptic species, e.g., Band-rumped and Monteiro's Storm Petrels *H. monteiroi* in the Azores (Bolton *et al*. 2008). Comparative analyses can provide useful insights into these mechanisms.

Gannets (order Pelecaniformes, family Sulidae, genus *Morus*) are temperate colonial seabirds that provide interesting subjects for studying the interplay of forces that promote versus inhibit

population differentiation and local adaptation. The genus includes three recently diverged species that are ecologically similar but differ in morphology, vocalizations, and distributions (Nelson 2010, Patterson *et al*. 2011): Cape Gannets *M. capensis* nest on islands off South Africa; Australasian Gannets *M. serrator* have colonies in southeastern Australia and New Zealand; and Northern Gannets *M. bassanus* breed along the coast of Atlantic Canada and coastal Europe (Nelson 2010) (Fig. 1). Several lines of evidence indicate that populations of these species should be genetically similar, at least at neutral genetic markers; other evidence reviewed below indicates, however, that local populations may differ genetically.

Dispersal patterns

Dispersal among breeding colonies can promote gene flow and homogenize populations. Northern Gannets have been recorded breeding at non-natal colonies within Europe (Brun 1972, Barrett 2008, Veron & Lawlor 2009); band returns indicate that low but significant natal dispersal occurs in Cape Gannets (Distiller *et al*. 2012); and some colonies of Northern and Australasian gannets are growing faster than rates predicted under self-recruitment, an indication that immigration may be occurring (Brun 1972, Montevecchi & Myers 1997, Moss *et al*. 2002, Pyk *et al*. 2013). Furthermore, mixed pairs of Cape and Australasian gannets have been found in both New Zealand and South Africa, and phenotypic hybrids have been reported (Dyer *et al*. 2001, Pizzey & Knight 2007, Robertson & Stephenson 2005, Robertson 2008, Ismar *et al*. 2011), an indication that interspecific gene flow might also occur.

In contrast, philopatry to natal colonies can potentially inhibit gene flow and lead to genetic differences among regional populations of seabirds (Friesen *et al*. 2007, Friesen 2015, Lombal *et al.* 2020). Despite extensive banding efforts (62 000 gannets banded in Europe, 14 000 banded in Canada; Gaston *et al*. 2008, Fifield *et al.* 2014), no Northern Gannets banded in Europe have been found breeding in Canada, and only one Northern Gannet banded in Canada has been found as a possible breeder in Europe. Similarly, no Australasian Gannets banded in southeastern Australia have been recovered breeding in New Zealand or vice versa (Ismar *et al*. 2011).

Historical distributions

Range expansions can homogenize populations genetically (e.g., Ibrahim *et al*. 1996, Hewitt 1999). Colonies of Australasian and Northern gannets currently occupy areas that were previously covered by Pleistocene glaciers (MacPhail 1979, Hewitt 2000) and so presumably represent recent range expansions (< ~10 000 years). Archaeological evidence and breeding bird surveys indicate that Northern Gannets have expanded their numbers and breeding distributions in Europe in the past 8 000 years, and in Canada in the past 30+ years, with many contemporary colonies being < 100 years old (Nettleship & Chapdelaine 1988, Montevecchi & Hufthammer 1990, Mitchell *et al*. 2004, Wanless & Harris 2004, Nelson 2010, Chardine *et al*. 2013, Barrett *et al*. 2017). In addition, Cape and Australasian gannets have both been recorded attempting to breed on Saint Paul Island in the Indian Ocean, thousands of kilometers outside their normal ranges (Lequette *et al*. 1995). Range expansions, combined with large population sizes (e.g., > 360 000 pairs of Northern Gannets; Nelson 2010) and long generation times (5–10 years; Nelson 2010), generally tend to homogenize populations (Wright 1951, 1978). Other seabird species with similar characteristics tend to show little, if any, population genetic structure (reviewed in Friesen *et al*. 2007, Friesen 2015).

In contrast, historical separation, such as in multiple glacial refugia, can promote population differentiation and has been reported for many species of plants and animals, including seabirds (Hewitt

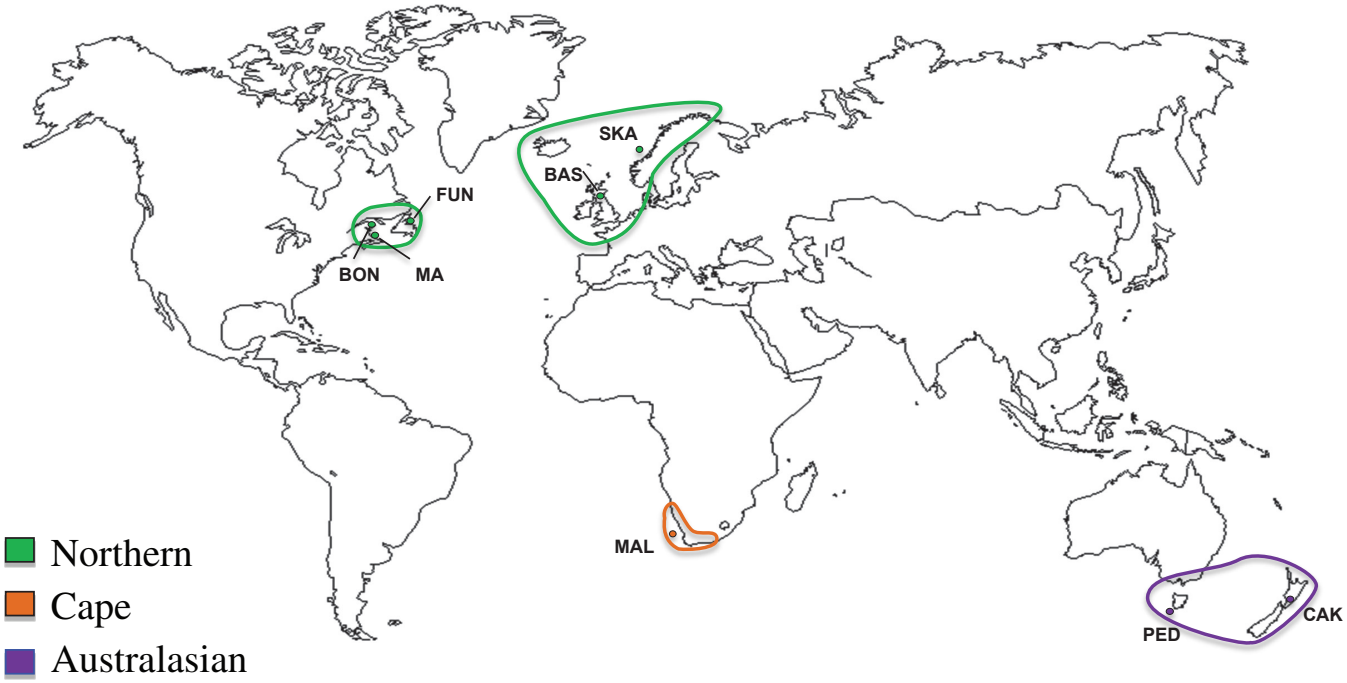

Fig. 1. Approximate breeding distributions of Northern *Morus bassanus*, Cape *M. capensis*, and Australasian *M. serrator* gannets (Nelson 2010), and sampling sites used in this study. Both Cape and Australasian gannets have also attempted to breed on St. Paul Island in the Indian Ocean (Lequette *et al*. 1995). See Table 2 for colony abbreviations.

2000, Friesen *et al*. 2007, Friesen 2015, Lombal *et al*. 2020). Although Pleistocene distributions of gannets are not known, many seabird species appear to have occupied two or more refugia each in Australasia and the North Atlantic during the Pleistocene glaciations (e.g., Morris-Pocock *et al*. 2008, Overeem *et al*. 2008, Peuker *et al*. 2009, Lombal *et al*. 2020).

Non-breeding and foraging distributions

Overlap among colonies in non-breeding and/or foraging distributions could enable breeding recruits to follow established breeders to non-natal colonies, promoting gene flow (Burg & Croxall 2001). Non-breeding and foraging distributions of Northern Gannets from different colonies within each side of the Atlantic often overlap (Fort *et al*. 2012, Wakefield *et al*. 2013, Fifield *et al*. 2014, Garthe *et al*. 2016). Juvenile Cape Gannets also may intermix along the western coast of Africa (Klages 1994). Adult Australasian Gannets tend to remain near breeding colonies year-round, but young gannets from New Zealand tend to disperse to southern and eastern Australia, often near Australian colonies (Wodzicki & Stein 1958, Nelson 1978, Ismar *et al*. 2010, 2011, Rodríguez Malagón 2019). These observations, combined with the fact that gannets are often social foragers (Thiebault *et al*. 2014), may promote gene flow among colonies.

In contrast, population genetic differentiation in birds, including seabirds, is often associated with migratory divides, i.e. separation of birds from different breeding regions in different non-breeding areas (Webster *et al*. 2002; seabirds reviewed in Friesen *et al*. 2007, Friesen 2015, Lombal *et al*. 2020; but with several exceptions, e.g., Quillfeldt *et al*. 2017). Data from band returns, data loggers, and satellite tracking indicate that, although gannets from different colonies may intermingle during the non-breeding season, gannets in Europe winter along the eastern Atlantic coast from the North Sea to West Africa (Kubetzki *et al*. 2009, Fort *et al*. 2012, Fifield *et al*. 2014, Garthe *et al*. 2016), whereas Northern Gannets from eastern North America tend to overwinter on the western North Atlantic, from southeast Newfoundland to the Gulf of Mexico (although some individuals from eastern Newfoundland have been tracked to West Africa; Huettmann & Diamond 2000, Nelson 2010, Montevecchi *et al*. 2012, Fifield *et al*. 2014).

Local adaptation

Extensive geographic variation in morphology has been reported in some seabird species, indicating possible genetic adaptation to local conditions or phenotypic plasticity (e.g., low food availability resulting in poor growth; Moen 1990). This is evident in Atlantic Puffin *Fractercula arctica* wing and bill lengths, which tend to increase with latitude, possibly due to local variation in food availability and ocean temperatures (Moen 1990). A similar pattern occurs in body size, bill length, and frequency of the dark morph in Northern Fulmars in the North Atlantic (reviewed in Mallory *et al*. 2020). Great Auks *Alca impennis* from Funk Island were larger than conspecifics in eastern Atlantic colonies (Burness & Montevecchi 1992), whereas Little Auks in the North Atlantic increase in several measurements from west to east in association with air temperature (Wojczulanis-Jakubas *et al*. 2011). Although little information on geographic variation in morphology, behaviour, or physiology is available for gannets, some colony-specific differences have been reported (Nelson 2010). Most colonies of all three species of gannets occur in warm water currents, and most non-breeding gannets occur in warm water (Nelson 2010); however, colonies of Northern Gannets off eastern Newfoundland and northern Iceland, and of Cape Gannets off western South Africa, occur in distinctly colder currents, patterns that may indicate the potential for differences in selection pressures among colonies (Friesen 2015).

Current study

To learn more about speciation in gannets, we assayed geographic variation in both mitochondrial DNA (mtDNA) and morphology in all three species of gannets to test mechanisms of population differentiation. We had three objectives, with five primary hypotheses (Table 1):

Objective 1: To test whether Cape and Australasian gannets represent biological species. Given evidence of hybridization between Cape and Australasian gannets, we did not expect them to be reproductively isolated (Hypothesis 1).

Objective 2: To determine the relative importance of contemporary gene flow, genetic drift, and historical distributions in shaping population genetic structure within Australasian and Northern gannets. We hypothesized that Australasian Gannets from southeastern Australia would not differ from those from New Zealand due to gene flow promoted by dispersal of juvenile gannets (Hypothesis 2), but Northern Gannets from Canada would differ from those from Europe due to genetic drift during prolonged isolation in separate Pleistocene refugia (Hypothesis 3). Given evidence of both on-going gene flow and recent range expansions (above), we did not expect to find differences among colonies of Northern Gannets within Canada or within Europe (Hypothesis 4).

Objective 3: To test for geographic variation in morphology. Given results for other seabird species (above), we expected that morphometric differences would exist among gannets breeding in cold water currents (Funk and Malgas islands) compared to conspecifics breeding elsewhere (Hypothesis 5).

We tested specific predictions for each hypothesis using statistical phylogeography for the mitochondrial variation, and multifactorial analyses for the morphometric data (Table 1). Statistical phylogeography provides a framework for explicit tests of evolutionary mechanisms based on the phylogenetic relationships and geographic distributions of genetic variants (Knowles 2009, Thomas *et al*. 2019). The mitochondrial control region is useful for analyses at the intraspecific and intrageneric levels because it has a low effective population size (making it a more sensitive indicator of genetic drift than nuclear loci), it is non-recombining (so it provides a clearer record of demographic history), and it has a relatively rapid rate of molecular evolution (e.g., Avise *et al*. 1987, Baker & Marshall 1997). Note, however, that the mitochondrial genome represents a single, maternally-inherited supergene, restricting inferences that can be drawn from it (see Discussion).

STUDY AREA AND METHODS

Sample collection and mitochondrial control region sequencing

We collected blood samples from 29 Cape, 61 Australasian, and 133 Northern gannets, including multiple colonies of Australasian and Northern gannets (Fig. 1, Table 2). We also collected blood

TABLE 1
Hypotheses (H) and predictions (P) tested in the present study

Hypothesis or prediction		Analysis
H1	*Cape and Australasian gannets are not reproductively isolated*	
P1.1	Haplotypes of Cape and Australasian gannets will not be monophyletic on the mtDNA gene tree	MRBAYES, TCS
P1.2	Estimates of gene flow between Cape and Australasian gannets will be significantly greater than 0	IMa
P1.3	A demographic model of migration between Cape and Australasian gannets will be more strongly supported than a model of isolation	DIYABC
H2	*Australasian Gannets from Australia versus New Zealand do not differ genetically*	
P2.1	Estimates of population differentiation between Australasian Gannets from Pedra Branca versus Cape Kidnappers will not be significantly greater than 0	AMOVA
P2.2	Estimates of gene flow between Australasian Gannets from Pedra Branca versus Cape Kidnappers will be significantly greater than 0	IMa
H2.1	*Genetic differences in H2 are due to isolation in separate glacial refugia*	
P2.1.1	Mitochondrial haplotypes will be phylogeographically structured	MRBAYES, TCS
P2.1.2	Population divergences will pre-date the last glacial maximum	IMa
P2.1.3	A demographic model of historical isolation between Australasian Gannets from Pedra Branca versus Cape Kidnappers will be more strongly supported than models of expansion from a single refugium	DIYABC
H3	*Northern Gannets from Canada versus Europe differ genetically*	
P3.1	Estimates of population differentiation between Northern Gannets from Canada versus Europe will be significantly greater than 0	AMOVA
P3.2	Estimates of gene flow between Northern Gannets from Canada versus Europe will not differ from 0	IMa
H3.1	*Genetic differences in H3 are due to isolation in separate glacial refugia*	
P3.1.1	Mitochondrial haplotypes will be phylogeographically structured	MRBAYES, TCS
P3.1.2	Population divergences will pre-date the last glacial maximum	IMa
P3.1.3	A demographic model of historical isolation between western versus eastern populations of Northern Gannets will be more strongly supported than models of expansion from single refugia	DIYABC
H4	*Northern Gannets do not differ genetically among colonies within either Canada or Europe*	
P4.1	Indices of population differentiation will not differ from 0 within either Canada or Europe	AMOVA
H4.1	*Lack of differentiation in H4 is due, at least in part, to recent population and range expansions*	
P4.1.1	The number of haplotypes will exceed neutral expectations under constant population size	Fu's F_S
P4.1.2	The genetically effective sizes of contemporary populations will be significantly greater than the ancestral population size	IMa, DIYABC
H5	*Morphology varies among colonies of Northern Gannets*	
P5.1	Morphology will differ between gannets breeding in cold water currents (Funk Island) versus warm water currents (elsewhere)	MFA, DA

samples from two gannets that were nesting within the Cape Gannet colony at Malgas Island but that had all the field marks of Australasian Gannets (small gular pouch, dark eye, white tail; Robertson & Stephenson 2005). With the exception of birds from the Magdalen Islands, which were chicks, all birds were breeding adults captured at nests. Several colonies were composed of two or more subcolonies that were separated from each other by areas with no breeding gannets (e.g., the "satellite" and "main" colonies on Funk Island; the "stack" and "mainland" colonies at Cape St. Mary's); where possible, we collected samples from all subcolonies, and obtained samples from throughout each nesting area.

We prepared DNA from blood samples using a standard protease K, phenol/chloroform extraction (Sambrook & Russell 2001). We

TABLE 2
Sampling site information, colony sizes (number of breeding pairs, at the approximate time of sampling), sample sizes, and estimates of Fu's F_S

Species	Colony	Abbreviation	Latitude	Longitude	Colony size (pairs)	*n*	Fu's F_S
Northern Gannet	Bonaventure I.	BON	48°50′N	064°17′W	21 000[a]	28	−6.69*
	Magdalen Is.	MAG	47°23′N	061°54′W	6 600[a]	22	−16.3*
	Funk I.	FUN	49°45′N	053°11′W	6 100[a]	29	−21.4*
	Bass Rock	BAS	56°04′N	002°38′W	22 000[b]	24	−11.2*
	Skarvklakken	SKA	69°09′N	015°41′E	700[c]	30	−24.2*
Australasian Gannet	Pedra Branca	PED	43°51′S	146°58′E	3 000[d]	28	−4.08*
	Cape Kidnappers	CAK	39°38′S	177°05′E	5 200[e]	33	−2.82
Cape Gannet	Malgas I.	MAL	33°03′S	017°55′E	44 000[f]	29	−23.9*
Total						225	

[a] Chardine 2013
[b] Wanless & Harris 2004
[c] Barrett & Folkestad 1996, now extinct
[d] Bunce *et al*. 2002
[e] Wodzicki *et al*. 1984
[f] Crawford 2007
* Significantly different from 0 at $\alpha = 0.05$.

amplified and sequenced a fragment of the mitochondrial control region for all samples; however, this process was complicated both by a co-amplifying nuclear copy ("numt"; Friesen & Anderson 1997, Ibarguchi *et al*. 2006) and by a duplication within the mitochondrial genome (see also Abbott *et al*. 2005, Morris-Pocock *et al*. 2010a, Eda *et al*. 2010, Rains *et al*. 2011). We characterized the mitochondrial duplication using methods similar to those employed in Morris-Pocock *et al*. (2010a). Briefly, preliminary amplifications using various combinations of primers indicated that the mitochondrial genomes of all three gannet species contain a tandem duplication encompassing genes for cytochrome *b*, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, and the control region. We chose to target the second control region, CR2 (hereafter referred to as "the control region") for amplification in all gannets because it is surrounded by the typical mitochondrial gene order and is used in other genetic studies of sulids (Morris-Pocock *et al*. 2010b, Taylor *et al*. 2011a, 2011b). We amplified a 674-base pair fragment of the control region using the primers SbMCR-L52 (5′-TTATTTTTATGTCCCTTGGG-3′; this study) and SbMCR-H800 (5′-CCAATACGTCAACCGTCTCAT-3′; Steeves *et al*. 2005a). These primers were located in regions that differed between the two copies, so that CR2 was amplified to the exclusion of CR1. Amplifications were performed in 25 µL reaction volumes (~5 ng of DNA, 1× Multiplex Mix [Qiagen, Mississauga, Ontario, Canada], and 0.7 mM of each primer). DNA was denatured at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 90 s, and a final extension at 72 °C for 3 min. PCR products were checked for successful amplification on agarose gels, then cleaned of impurities using AMPure® paramagnetic beads (Beckman Coulter, Mississauga, ON) and sequenced in both directions using a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA) at the Genome Quebec Innovation Centre (McGill University, Montreal, QU).

We aligned control region sequences using CLUSTALW (Thompson *et al*. 1994) as implemented in BIOEDIT (version 7.0.5.3, Hall 1999). Some sequences ($n = 78$) had ambiguous sites (two bases that were present at the same site in the chromatograms in both sequencing directions). Ambiguous sites have also been found in the control regions of other seabird species that are closely related to gannets (Steeves *et al*. 2005a; Morris-Pocock *et al*. 2010a, 2010b; Taylor *et al*. 2011a, 2011b). As in these previous studies, and given the steps taken to avoid co-amplification of nuclear homologues, we attribute the presence of ambiguous sites to true mitochondrial heteroplasmy (see Morris-Pocock *et al*. 2010b for more details). Following Steeves *et al*. (2005a), we resolved ambiguous sites using the following conservative rules: (i) if the sequence with the ambiguous site was otherwise identical to another observed haplotype, we assigned the ambiguous site to the nucleotide present in the observed haplotype, rather than infer a new haplotype; (ii) if the sequence with the ambiguous site was identical to two different observed haplotypes, we assigned the ambiguous site to the nucleotide present in the more common haplotype; (iii) if assignment of the ambiguous base to either nucleotide resulted in a novel (singleton) haplotype, we assigned the ambiguous nucleotide randomly. We also re-ran downstream analyses after excluding all individuals that had ambiguous sequences: results were similar and did not change the main conclusions of the study. We report the results that include all individuals.

To test the basic assumption that variation in the control region is evolving under neutral expectations, we tested whether Tajima's D (Tajima 1989) and Fu's F_S (Fu 1996) differed significantly from zero using ARLEQUIN (version 3.1.1; Excoffier *et al*. 2005).

Phylogeographic and population genetic structure

To test for phylogeographic structure (Predictions 1.1, 2.1.1 and 3.1.1 in Table 1), we estimated an unrooted mitochondrial gene tree of all gannet control region haplotypes using MRBAYES (version 3.1.2, Huelsenbeck & Ronquist 2001). The tree could not be rooted

because gannet control region sequences could not be reliably aligned with control region sequences from candidate outgroup species (i.e., boobies *Sula* spp., Patterson *et al*. 2011). We used the Akaike Information Criterion (AIC) in JMODELTEST to determine the nucleotide substitution model that best fit the data (Guindon & Gascuel 2003, Posada 2008), then estimated the actual parameters of the nucleotide substitution model using MRBAYES. We used one cold chain and three incrementally heated chains to explore parameter space and ran the analysis for 2.0×10^7 generations, sampling trees every 100 generations and discarding the first 50 000 trees (25% of all sampled trees) as burn-in. We evaluated convergence of the Markov chain Monte Carlo (MCMC) process by monitoring the average standard deviation of split frequencies between two simultaneous runs, inspecting the trend plots of all parameters, and running three replicate analyses with different random starting seeds. We also estimated intraspecific haplotype networks for both Australasian and Northern gannets using TCS (version 1.21; Clement *et al*. 2000).

We used analyses of molecular variance (AMOVA; Excoffier *et al*. 1992) in ARLEQUIN to estimate global population genetic structure (Φ_{st}) within Australasian and Northern gannets (Predictions 2.1, 3.1 and 4.1 in Table 1). For Northern Gannets, we also estimated pairwise Φ_{st} between all pairs of colonies. We used Kimura's two-parameter model of nucleotide substitution (K2P; Kimura 1980) and parameterized the model using the shape parameter of the gamma distribution that was determined in JMODELTEST. We evaluated the significance of Φ_{st} estimates using 10 000 random permutations of the data. We did not correct for multiple tests due to the small number of pairwise comparisons within species. (Use of a Benjamini-Yekutieli correction [Narum 2006] would have altered the significance of only one comparison.) Too few colonies were sampled to test for isolation by distance within any species.

Demographic history

We used an Isolation with Migration model (where "migration" in this context means gene flow rather than seasonal movements) implemented in the software IMa (Hey & Nielsen 2004, 2007) to estimate divergence times and long-term rates of gene flow between (i) Cape and Australasian gannets (excluding the two putative Australasian Gannets from Malgas Island); (ii) southeastern Australian and New Zealand Australasian Gannets; and (iii) eastern and western North Atlantic Northern Gannets (Predictions 1.2, 2.2, 2.1.2, 3.2, 3.1.2, and 4.1.2 in Table 1). IMa assumes that (i) the two populations are sister taxa; (ii) genetic substructure does not exist within either population; and (iii) the populations do not exchange genes with any unsampled, genetically different population (Hey & Nielsen 2004, 2007). Our IMa analyses were designed to avoid or minimize violations to these assumptions: IMa parameter estimation is robust to minor violations of the above assumptions (Strasburg & Rieseberg 2010); Cape and Australasian gannets are well established sister species (Friesen & Anderson 1997, Patterson et al. 2011); and our results (below) suggest that only two genetic populations exist within either Australasian or Northern gannets. Note, however, that Northern Gannets from Iceland, and Australasian Gannets from Norfolk Island, which were not sampled in this study, may differ from conspecifics elsewhere.

IMa estimates six parameters: divergence time ($t = T\mu$, where T is the divergence time in generations and μ is the mutation rate per generation for the entire locus), two gene flow rates ($m_{ij} = M_{ij}/\mu$, where M_{ij} is the effective rate of maternal gene flow from population j into population i), and three population diversity parameters Θ_1, Θ_2, and Θ_A (where $\Theta_i = 2N_{fi}\mu$, N_{fi} is the female effective population size for population i and Θ_A is the parameter for the ancestral population). The mutation rate for the mitochondrial control region of gannets is not known. Published estimates for other bird species range from 0.14% (Pereira *et al*. 2004) to 10% (Quinn 1992), and vary both across the control region, as well as within and among species. We used a mutation rate of 1% per million years (MY), which is based on comparisons of complete control region sequences of geese *Anser* spp. with divergence dates based on fossil evidence (Ruokonen *et al*. 2000), and which is within the range of estimates for complete control regions for other species of birds (3.1% for grouse, Drovetski 2003; 0.7% for peafowl, Pereira *et al*. 2004; 0.5%–1.8% for parids, Packert *et al*. 2007). Given the uncertainty in the mutation rate, we consider estimates of divergence time and effective population size to be approximate. Most importantly, the above estimates represent interspecific comparisons, whereas several of the parameters we wished to estimate are intraspecific. Intraspecific rates are likely to be higher than interspecific rates (e.g., Ho & Larson 2006); however, rates even 10 times higher than the rate we used would still place population and species divergence before recession of the last glaciations (see Results).

We ran all IMa analyses in "M" mode, applied the Hasegawa-Kishino-Yano nucleotide substitution model (HKY; Hasegawa *et al*. 1985), and initially set broad, biologically realistic priors on all parameters. We then adjusted priors so that the posterior distributions of all parameters reached a distinct peak and then descended again. We ran analyses for at least 1.0×10^8 generations (1.0×10^6 generations discarded as burn-in), and we used 20 geometrically heated (g1 = 0.96, g2 = 0.90) Markov chains to explore parameter space. We monitored convergence by ensuring that all effective sample sizes (ESS) were at least 50, monitoring trend plots, and running all analyses three times with different random starting seeds.

We used the program DIYABC (version 2.1.0; Cornuet *et al*. 2014) to test alternative models of population history (Predictions 1.3, 2.1.3, 3.1.3, and 4.1.2 in Table 1). DIYABC uses coalescent theory to simulate a large number of genetic datasets for alternative demographic models, and it computes summary statistics for each model. The summary statistics for a model can then be compared to the observed dataset to determine the posterior probability of a given model (Cornuet *et al*. 2014).

To test whether Cape and Australasian gannets are genetically isolated (Prediction 1.3 in Table 1), we ran an initial test of three models: 1) Cape origin, spread to Australasia; 2) Australasia origin, spread to the Cape; and 3) isolation of Cape and Australasia populations from a common ancestral population. We then tested the most probable model (Model 2, see Results) with and without gene flow ("admixture") after separation.

To test whether Australian and New Zealand Gannets diverged in two separate refugia (Prediction 2.1.3), we ran an initial test of three models: 1) Australian origin, spread to New Zealand; 2) New Zealand origin, spread to Australia; and 3) historical isolation of gannets in New Zealand and Australia. We found that all of the above models had similar probabilities (see Results), so we tested each one with and without gene flow to determine if gene flow was generally more probable during divergence.

Finally, to test whether Northern Gannets were separated in two glacial refugia (Prediction 3.1.3 in Table 1), we ran an initial test of three models: 1) isolation in the western North Atlantic and spread to the east; 2) isolation in the eastern North Atlantic and spread to the west; and 3) isolation of western and eastern North Atlantic populations in separate refugia. We then tested the most probable historical demography from these initial comparisons (Model 3, see Results) with and without gene flow after separation.

For all three sets of analyses, we generated a reference table containing 1×10^6 simulated datasets for each scenario and used the 0.01% of simulated datasets closest to the observed genetic dataset to estimate posterior probabilities for each scenario. We used uniform priors ranging from 1×10^1 to 1×10^9 for both divergence time and effective population sizes, and a uniform prior for the mutation rate ranging from 1% per 100 000 years to 1% per 10 million years. The mutation rate, divergence times, and effective population sizes of the mitochondrial control region are unknown for gannets, so we used broad uninformative priors. Our priors for the mtDNA mutation rate span empirical estimates of the control region mutation rate (Pereira *et al*. 2004, Quinn 1992), and our priors for divergence times and effective population sizes allowed for recent or ancient timing of events and encompassed global and local gannet populations sizes (Carboneras *et al*. 2020a, 2020b, Mowbray 2020). The posterior probability of a scenario was estimated as the intercept of a logistic regression, where the proportion of the closest datasets made up by a model was predicted by the differences between the observed and simulated summary statistics (i.e., the "logistic approach" using DIYABC; Cornuet *et al*. 2014). If two scenarios were equally probable (their high probability densities [HPD] intervals overlapped), we estimated the posterior distributions of parameters from the combined posterior distribution of the most probable scenarios. The most probable models were used to estimate gene flow (if any), the timing of recent gene flow (if any), effective population sizes, and the timing of divergence.

Morphometrics

All adults that we captured for blood sampling were measured for 10 morphological traits: wing length (relaxed cord), half wingspan (both measured with a meter stick), tarsus, middle toe length, bill width at gonys, bill depth at gonys, bill depth at culmen, bill length (culmen to tip), head plus bill length, and head width (all measured with calipers). VLF measured all gannets except for Cape Gannets (measured by M. Peck, Royal Ontario Museum). Subsequently, both VLF and M. Peck measured all gannets at Bonaventure and adjusted measurements of Cape Gannets by the mean difference between measurements made by VLF and M. Peck at Bonaventure. We sexed Cape Gannets by the length of the gular stripe (Rishworth *et al*. 2014), whereas Northern and Australasian gannets were sexed using PCR primers and protocols described by Fridolfsson & Ellegren (1999).

We standardized individual measures for each of the 10 morphological variables by subtracting the mean value for each trait from the individual values and dividing the difference by the standard deviation. To replace blanks in the data, we interpolated missing values based on the regression between a particular morphological variable and its highest correlating alternative variable (all such regressions had $R^2 > 0.5$). No more than three values per variable were replaced in that way and so were unlikely to have any impact on the results. Nonetheless, because principal component analyses require values for all variables, not including those data would have substantially reduced our sample size. To test for differences in morphology among colonies, we ran a principal components analysis (PCA) on the 10 variables, and the first three principal components were inserted into a multi-factorial analysis (MFA) of variance with sex, subcolony, and colony as factors. We used Tukey's HSD criterion for post-hoc tests, and a Bonferroni correction (adjusted $\alpha = 0.01$) to account for multiple comparisons. We also used the first three principal components to conduct a multi-group discriminant analysis (DA) with colony as a factor. All analyses were completed within the "vegan" package of R 3.0 (Oksanen *et al*. 2013, R Core Team 2020).

RESULTS

Mitochondrial control region variation

We obtained 674 base pairs of mitochondrial control region sequence from 225 gannets (GenBank Accession numbers OK144288–OK144463; Table S1, Appendix 1, available on the website). Several features of the sequences supported the assumption that the amplified fragment is the true mitochondrial gene, as opposed to a nuclear homolog: (i) regions that were similar to conserved avian sequence blocks were detected in sequences from all three species (F, D, and C boxes; Baker & Marshall 1997), (ii) base-pair composition on the L-strand was biased against Gs, and (iii) variable sites were more frequent in Domain I than in Domain II (Fig. S1, Appendix 1).

We found 137 control region haplotypes, which were defined by 91 variable sites (86 sites with transitions, one site with a transversion, and four sites with both transitions and transversions. Estimates of Tajima's D did not differ from 0, providing no evidence of deviations from neutrality. However, most estimates of Fu's F_S were significantly less than 0 (Table 2). Negative values of F_S indicate an excess of haplotypes compared to neutral expectations, suggestive of population expansion (Fu 1996).

Phylogeographic and population genetic structure

No haplotypes were shared among species. Haplotypes from each of the three gannet species were monophyletic on the Bayesian gene tree, and these relationships were well supported (posterior probabilities of 0.86, 1.00, and 1.00; Fig. 2, see Fig. S2, Appendix 1 for the full tree with haplotype names and all posterior probabilities). Both of the putative Australasian Gannets from Malgas Island had a haplotype that was found only in other Australasian Gannets and clearly grouped with all other haplotypes from Australasian Gannets and no other Cape Gannets (Fig. S2). These two samples were excluded from further analyses.

Very little phylogenetic resolution existed within species, and no obvious geographic clades were present within either Australasian or Northern gannets (Fig. S2, Appendix 1). TCS networks also lacked obvious geographic structure (Supplementary Figs. S3–S5, Appendix 1).

Both Australasian and Northern gannets exhibited moderate population genetic structure across their ranges. Within Australasian Gannets, most haplotypes were private (10 each to Pedra Branca and Cape Kidnappers), often at high frequency (Table S1, Appendix 1); only four haplotypes were found in individuals from both colonies. The estimates of Φ_{st} and δ between Pedra Branca and Cape Kidnappers were both significantly greater than 0 ($\Phi_{st} = 0.08$, $\delta = 0.056$, both

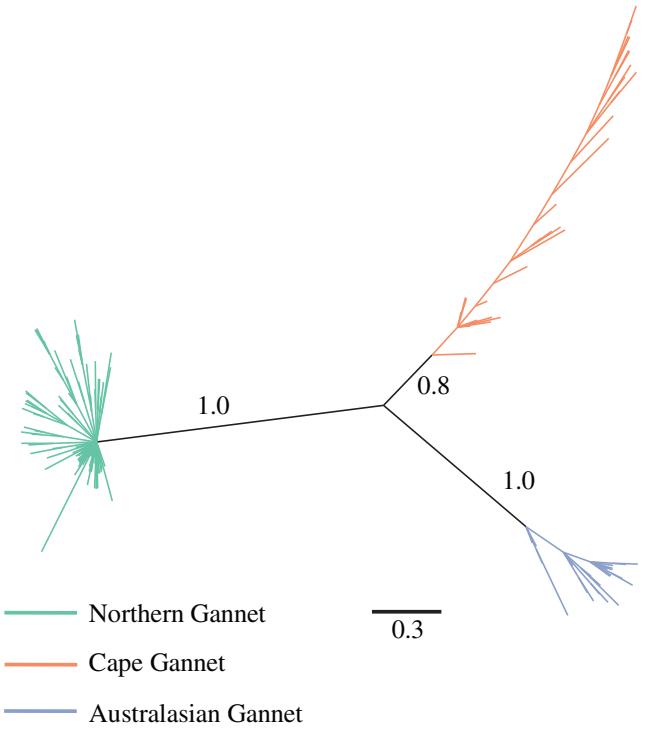

Fig. 2. Unrooted gene tree for gannet control region sequences from MrBayes, for Northern *Morus bassanus*, Cape *M. capensis*, and Australasian *M. serrator* gannets. Support values for the major nodes are Bayesian posterior probabilities.

$P < 0.01$). Within Northern Gannets, 19 haplotypes were shared among colonies. Of the shared haplotypes, six were shared between eastern and western Atlantic colonies, 10 were restricted to western Atlantic colonies (Funk, Bonaventure, and Magdalen islands), and three were restricted to eastern Atlantic colonies (Bass Rock and Skarvklakken; Table S1, Appendix 1). Sixty-six haplotypes were private to a single colony, but most of these haplotypes were found in only a single individual (Table S1, Appendix 1). Global population genetic structure in Northern Gannets was significantly greater than 0 (Φst = 0.06, $P < 0.0001$), and appeared to be driven primarily by genetic differentiation between the eastern versus western colonies: all pairwise estimates of Φ_{ST} were higher between colonies on opposite sides of the Atlantic (range: 0.04 to 0.12) than between colonies on the same side (range: –0.01 to 0.02, Table 3). Moreover, all Φ_{ST} estimates between colonies on opposite sides of the Atlantic were statistically significant, while no comparisons within either the eastern or western Atlantic were significant (Table 3).

TABLE 3
Estimates of Φ_{st} (below diagonal) and δ (as a %, above diagonal) between Northern Gannet *Morus bassanus* colonies based on mitochondrial control region variation[a]

	BON	MAG	FUN	BAS	SKA
BON		–0.01	0.02	0.11[b]	0.05[b]
MAG	–0.01		0.02	0.08[b]	0.03[b]
FUN	0.02	0.02		0.12[b]	0.08[b]
BAS	0.12[b]	0.09[b]	0.12[b]		0.01
SKA	0.05[b]	0.04[b]	0.09[b]	0.02	

[a] Population abbreviations are given in Table 2.
[b] Significantly greater than 0 at $\alpha = 0.05$.

Demographic history

The posterior distribution of the divergence time parameter (t) from IMa for Cape versus Australasian gannets peaked at 1.97 (Table 4; Fig. S6a, Appendix 1). Assuming a mutation rate of 1%/MY, this corresponds to a divergence time of approximately 290 000 years ago (90% HPD = 190 000 to 570 000 years). No gene flow appears to occur between Cape and Australasian gannets: the posterior distributions of both gene flow parameters peaked at zero (Table 4; Fig. S6a, Appendix 1).

For the two Australasian Gannet populations, the posterior distribution of t peaked at 1.83, which corresponds to an approximate divergence date of 270 000 years. However, a reliable HPD could not be estimated (Fig. S6c, Appendix 1). All other parameters were well estimated (Fig. S6c). Estimated gene flow between Pedra Branca and Cape Kidnappers did not differ from zero in either direction (Table 4; Fig. S6c, Appendix 1).

In the IMa analysis of eastern versus western Atlantic Northern Gannet colonies, the posterior distribution of t peaked at 1.46, corresponding to approximately 220 000 years before present (Table 4; Fig. S6b, Appendix 1). Long-term gene flow between the eastern and western Atlantic was not statistically different from zero in either direction (Table 4; Fig. S6b, Appendix 1).

Results from DIYABC for the Cape versus Australasian samples indicated that an origin from an Australasian ancestral population (Model 2) was more probable than either origin from a Cape gannet population, or vicariance within a widespread ancestral population (Models 1 and 3; Table 5). Given an Australasian origin, no gene flow after separation (Model 2.1) was more probable than recent gene flow (Models 2.2, 2.3; Table 5). Estimates of effective population size were larger than or similar to estimates from IMa, and estimates of the timing of divergence were older than or similar to estimates from IMa (Table 6).

Within Australasian gannets, DIYABC analyses indicated that historical vicariance of a common ancestor (Model 3) was more probable than an origin from either a New Zealand or Australian ancestral population (Model 1 or 2; Table 5). However, posterior probabilities of all three scenarios were all very similar, so we evaluated gene flow for each of our initial models (Model 1, 2, and 3). For each analysis, models that included recent gene flow were more probable than any of the initial models that did not include gene flow (Table 5). Given the uncertainty in model choice, we did not estimate the timing of divergences or migration rates between Australian and New Zealand gannets.

Results from DIYABC indicated that within Northern Gannets, historical vicariance (Model 3) was more probable than isolation within either the western or eastern North Atlantic (Models 1 or 2; Table 5). Given historical separation, models that included recent gene flow were more probable than models without recent gene flow (although the 95% HPDs overlapped for Models 3.2 and 3.3; Table 5). Estimates of effective population size in the Atlantic, and the timing of divergence between the eastern and western Atlantic, were on the same order of magnitude as estimates from IMa (Tables 4, 6).

TABLE 4
Estimates of model parameters and 90% highest posterior densities (HPD, given in square brackets) from IMa analyses

	Model parameter[a]	Estimate[b]	Estimated divergence time (y) or effective population size (females)	
			Most likely	90% HPD
Cape Gannets vs. Australasian Gannets	t	1.97 [1.30–3.84]	290 000	190 000–570 000
	$\Theta_{\text{Cape Gannet}}$	undefined	-	
	$\Theta_{\text{Australasian Gannet}}$	8.41 [5.26–13.1]	62 000	37 000–92 000
	$\Theta_{\text{Ancestral}}$	9.60 [4.17–19.6]	68 000	29 000–140 000
	$m_{\text{Cape Gannet -> Australasian Gannet}}$	0.00 [0.00–0.11]		
	$m_{\text{Australasian Gannet -> Cape Gannet}}$	0.00 [0.00–0.07]		
Australasian Gannets: Pedra Branca vs. Cape Kidnappers	t	1.83 [undefined]	270 000	-
	$\Theta_{\text{Pedra Branca}}$	3.61 [0.61–10.2]	27 000	4 300–72 000
	$\Theta_{\text{Cape Kidnappers}}$	5.87 [1.11–16.0]	41 000	7 800–110 000
	$\Theta_{\text{Ancestral}}$	1.79 [0.02–32.6]	13 000	140–230 000
	$m_{\text{Pedra Branca -> Cape Kidnappers}}$	0.00 [0.00–7.43]		
	$m_{\text{Cape Kidnappers -> Pedra Branca}}$	0.19 [0.00–11.19]		
Northern Gannets: eastern vs. western Atlantic	t	1.46 [0.94–2.26]	220 000	140 000–340 000
	Θ_{Eastern}	49.6 [25.9–101]	350 000	183 000–720 000
	Θ_{Western}	42.5 [25.9–67.7]	300 000	183 000–480 000
	$\Theta_{\text{Ancestral}}$	6.29 [1.86–14.1]	44 000	13 000–100 000
	$m_{\text{Eastern -> Western}}$	0.00 [0.00–0.63]		
	$m_{\text{Western -> Eastern}}$			

[a] Estimates of m are interpreted forwards in time (e.g., $m_{1->2}$ is interpreted as the migration rate from population 1 to 2).
[b] Estimates of t and Θ that changed depending on their prior distributions are referred to as undefined.

Morphometrics

The first principal component explained 61% of the variance in morphometrics, and all 10 factors loaded positively on this component. We therefore labeled that component "size". The second principal component explained 14% of the variance, and only tarsus and toe size showed significant loadings on that axis (therefore labeled "relative leg size"). The third principal component explained 7% of the variance, and only wingspan and wing chord showed significant loadings on that axis (labeled "relative wing size"). Size (PC1) depended on colony ($F_{6,189} = 151$, $P < 0.0001$) but not subcolony ($F_{2,189} = 0.33$, $P = 0.57$) or sex ($F_{2,189} = 4.05$, $P = 0.02$). Each species grouped separately for size using Tukey's HSD. Relative leg size (PC2) depended on colony ($F_{6,189} = 70.3$, $P < 0.0001$) and sex ($F_{2,189} = 6.53$, $P = 0.002$) but not subcolony ($F_{2,189} = 0.00$, $P = 0.95$). Males had smaller relative leg size (PC2) than females, while gannets from Funk Island, Cape Kidnappers, and Malgas Island all had different leg sizes relative to each other, with all remaining colonies grouping together. PC3 depended on colony ($F_{6,189} = 11.9$, $P < 0.0001$) but not subcolony ($F_{2,189} = 0.30$, $P = 0.59$) or sex ($F_{2,189} = 1.27$, $P = 0.28$). Given these results, we excluded subcolony from all subsequent analyses. Australasian Gannets and Northern Gannets from Bonaventure grouped together on relative wing size, with all other gannets in a separate group. Within the multivariate discriminant analyses, the three species clustered separately, with Northern Gannets being larger than the two other species, and Cape Gannets having relatively longer legs than Australasian Gannets (Fig. 3). Individual colonies also showed some significant discrimination, with gannets from Funk Island having relatively longer legs than other Northern Gannets, and Australasian gannets from Pedra Branca being larger with relatively longer legs than gannets from Cape Kidnappers.

DISCUSSION

Reproductive isolation of Cape and Australasian gannets

The species status of Cape versus Australasian gannets has been uncertain due to the presence of mixed pairs and mature hybrid individuals (reviewed in Dyer *et al*. 2001, Robertson & Stephenson 2005, Ismar *et al*. 2011). There are several reports of Cape and Australasian gannets nesting together in both South Africa and New Zealand, and chicks, fledglings, and mature hybrids have been documented (Dyer *et al*. 2001, Robertson & Stephensen 2005 and references therein, Ismar *et al*. 2011). Two phenotypic Australasian Gannets that were sampled nesting at the Cape Gannet colony at Malgas Island during the present study possessed a mitochondrial control region haplotype otherwise found only in Australasian Gannets, supporting their identity as Australasian Gannets. Whether these two birds had mates or fledged chicks was not recorded. However, our results did not support any of our predictions of introgression between these two species (H1 in Table1) because

TABLE 5
Historical models tested using DIYABC, posterior probabilities, and 95% highest posterior densities (HPD) for posterior probabilities

Model	Posterior probability	95% HPD
Cape and Australasian gannets are reproductively isolated (Hypothesis 1 from Table 1)		
1. Cape origin, spread to Australasia	0.25	0.23–0.26
2. Australasia origin, spread to the Cape	0.46	0.45–0.48
2.1. No recent gene flow	0.89	0.87–0.92
2.2. Recent gene flow from Cape to Australasian gannets	0.04	0.03–0.04
2.3. Recent gene flow from Australasian to Cape gannets	0.08	0.07–0.09
3. Vicariance within a widespread ancestral population	0.28	0.27–0.30
Australian and New Zealand Australasian Gannets diverged in separate refugia (Hypothesis 2.1 in Table 1)		
1. Refugium in Australia, spread to New Zealand	0.30	0.29–0.31
1.1 No recent gene flow	0.09	0.07–0.10
1.2 Recent gene flow from Australia to New Zealand	0.38	0.36–0.40
1.3 Recent gene flow from New Zealand to Australia	0.54	0.52–0.55
2. Refugium in New Zealand, spread to Australia	0.33	0.32–0.34
2.1 No recent gene flow	0.10	0.09–0.11
2.2 Recent gene flow from New Zealand to Australia	0.46	0.45–0.47
2.3 Recent gene flow from Australia to New Zealand	0.44	0.43–0.45
3. Separate refugia	0.36	0.35–0.38
3.1 No recent gene flow	0.11	0.10–0.12
3.2 Recent gene flow from New Zealand to Australia	0.42	0.40–0.43
3.3 Recent gene flow from Australia to New Zealand	0.47	0.46–0.48
Western and eastern North Atlantic Northern Gannets diverged in separate refugia (Hypothesis 3.1 in Table 1)		
1. Western North Atlantic refugium, spread to the east	0.23	0.22–0.24
2. Eastern North Atlantic, spread to the west	0.25	0.24–0.26
3. Two North Atlantic refugia	0.52	0.50–0.53
3.1. No recent gene flow	0.11	0.10–0.12
3.2. Recent gene flow from western to eastern Atlantic	0.52	0.42–0.62
3.3. Recent gene flow from eastern to western Atlantic	0.39	0.30–0.48

TABLE 6
Estimates of model parameters and 95% highest posterior densities (HPD) from DIYABC analyses

Model	Model parameter[a]	Estimated divergence time (y) or effective population size (females)	
		Mode	95% HPD
2.1 Origin of Cape Gannets from Australasian Gannets, no gene flow	t	377 000	157 000–794 000
	$\Theta_{\text{Cape Gannet}}$	645 000	278 000–1 200 000
	$\Theta_{\text{Australasian Gannet}}$	276 000	107 000–658 000
3.1 Isolation of Northern Gannets in two North Atlantic refugia	t	193 000	14 000–666 000
	Θ_{Eastern}	627 000	178 000–1 320 000
	Θ_{Western}	624 000	157 000–1 290 000
	$\Theta_{\text{Ancestral}}$	203 000	48 000–776 000
3.2 Recent gene flow from the western to eastern North Atlantic	$t_{\text{Eastern -> Western}}$	574	0–1 230
	$m_{\text{Eastern -> Western}}$	0.91	0.09–0.98
3.3 Recent gene flow from the eastern to western North Atlantic	$t_{\text{Western-> Eastern}}$	431	0–1 010
	$m_{\text{Western -> Eastern}}$	0.69	0.08–0.98

[a] Estimates of m are interpreted forwards in time (e.g., $m_{1->2}$ is interpreted as the migration rate from population 1 to 2).

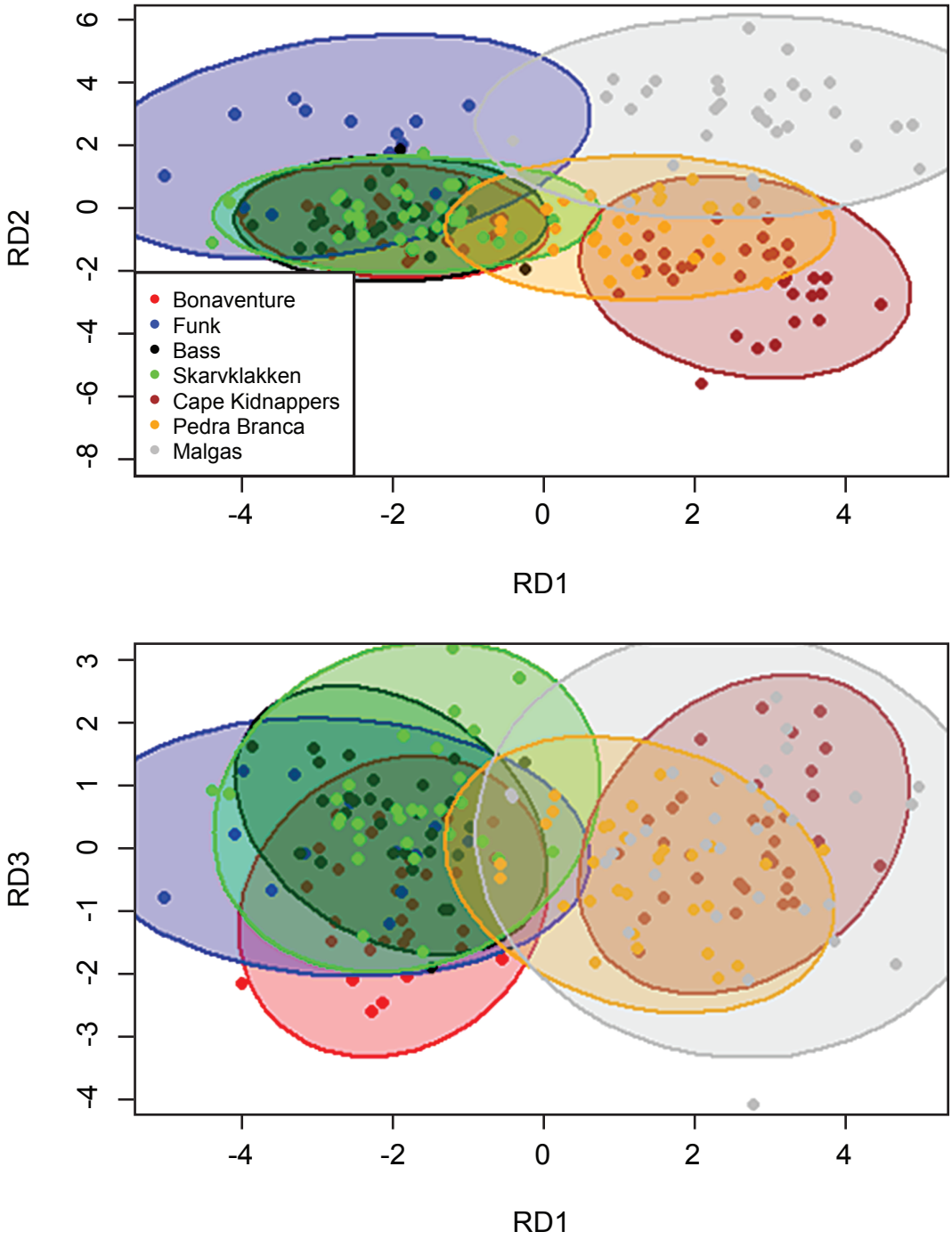

Fig. 3. Redundancy analysis of 10 morphometric traits for gannets. RD1, RD2, and RD3 are the first, second, and third major discriminant axes. Bonaventure, Funk, Bass, Skarvklakken = Northern Gannets *Morus bassanus*; Cape Kidnappers, Pedra Branca = Australasian Gannets *Morus serrator*; Malgas = Cape Gannets *Morus capensis*.

none of the phenotypic Cape or Australasian gannets had mtDNA haplotypes found in heterospecific clades; estimates from IMa of female-mediated gene flow between these species did not differ from zero; and DIYABC did not support models of migration between Cape and Australasian gannets. Nupen (2014) also did not find evidence of introgression in her survey of mitochondrial and intron sequences of Cape Gannets sampled throughout the species' range. For example, of 94 Cape Gannets sequenced from six colonies, none possessed mtDNA haplotypes characteristic of Australasian Gannets. Thus, hybridization must be rare, or it must result in either infertile F1 hybrids or inviable backcross offspring (i.e., post-zygotic isolation). Discrimination among these possibilities, and confirmation that Cape and Australasian gannets represent reproductively isolated species despite occasional hybridization, will require analysis of nuclear genes in large numbers of individuals from multiple colonies of both species.

Estimates of divergence dates for mtDNA lineages of Cape and Australasian gannets from IMa and DIYABC were similar (i.e., had overlapping HPDs; Tables 4, 6) at 290 000 and 377 000 years ago,

respectively. Based on smaller numbers of samples, but including one mitochondrial and five nuclear genes, Patterson *et al*. (2011) estimated that these two species diverged 500 000–700 000 years ago. The slight discrepancy between our estimates and those of Patterson *et al*. may be because of uncertainty in mutation rates, mitochondrial introgression after speciation, or both. Regardless, Cape and Australasian gannets appear to be young lineages that originated during the climatic and oceanographic changes of the late Pleistocene.

Population genetic differentiation within Australasian and Northern gannets

Despite evidence of dispersal of young gannets from New Zealand to Australian waters, potentially promoting gene flow (see Introduction), we found significant differentiation in mtDNA between Australasian Gannets from Australia versus New Zealand (H2 in Table 1), although some female-mediated gene flow may be occurring. These results are similar to findings for other species of Southern Hemisphere seabirds (e.g., Little Penguins: Overeem *et al*. 2008, Peuker *et al*. 2009; reviewed in Friesen *et al*. 2007, Friesen 2015, Lombal *et al*. 2020). Although neither IMa nor DIYABC could estimate *t* with confidence for these populations, both supported a model of historical separation (H2.1 in Table 1).

Although not phylogeographically structured, mtDNA variation in Northern Gannets from the western versus eastern North Atlantic was significantly differentiated (H3 in Table 1), with little or no female-mediated gene flow. These results are consistent with band returns, which indicate little trans-Atlantic gene flow in Northern Gannets, as well as differences in non-breeding and foraging distributions between western and eastern populations (see Introduction). From our results, we suggest that these differences may have arisen during isolation in separate glacial refugia (H3.1 in Table 1), because estimates from IMa and DIYABC indicate that mtDNA lineages of these populations diverged ~200 000 years ago, which pre-dates recession of the Laurentide and Scandanavian ice sheets (Hewitt 1996). Although the location of a western Atlantic refugium is unknown, fossil evidence indicates that gannets occurred in the Mediterranean Sea from 30 000–40 000 years ago until 2 700 years ago (Tyrberg 1999, Oros Sršen *et al*. 2017). Phylogeographic variation indicates that several other species of North Atlantic seabirds also survived the Pleistocene in multiple refugia (e.g., European Storm petrel *Hydrobates pelagicus*, Cagnon *et al*. 2004; Scopoli's Shearwater *Calonectris diomedea*, Gómez-Díaz *et al*. 2006; Common Murre, Morris-Pocock *et al*. 2008; European Shag *Phalacrocorax aristotelis*, Thanou *et al*. 2016; reviewed in Friesen 2015, Lombal *et al*. 2020).

In contrast, we found no evidence of population structure in mtDNA in Northern Gannets within either Canada or Europe (H4 in Table 1). This finding is consistent with evidence from band returns indicating on-going gene flow within each of these regions (see Introduction), and overlapping non-breeding distributions. Our results also support the hypothesis that Northern Gannets have undergone recent population expansions (H4.1 in Table 1), in that the number of haplotypes exceeded neutral expectations for most colonies (Fu's F_S Table 2), and estimates of effective sizes of contemporary populations exceeded those for the ancestral populations in both IMa and DIYABC. These results are consistent with colony surveys indicating population expansion in Europe and Canada in the past 100 years (Wanless & Harris 2004, Mitchell *et al*. 2004, Chardine *et al*. 2013, Barrett *et al*. 2017) and archaeological evidence of expansion in Norway in the past 8 000 years (Montevecchi & Hufthammer 1990). Patterns of genetic variation indicate that several other North Atlantic species of seabirds underwent recent (Holocene) range expansions (e.g., Common Murres, Moum & Arnason 2001, Morris-Pocock *et al*. 2008; reviewed in Friesen *et al*. 2007, Friesen 2015). Interestingly, Nupen (2014) also did not find any differentiation in either mtDNA or two nuclear introns among Cape Gannets sampled from throughout their breeding range in South Africa and Namibia.

Geographic variation in morphology

Much of the geographic variation in morphology uncovered in this study was explained by differences in size and shape between species. However, significant intraspecific variation in shape was also detected, especially in leg and wing dimensions: Australasian Gannets from Cape Kidnappers had shorter legs relative to overall body size compared to gannets from Pedra Branca; Northern Gannets from Bonaventure had different wing dimensions from Northern Gannets elsewhere; and Northern Gannets from Funk Island had relatively longer legs, although the differences were slight and may not be biologically meaningful (Fig. 3). Geographic variation in morphology within species may result from phenotypic plasticity or local adaptation. Funk Island is in a colder ocean current than the other colonies in this study, and so gannets breeding there may experience slightly different selective pressures (H5 in Table 1). Several other species of seabirds in the North Atlantic also exhibit geographic variation in morphology (see Introduction), and Cape Gannets from Malgas Island (another island situated in a cold-water current) also had long legs relative to body size. Whether the morphological variation represents phenotypic plasticity or genetic adaptation requires more rigorous testing, e.g., including measurements of gannets from other colonies in the Labrador and East Greenland currents.

Mechanisms of diversification

Based on our analyses, we suggest that historical vicariance is the strongest predictor of population genetic structure in gannets, in agreement with the conclusions of Lombal *et al*. (2020) for other seabird species. We found no evidence of selection on mtDNA sequence variation in this study, indicating that differentiation is most likely due to genetic drift as a function of the duration of isolation and the genetically effective population size, rather than selection on the control region or linked loci (e.g., Avise *et al*. 1987). Genetic differentiation initiated by isolation in separate refugia could be maintained or promoted by non-overlapping non-breeding distributions or geographic distance, as follows:

Non-breeding distributions—Northern Gannets from Canada versus Europe (including Iceland) winter on opposite sides of the Atlantic (Fort *et al*. 2012, Fifield *et al*. 2014, Garthe *et al*. 2016), which could reduce gene flow between these regions. However, a few Northern Gannets breeding in eastern Canada have been tracked to western Africa in winter (Fifield *et al*. 2014), and distributions of juvenile Australasian Gannets from New Zealand overlap with breeding areas in Australia (see Introduction). Thus, non-breeding distributions are unlikely to be maintaining the regional population genetic structure in gannets. Lombal *et al*. (2020) also did not find differences in non-breeding distributions to be a significant predictor of population genetic structure in a multivariate analysis of geographic variation in mtDNA in seabirds.

Geographic distance—Although we could not explicitly test for isolation by distance, the most proximate Canadian and European colonies of Northern Gannets (Funk Island and Eldey, Iceland) are separated by ~2 400 km of open ocean. Similarly, the most proximate Southeastern Australian versus New Zealand colonies of Australasian Gannets (Eddystone Island versus Farewell Spit) are separated by 2 050 km of open ocean. Thus, large expanses of open ocean may help maintain genetic isolation in gannets. The observation that juvenile Australasian Gannets from New Zealand migrate to Australia, yet little to no gene flow occurs, remains to be explained.

Because mtDNA represents a single supergene that reflects primarily female-mediated gene flow, rigorous testing of the role of these mechanisms in promoting and maintaining population differentiation in gannets will require analysis of a large number of nuclear loci, including samples from a large number of colonies of Northern and Australasian gannets.

Conservation implications

Populations of Australasian and Northern gannets appear to be stable or increasing (www.iucnredlist.org), apparently due to cessation or lack of direct and indirect persecution; long-term (century-scale) increases in sea-surface temperatures and prey in southeastern Australia and the North Atlantic; and possibly removal of gillnets associated with ocean-basin scale fishery closures in eastern Canada during the early 1990s (Nettleship & Chapdelaine 1984, Wodzicki *et al*. 1984, Montevecchi & Myers 1997, Bunce *et al*. 2002, Mitchell *et al*. 2004, Barrett 2008, Nelson 2010, Chardine *et al*. 2013, Regular *et al*. 2013, Murray *et al*. 2014, Barrett *et al*. 2017, Montevecchi *et al*. 2021). Cape Gannets, however, are declining, possibly owing to climate and fishing influences on prey availability (Crawford *et al*. 2007, 2014, Green *et al*. 2014), and are listed as Vulnerable by the International Union for the Conservation of Nature. Furthermore, seasonal migrants, such as gannets, are exposed to different threats at their breeding, migration, and non-breeding areas (Montevecchi *et al*. 2012). Genetic data can help delineate appropriate population units for conservation (e.g., Moritz 1994, Barbosa *et al*. 2018). Evidence of population genetic structure, but not phylogeographic structure, within both Australasian and Northern Gannets suggests that each of these species comprises a single evolutionarily significant unit with two management units (*sensu* Moritz 1994): Southeastern Australia versus New Zealand for Australasian Gannets; and Canada versus Europe for Northern Gannets. These results should be confirmed through the analysis of nuclear DNA from samples from a larger number of colonies of both species, including Australasian Gannets from Norfolk Island and Northern Gannets from Iceland. Band returns and wintering distributions of Northern Gannets from Iceland (Scotland to Northwestern Africa, Garthe *et al*. 2016) suggest they are likely more similar genetically to Northern Gannets from Europe versus North America.

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