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Evidence for strong assortative mating, limited gene flow, and strong differentiation across the blue-footed/Peruvian booby hybrid zone in northern Peru

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Hybrid zones represent natural laboratories in which the processes of divergence and genetic isolation can be examined. The generation and maintenance of a hybrid zone requires mispairing and successful reproduction between organisms that differ in one or more heritable traits. Understanding the dynamics of hybridization between two species requires an understanding of the extent to which they have diverged genetically, the frequency of misparing and hybrid production, and the extent of introgression. Three hundred and twenty one blue-footed *Sula nebouxii* and Peruvian *S. variegata* boobies from the eastern tropical Pacific Ocean were analyzed using 19 putatively neutral genetic markers to evaluate interspecific differentiation, to classify morphological hybrids using Bayesian assignments, and to characterize hybridization using cline theory and Bayesian assignments. The species were well differentiated at mitochondrial and nuclear microsatellites, the hybrid zone was bimodal (contained a high frequency of each parental species but a low frequency of hybrids), and morphologically intermediate individuals were most likely F1 hybrids resulting from mating between female Peruvian boobies and male blue-footed boobies. Clines in allele frequency could be constrained to share a common geographic centre but could not be constrained to share a common width. Peruvian and blue-footed boobies hybridize infrequently, potentially due to strong premating reproductive isolation; however, backcrossing appears to facilitate introgression from blue-footed to Peruvian boobies in this hybrid system.

Hybridization can be defined operationally as the 'the interbreeding of individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters' (Harrison 1990). Understanding the causes and consequences of hybridization has been recognized as an important avenue of research in evolutionary biology for more than a century (Endler 1977, Harrison 1993, Arnold 1997, Jiggins and Mallet 2000, Alexandrino et al. 2005, Gay et al. 2007, 2008, Ruegg 2008, Brelsford and Irwin 2009, Aboim et al. 2010). Hybridization between closely related species can have numerous outcomes ranging from no reproductive output to the production of fertile offspring that are not reproductively isolated from either parental population (reviewed by Allendorf et al. 2001), and often produces a stable hybrid zone between the interacting species (Barton 1983, Barton and Hewitt 1985, Hewitt 1988, Kruuk et al. 1999). Hybrid zones are regions where closely related species coexist, mate, and produce hybrid offspring (Barton and Hewitt 1985).

Hybridization is a common phenomenon in natural systems (Hewitt 1988, Harrison 1993, Arnold 1997), and many hybrid zones have been characterized using morphology (Gay et al. 2007, 2008, Ruegg 2008, Brelsford and Irwin 2009, Irwin et al. 2009), behaviour (Toews and Irwin 2008), and DNA (Syzmura 1976, Pierce and Mitton 1980, Gay et al. 2007, 2008, Carling and Brumfield 2008, Ruegg 2008, Toews and Irwin 2008, Brelsford and Irwin 2009, Pereira and Wake 2009, Aboim et al. 2010).

Understanding the dynamics of gene flow in hybrid zones, and the relative importance of endogenous (i.e. genetic incompatibility) versus exogenous (i.e. ecological) isolating mechanisms can provide insight into speciation processes (Barton and Hewitt 1985, Hewitt 1988, Jiggins and Mallet 2000). For example, hybridization may occur upon secondary contact between two previously physically isolated and genetically differentiated species. An examination of gene flow under these circumstances can shed light on the process of reinforcement, and on the importance of genetic incompatibility (endogenous factors) in the maintenance of hybrid zones and the process of speciation. Alternatively, hybridization may occur between species that have diverged through ecological speciation in the absence of physical isolation, and an examination of gene flow should identify exogenous factors important in divergence and speciation processes.

Both endogenous and exogenous factors generate and maintain hybrid zones, and differentiating the roles of these factors in a given hybrid zone can be challenging (Hewitt 1988, Kruuk et al. 1999, Jiggins and Mallet 2000). Given the diversity of possible outcomes when hybridization occurs, a number of models have been developed to understand the dynamics operating in hybrid zones. The models include the neutral diffusion model (Endler 1977, Barton and Hewitt 1985, Barton and Gale 1993), the bounded hybrid superiority model (May et al. 1975, Moore 1977), and the tension zone model (Key 1968, Barton and Hewitt 1985). Prior to determining the most appropriate model for a given hybrid zone one must first examine interspecific differentiation, frequency of hybridization, and level of introgression.

Blue-footed *Sula nebouxii* and Peruvian *S. variegata* boobies breed within the eastern tropical and subtropical Pacific Ocean, and both species are capable of long distance dispersal (Fig. 1; Nelson 1978, Aid et al. 1985, Simeone et al. 2002). They are parapatric (Nelson 1978), hybridize on two islands, Lobos de Tierra and Lobos de Afuera in northern Peru (Ayala 2006, Figueroa and Stucchi 2008, Taylor et al. 2010b), and diverged recently (0.25–0.45 mya) (Friesen and Anderson 1997, Patterson et al. 2010). Blue-footed boobies outnumber Peruvian boobies on Lobos de Tierra by an order of magnitude (Zavalaga unpubl.), with a total population estimate for both species of approximately 300 000 individuals (~270 000 blue-footed boobies, 3000 Peruvian boobies) (Zavalaga et al. 2011). On Lobos de Afuera the species exist at relatively equal numbers: in 2004, Figueroa and Stucchi recorded 35 000 blue-footed boobies and 20 000 Peruvian boobies, and population sizes on both islands have been relatively stable since 2000 (Zavalaga unpubl.).

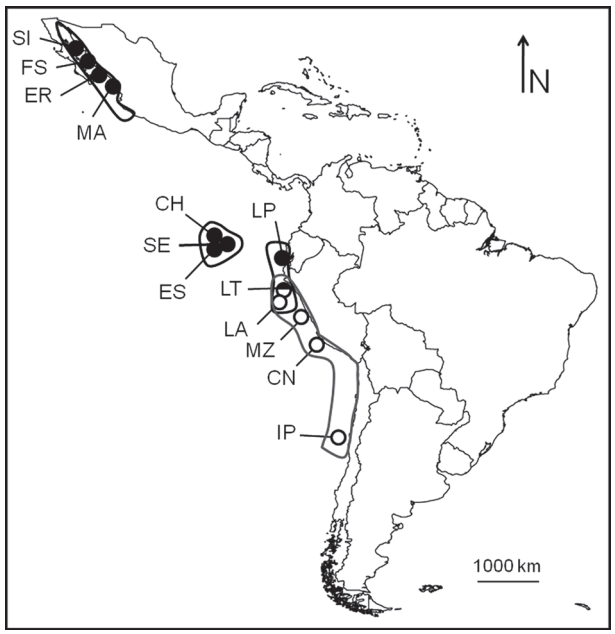

Figure 1. Map of sampling locations. Approximate breeding distributions outlined in black for blue-footed boobies and in dark grey for Peruvian boobies; blue-footed booby sample sites indicated by black circles, Peruvian booby sample sites indicated by white circles. Colony codes as in Table 1.

Aberrant individuals possessing characteristics intermediate between blue-footed and Peruvian boobies have been recorded on both islands where the species co-occur (Ayala 2006, Figueroa and Stucchi 2008, Taylor et al. 2010b, Zavalaga unpubl.). These intermediate individuals are commonly observed in breeding pairs, and have thus far been reported as females paired to male blue-footed boobies (Ayala 2006, Taylor et al. 2010b), or males courting female blue-footed boobies (Figueroa and Stucchi 2008). The majority of intermediate females were observed successfully laying eggs and raising chicks (Ayala 2006), or incubating eggs (Taylor et al. 2010b), indicating that backcrossing between these species occurs.

Both blue-footed and Peruvian boobies exhibit low population genetic structure throughout their respective ranges (Taylor et al. 2010a, b, 2011a, b), and the islands where they are sympatric are situated close to, and are influenced by, the transition between cold nutrient-rich waters of the Peruvian upwelling system and warmer nutrient-poor waters of the North Equatorial Countercurrent (Pennington et al. 2006). Sulids have occupied the eastern tropical Pacific, including the Peruvian upwelling and islands within the North Equatorial Countercurrent, since the middle Miocene (Stucchi and Devries 2003), and the islands where both blue-footed and Peruvian boobies breed have been occupied by both species at least since recorded history, and probably for thousands of years prior to European discovery (Murphy 1925). The Peruvian upwelling was established well before the divergence of blue-footed and Peruvian boobies from their common ancestor (Hartley et al. 2005, Patterson et al. 2010), and both species regularly travel long distances within the eastern tropical Pacific, increasing their potential to encounter heterospecifics and making these seabirds excellent candidates for the examination of hybridization between recently diverged marine vertebrates capable of long distance dispersal.

Using classic population genetic methods and Bayesian assignment tests, we sought to evaluate interspecific differentiation between blue-footed and Peruvian boobies, to assess linkage disequilibrium within the area of sympatry, and to determine hybrid classes of intermediate individuals and frequency of hybrids within the area of sympatry. Additionally, using theory developed by Barton and Hewitt (1985), we characterized changes in species specific allele frequencies across the geographic range of both species, including the region of overlap, to determine if hybridization was resulting in introgression. To accomplish this we used 19 putatively neutral genetic markers and samples from 321 individuals, including 5 morphologically intermediate individuals.

Methods

Sampling and DNA extraction

Blood samples were collected (as described in Müller et al. 2008, Zavalaga et al. 2009) from throughout the breeding distributions of blue-footed and Peruvian boobies (Fig. 1, Table 2). Original samples are archived at Queen's Univ. DNA was extracted using either a PureLink extraction

Table 1. Colony locations, distances from Isla Pajaros, and sample sizes for sampled colonies of blue-footed (BFBO) and Peruvian (PEBO) boobies and morphological hybrid individuals. mtDNA = number of individuals genotyped at mitochondrial control region, nDNA = number of individuals genotyped at microsatellite loci.

Species	Colony	Code	Location	Distance from Isla Pajaros (km)	N (mtDNA/nDNA)
BFBO	Isla San Ildefonso, Mexico	SI	26°43′N, 111°29′W	7485	10/10
	Farallon de San Ignacio, Mexico	FS	25°24′N, 108°50′W	7318	15/15
	El Rancho, Mexico	ER	25°06′N, 108°22′W	7267	13/14
	Islas Marietas, Mexcio	MA	21°33′N, 106°23′W	7267	03/03
	Seymour Island, Galapagos	SE	00°23′S, 90°17′W	3862	06/11
	Champion Island, Galapagos	CH	01°13′S, 90°21′W	3862	06/10
	Espanola Island, Galapagos	ES	01°21′S, 89°41′W	3862	03/10
	Isla La Plata, Ecuador	LP	01°16′S, 81°03′W	3319	54/47
	Lobos de Tierra, Peru	LT	06°26′S, 80°51′W	2766	44/48
PEBO	Lobos de Tierra, Peru	LT	06°26′S, 80°51′W	2766	32/30
	Lobos de Afuera, Peru	LA	06°57′S, 80°41′W	2705	30/30
	Isla Mazorca, Peru	MZ	11°23′S, 77°43′W	2135	27/29
	Isla Chincha Norte, Peru	CN	13°38′S, 76°22′W	1851	32/30
	Isla Pajaros, Chile	IP	29°37′S, 71°24′W	0	25/29
Hybrid	Lobos de Tierra, Peru	LT	06°26′S, 80°51′W	2766	04/04
	Lobos de Afuera, Peru	LA	06°57′S, 80°41′W	2705	01/01

kit (Invitrogen: Burlington, Canada) according to the manufacturer's recommended protocol, or a standard protease-K phenol/chloroform technique (Sambrook and Russell 2001). As in Taylor et al. (2010b) samples from Islas Marietas were combined with samples from nearby El Rancho for all analyses because the sample size from Islas Marietas was small, and no significant genetic differences ($p > 0.05$) were found between Isla Marietas and El Rancho. Samples from islands within the Galápagos archipelago were also combined for all analyses because they were not significantly differentiated ($p > 0.05$; Taylor et al. 2011a, b). Although there are two sites of sympatric breeding, permits to sample blue-footed boobies on Lobos de Afuera could not be obtained.

Five individuals identified by morphology as hybrids (Taylor et al. 2010b) were captured intentionally and included in the sampling for the present study: these individual were not among the birds caught randomly for population genetic analyses. These individuals possessed plumage and bare-part colouration intermediate between blue-footed and Peruvian boobies, including orange irises, mottled blue-grey feet, and intermediate crown and nape feather structure and colouration, and were very distinct from either parental species (see Fig. 2 and 3 in Taylor et al. 2010b): blue-footed boobies possess yellow irises, blue feet, and predominantly brown crown and nape feathers, while Peruvian boobies possess red irises, grey feet, and white head and nape feathers.

Table 2. Percent of simulated birds assigned to each of five hybrid categories by Structure and Newhybrids. Rows sum to 100% for each program. First number is result from Structure and second number is result from Newhybrids.

Simulated genotype	% assigned to				
	BFBO	PEBO	F1	F1 × BFBO	F1 × PEBO
BFBO	100/82	0/0	0/0	0/8	0/0
PEBO	0/0	100/64	0/0	0/0	0/36
F1	0/0	0/0	96/68	4/0	0/32
F1-BFBO	16/4	0/0	8/0	76/96	0/0
F1-PEBO	0/0	16/0	12/0	0/0	72/100

Laboratory protocols: mitochondrial DNA

DNA was extracted and a 540 base pair (bp) region including all of domains I and II and part of domain III of the mitochondrial control region was sequenced from 305 individuals using the primers SdMCR-H750 and SlMCR-L160A as described in Taylor et al. (2011a, b). Data were previously published in Taylor et al. (2011a, b); however, the analyses presented here are entirely new.

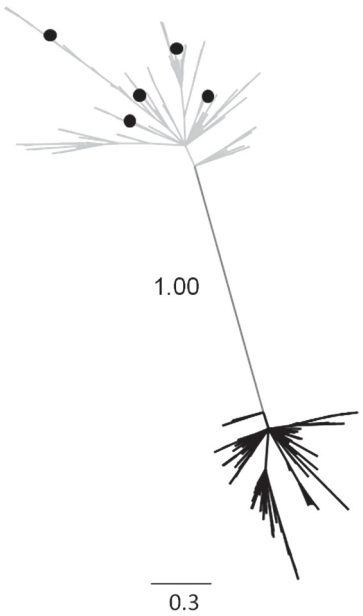

Figure 2. Unrooted Bayesian mitochondrial control region gene tree. Support for major clades indicated by posterior probability. Peruvian booby haplotypes indicated by grey lines; blue-footed booby haplotypes indicated by black lines. Black circles indicate Peruvian booby mitochondrial control region haplotypes possessed by morphological hybrids.

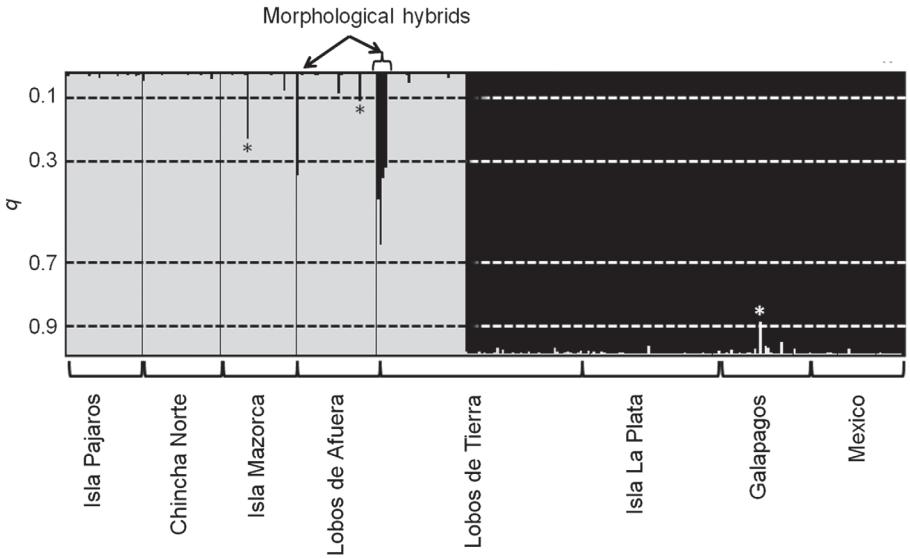

Figure 3. Probability of assignment to each of two genetic populations for individual blue-footed (in black) and Peruvian (in grey) boobies as inferred by Structure. q = the probability of assignment to the genetic population displayed in black. Dashed lines indicate threshold q values used to classify individuals (see text). Sampling sites are given on the x-axis, q values are given on the y-axis. *indicates individuals identified as pure parentals by morphology, but as hybrids by Structure.

Laboratory protocol: microsatellites

Eighteen microsatellite loci were amplified from 321 individuals using the PCR methods described in Taylor et al. (2010a, 2011a, b). Thirteen loci were combined for amplification in five multiplexes and five loci were amplified in single locus PCRs ([1] Sn2B-68, Sv2A-53 Ss1B-100; [2] Ss1B-88, Sn2B-83, Sv2A-47; [3] BOOB-RM4-C03, BOOB-RM4F11, BOOB-RM4-G03; [4] BOOB-RM4-D07, BOOB-RM4-E10; [5] BOOB-RM2-F07, BOOB-RM4-E03; [6] Ss2B-138; [7] BOOB-RM3-F11; [8] BOOB-RM4-B03; [9] BOOB-RM3-D07; [10] Sn2A-36). Thirteen of the primer pairs were designed from blue-footed booby genomic libraries (Faircloth et al. 2009, Taylor et al. 2010a), two were designed from a Peruvian booby genomic library (Taylor et al. 2010a), and three were designed from a red-footed booby *Sula sula* genomic library (loci Ss1b-88, Ss1b-100, Ss2b-138) (Molecular Ecology Resources Primer Development Consortium 2010). Primers for locus Ss1B-88 and locus Ss1B-100 were redesigned for blue-footed boobies (Taylor et al. 2011a).

Amplification, PCR confirmation, and fragment sizing were performed as described in Taylor et al. (2010a). A total of 180 randomly chosen locus-specific genotypes, about 3% of the total sample, was re-amplified and rescored at randomly selected loci to check for repeatability in allele size scoring. Data for eight microsatellite loci were published previously (Taylor et al. 2010b, 2011a, b); however, data for ten loci and the analyses presented here are entirely new.

Data analyses

Unless stated otherwise, data analyses were carried out on the full 321 individual dataset.

Interspecific differentiation

To examine evolutionary relationships among sequences of blue-footed and Peruvian booby control regions, an unrooted Bayesian phylogenetic tree was generated in MrBayes (ver. 3.1.2; Ronquist and Huelsenbeck 2003). The tree could not be rooted due to the extreme divergence in potential outgroups. MrModelTest (ver. 2.3; Nylander 2004) was used to determine the appropriate nucleotide substitution model and the parameters of the substitution model were allowed to vary during MrBayes analyses. MrBayes runs used the default parameters, consisting of one cold chain and three incrementally heated chains, ran for 1.0×10^7 generations, and were sampled every 100 generations. Runs were considered to have converged when the standard deviation of the split frequencies fell below 0.01. Convergence was also assessed by examining traces of parameter estimates in Tracer (ver. 1.5; Rambaut and Drummond 2007). A burn-in of 25% was discarded after MCMC chains had converged. The analysis was repeated three times to assess reliability, and results of a single run were drawn using FigTree (ver. 1.3.1; Rambaut 2009).

Interspecific differentiation at the mitochondrial control region and at nuclear microsatellite loci was indexed using analyses of molecular variance (AMOVA) in Arlequin (ver. 3.11; Excoffier et al. 2005). Individuals were grouped by species and sympatric populations were excluded from the analysis to ensure interspecific differentiation was not underestimated. Mitochondrial divergence estimates were corrected using Kimura's two-parameter substitution model (Kimura 1980) with a rate parameter (α) of 0.45 as determined using MrModelTest (ver. 2.3; Nylander 2004). Significance of all Arlequin analyses was determined by comparing the results to 10 000 random permutations of the data at an α level of 0.05.

Interspecific differentiation of nuclear microsatellites was further examined using Structure (ver. 2.3.1; Pritchard

et al. 2000). Structure analyses were performed using an admixture model and correlated allele frequencies, a burn-in of 50 000 cycles, and 500 000 additional cycles (determined from test runs to be sufficient for parameter stabilization). Analyses were repeated 20 times for K = 1 to 4 genetic populations. K was constrained to a maximum of four genetic clusters because previous work on blue-footed boobies showed a high likelihood of two genetic clusters (Taylor et al. 2011a), while previous research on Peruvian boobies showed the highest likelihood of one genetic cluster (Taylor et al. 2011b). The posterior probability, Ln[P(D)], as well as the method of Evanno et al. (2005) were used to infer the most likely number of genetic clusters. Figures from Structure analyses were generated using Distruct (ver. 1.1; Rosenberg 2004).

Genetic cline analysis

To investigate introgression using cline theory the program Cfit (ver. 0.6; Gay et al. 2008) was used. The program can use up to six parameters to fit different cline shapes for allele or haplotype frequencies, and estimates the shape parameters of each cline by modeling the relationship between allele or haplotype frequency and geographic location for each population; the cline shapes modeled by Cfit are a function of distance (x) and describe allelic frequency ($f(x)$) (Gay et al. 2008). The shape parameters of each cline are estimated using three equations developed by Syzmura and Barton (1986, 1991) and outlined in Gay et al. (2008). Briefly, one equation describes the centre of the cline as a symmetrical sinusoidal shape and includes the cline centre (c), cline width (w), height, and geographic location (x) as parameters. The other two equations describe the change in allele frequencies in the left and right tails of the cline and include the same parameters as the previous equation, in addition to parameters for the exponential decay of allele frequencies away from the centre of the cline (Carling and Brumfield 2008, Gay et al. 2008). From these equations the cline centre and cline width (where cline width is equal to 1/maximum slope) can be estimated and compared between loci. The cline centre is the geographic location along the sampling transect where allele frequencies change most rapidly, and the width is the geographic distance over which the change in allele frequencies occurs (Carling and Brumfield 2008). For this study geographic location was defined as the distance in kilometres from Isla Pajaros, Chile, the southernmost colony sampled (Table 2). Distances were calculated as the great-circle distance between two latitude/longitude points using the Haversine formula (<www.movable-type.co.uk/scripts/latlong.html>; Sinnott 1984).

Using Cfit, clines were fitted for the 18 microsatellite loci and mitochondrial control region haplotypes. The alleles at each microsatellite locus were reduced to two-allele systems using species-specific compound alleles (Gay et al. 2008, Kawakami et al. 2009), and each allele was designated as either a blue-footed booby allele or a Peruvian booby allele based on its coordinates on the first axis of a multiple correspondence analysis (MCA) executed with Genetix (ver. 4.05.2; Belkhir et al. 1998). The species showed moderate to strong differentiation at the loci analyzed, appearing as discrete groups on the MCA (eigenvalue of first axis = 0.39, see below; Supplementary material Appendix 1, Fig. A2). Morphologically intermediate individuals were included in this analysis and appeared as their own discrete group halfway between the blue-footed and Peruvian booby clusters. A number of alleles were positioned very close to the limit between the species on the MCA: however, the cline shape for shared alleles is flat, and incorrect assignment of alleles would further flatten the cline in a conservative manner (Gay et al. 2008).

To determine the best model for each locus, maximum likelihood methods and Akaike information criterion (AIC; Akaike 1974) were used to compare the fit of a three parameter logit model that generates a sigmoid curve using centre, slope, and height, with a six parameter full piecewise model that assumes an asymmetrical stepped cline using centre and slope as well as the positions and slopes of the exponential tails, for all loci. For the diploid genetic data, the Hardy-Weinberg correction implemented in Cfit was fitted for each population (Gay et al. 2008).

Cfit was also used to assess cline coincidence (common cline centre) and concordance (common cline width). AIC was used to compare an unconstrained model for both the logit and full piecewise models to three models 1) a centre constrained model, 2) a width constrained model, and 3) a centre and width constrained model.

Linkage disequilibrium, hybrid class and detection of introgression

To evaluate the level of introgression between blue-footed and Peruvian boobies the program Genetix was used to estimate three sets of values: the number of locus pairs that deviated from linkage equilibrium in each population, the average standardized linkage disequilibrium across locus pairs for each population and the average standardized linkage disequilibrium between each locus pair. As in Kawakami et al. (2009), all of these analyses used the compound allele dataset.

To test the power of the microsatellite data for detecting nuclear introgression across the hybrid zone and for determining the hybrid category of each individual, the programs Hybridlab (ver. 1.0; Nielsen et al. 2006), Structure and Newhybrids (ver. 1.1 beta; Anderson and Thompson 2002) were used as described in Taylor et al. (2010b). In brief, multilocus genotypes for 25 blue-footed boobies, 25 Peruvian boobies, 25 F1 hybrids, 25 blue-footed booby backcross hybrids, and 25 Peruvian booby backcross hybrids were simulated from existing data using Hybridlab, and simulated data were subsequently analyzed in Structure and Newhybrids. For Structure analyses an individual was recorded as a pure Peruvian booby if its estimated membership coefficient $q \leq 0.1$, and as a pure blue-footed booby if $q \geq 0.9$ (Aboim et al. 2010). In addition, an individual was classified as an F1 hybrid if $0.7 \geq q \geq 0.3$, as a Peruvian booby backcross hybrid if $0.3 > q > 0.1$, and as a blue-footed booby backcross hybrid if $0.9 > q > 0.7$. For Newhybrids analyses, an individual was recorded as belonging to a certain hybrid class if its probability of belonging to a category $q_i > 0.5$ (Anderson and Thompson 2002, Aboim et al. 2010). No F2 hybrids were simulated for this power test because of the probable rarity of this hybrid class in nature (see below).

Subsequently, the real multilocus genotypes (18 microsatellite loci) for all 321 individuals were analyzed using both Structure and Newhybrids to assess levels of introgression, and to determine the hybrid class of morphological hybrids. Structure was run as above with $K = 2$ and not using species as prior information. Newhybrids was run as described in Taylor et al. (2010b), but using five genotype frequency classes. Given the rarity of hybrids, the likelihood of sampling F2 hybrids was deemed low and the F2 genotype frequency class was removed from the analysis as suggested by Anderson and Thompson (2002). The same limits for determining individual classification (as hybrids or parental for Structure, or for the various hybrid classes for Newhybrids) were used as during power analysis.

Results

Intraspecific variability

Microsatellite loci had between 4 and 35 alleles with an average of 11 alleles per locus for blue-footed boobies and between 4 and 55 alleles with an average of 15 alleles per locus for Peruvian boobies (Supplementary material Appendix 1, Table A1). Allele size scoring was highly repeatable with a 99% success rate. Genotype frequencies showed no significant deviations from HWE either at individual loci across multiple colonies or at individual colonies across multiple loci for blue-footed boobies ($p > 0.05$; Supplementary material Appendix 1, Table A1). Genotype frequencies for locus RM3F11 showed significant deviations from HWE for Peruvian boobies in four of the five Peruvian booby colonies ($p < 0.05$). No other genotype frequencies showed significant deviations from HWE either at a single locus across colonies or at a single colony across loci for Peruvian boobies (all $p > 0.05$; Supplementary material Appendix 1, Table A1). Tests for within species linkage disequilibrium did not detect any deviations from equilibrium for any pair of loci within any colony for either species (all $p > 0.05$).

Interspecific differentiation

Control region sequences and nuclear microsatellites of blue-footed versus Peruvian boobies were well differentiated ($\Phi_{CT} = 0.98$, $p = 0.001$; $F_{CT} = 0.17$, $p = 0.018$). Control region haplotypes from Peruvian and blue-footed boobies formed separate, strongly supported clades on the gene tree (Fig. 2; GenBank accession numbers HQ334018-HG334172 and HQ592377-HQ592522). Results from Structure indicated that the most likely number of genetic populations was three using the posterior probability (AVG Ln $P(K = 3) = -19058.2$, $p = 1.00$) or two using Evanno's method ($\Delta K(K = 2) = 128$) (Fig. 3; Supplementary material Appendix 1, Table A2), and AMOVA revealed similar weak but significant intraspecific population genetic differentiation at mitochondrial and nuclear microsatellites ($\Phi_{SC} = 0.02$, $p < 0.0001$, $\Phi_{ST} = 0.97$, $p < 0.0001$; $F_{SC} = 0.02$, $p < 0.0001$, $F_{ST} = 0.11$, $p < 0.0001$). This pattern (three genetic populations) was expected and is driven by weak but significant population differentiation across the range of the blue-footed booby ($p < 0.001$; Taylor et al. 2011a). Peruvian boobies exhibit genetic panmixia across their entire range (Taylor et al. 2011b).

Table 3. Estimates of cline centre location and widths, and Ln (likelihood) values from Cfit for the unconstrained logit model and for four full piecewise models. Smallest AIC value is in bold and indicates the model that best fits the data. p = number of parameters in the model, N/A indicates locus was not included in full model. *indicates significantly non-concordant locus from locus by locus likelihood ratio tests.

	(A) Unconstrained logit model			(B) Unconstrained full piecewise model			(C) Centre constrained centre = 2790	(D) Slope constrained width = 6250	(E) Centre and Slope constrained centre = 2905 width = 6600
Locus	Centre	Width	Likelihood	Centre	Width	Likelihood	Width	Centre	
Ss1b88	1513	1250	−225	1756	67	−187	3300	3046	
Ss1b100*	2333	3333	−316	2695	2631	−317	1667	2687	
Ss2b138	3980	2941	−282	2783	50	−213	53	2815	
Sn2b68	4119	2500	−300	2800	476	−274	53	3320	
Sv2a53*	2222	2778	−311	2330	2000	−309	500	1772	
Sn2b83*	1645	2500	−292	2415	3300	−280	270	2380	
RM4F11*	7690	2500	−271	2801	>7000	−273	N/A	N/A	
RM4C03	2716	5880	−224	3096	58	−192	57	2833	
RM4D07*	1025	2564	−283	2803	>7000	−277	N/A	N/A	
RM4E10*	3171	2703	−316	3096	2500	−316	53	3319	
RM2F07	6809	5000	−296	2804	50	−260	53	4112	
RM3F11	2660	1429	−273	2752	212	−225	111	2518	
RM4B03*	4928	2564	−283	4670	2500	−282	53	6672	
RM3D07*	4500	2564	−304	2793	660	−293	53	3566	
Sn2a36	2813	625	−171	2762	51	−108	167	2743	
mtDNA	2770	50	−65	2760	50	−57	50	50	
Ln (likelihood)			−3752			−3359	−3336	−3535	−3638
p			36			88	76	76	64
AIC			7576			6894	6823	7222	7403

Genetic cline analysis

Blue-footed and Peruvian boobies were well differentiated at most loci along the first axis of the MCA (eigenvalue of first axis = 0.39). The eigenvalue of the first axis is analogous to a partial F_{ST} estimate and is congruent with F_{ST} and Φ_{ST} estimates. For three loci, RM4E03, RM4G03, and Sv2A47, the two species shared common alleles and no clear differentiation between rare alleles; these loci were excluded from further cline analyses. Two other loci, RM4F11 and RM4D07, did not exhibit clinal variation in species specific alleles and were excluded from estimates of combined microsatellite cline shape parameters (Table 3). All other microsatellite loci (13) showed clinal variation in species-specific compound alleles. Mitochondrial haplotype frequencies also exhibited clinal variation and were best modeled by the full piecewise model (Table 3, Fig. 4). The majority of the locus-specific clines (10 of 14) were better modeled by the full piecewise model than the logit model. This indicates that, while the species-specific allele frequencies do fit the sinusoidal shape modeled by both the logit and full piecewise models at the centre of the cline, allele frequencies for some loci do not increase or decrease exponentially in the tails of the cline (Table 3).

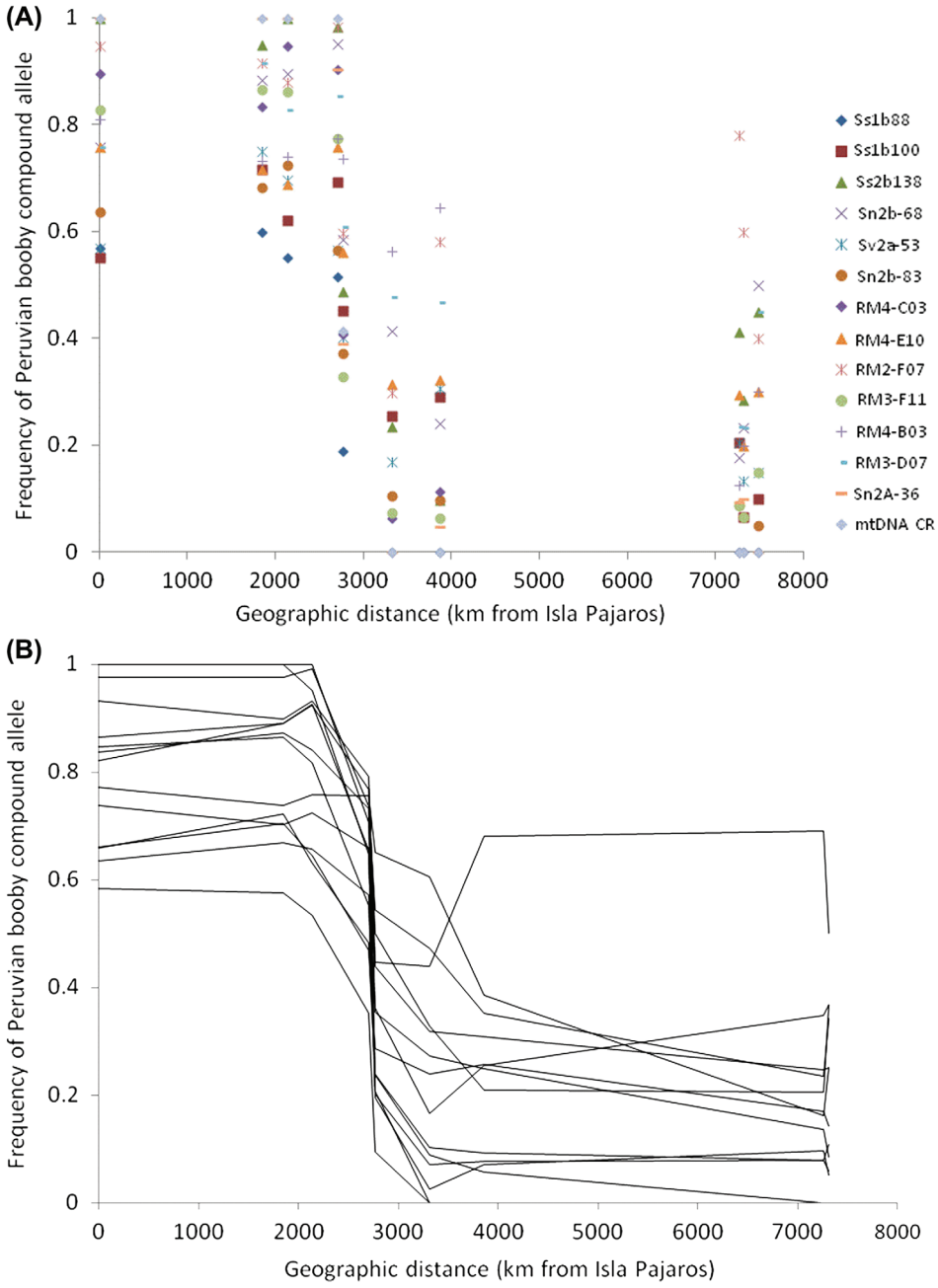

Figure 4. (A) Frequencies of Peruvian booby compound alleles for each microsatellite locus and for the mitochondrial control region. (B) Moving average frequency trend lines of Peruvian booby compound alleles for each microsatellite locus and for the mitochondrial control region.

Because the majority of clines fit the full piecewise model, and the unconstrained full piecewise model was a significantly better fit to the total nuclear microsatellite and mitochondrial datasets than the unconstrained logit model, the full piecewise model was used to describe the shape of the dataset ($p < 0.05$; Table 3).

For the total dataset the centre constrained model gave a significantly better fit to the data than the unconstrained model did, indicating that the clines were coincident ($p < 0.05$; Table 3). The unconstrained model gave a better fit to the data than either the slope constrained model or the slope and centre constrained model, indicating that the clines were not concordant (i.e. the clines were not all equal in width) (Table 3). An absence of cline concordance could be the result of one of three factors: stochastic processes, homoplasy, or differential selection (see below). The estimated centre of the cline was 2790 km north of Isla Pajaros, Chile; this location is approximately 50 km north of Lobos de Tierra, the northernmost island where the species are sympatric. The maximum estimated cline width from the centre constrained model was 3300 km, and the minimum width was 50 km; the minimum width equated to the distance between Lobos de Tierra and Lobos de Afuera (Table 3). Using the full piecewise model, the mitochondrial cline's centre was estimated to be 2760 km from Isla Pajaros, in agreement with the estimate from the nuclear dataset and total dataset. The cline's width was estimated to be 50 km, which was equal both to the narrowest estimates of cline width from the nuclear dataset and to the distance between Lobos de Tierra and Lobos de Afuera.

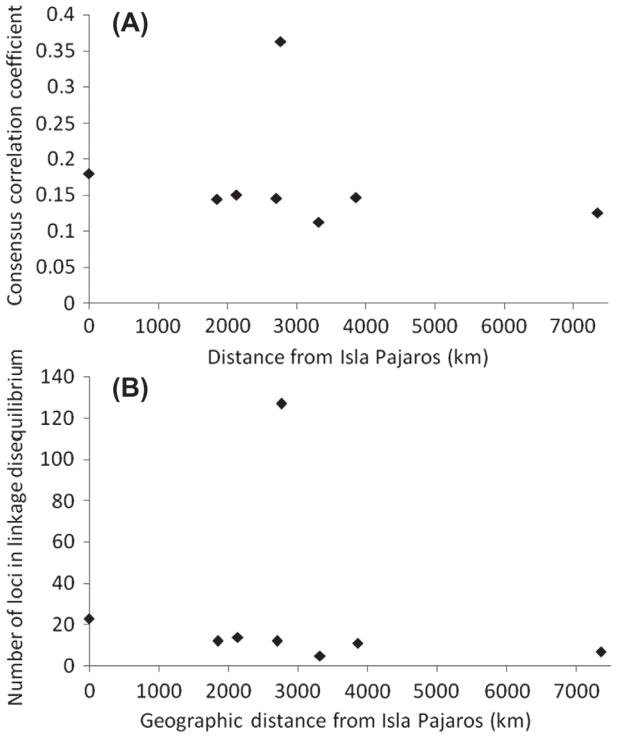

Figure 5. (A) Consensus correlation coefficient of linkage disequilibrium across loci for each sampling site. (B) Number of locus pairs in linkage disequilibrium in each population. Diamonds indicate population value.

Table 4. Average standardized linkage disequilibrium between each locus pair: all comparisons are significant ($p < 0.05$).

Locus	Ss1b 88	Ss1b 100	Ss2b 138	Sn2b 68	Sv2a 53	Sn2b 83	Sv2a 47	RM4 G03	RM4 F11	RM4 CO3	RM4 D07	RM4 E10	RM4 E03	RM2 F07	RM3 F11	RM4 B03	RM3 D07	Sn2A 36
Ss1b88	–																	
Ss1b100	0.439	–																
Ss2b138	0.648	0.485	–															
Sn2b68	0.484	0.445	0.596	–														
Sv2a53	0.448	0.294	0.435	0.379	–													
Sn2b83	0.510	0.312	0.553	0.372	0.317	–												
Sv2a47	0.417	0.319	0.436	0.361	0.267	0.367	–											
RM4G03	0.399	0.363	0.441	0.396	0.331	0.337	0.257	–										
RM4F11	0.349	0.261	0.410	0.254	0.257	0.251	0.209	0.245	–									
RM4CO3	0.682	0.548	0.773	0.614	0.493	0.589	0.476	0.489	0.399	–								
RM4D07	0.268	0.289	0.255	0.247	0.262	0.187	0.211	0.262	0.205	0.331	–							
RM4E10	0.361	0.338	0.489	0.343	0.240	0.378	0.335	0.256	0.270	0.520	0.155	–						
RM4E03	0.454	0.377	0.516	0.434	0.375	0.405	0.326	0.310	0.316	0.578	0.188	0.304	–					
RM2F07	0.481	0.322	0.576	0.364	0.375	0.424	0.342	0.321	0.241	0.571	0.215	0.314	0.344	–				
RM3F11	0.661	0.446	0.720	0.569	0.447	0.573	0.442	0.424	0.391	0.740	0.280	0.458	0.528	0.528	–			
RM4B03	0.317	0.253	0.246	0.269	0.202	0.274	0.221	0.262	0.162	0.401	0.198	0.253	0.241	0.132	0.305	–		
RM3D07	0.412	0.391	0.394	0.405	0.205	0.339	0.334	0.267	0.173	0.515	0.270	0.286	0.386	0.242	0.486	0.283	–	
Sn2A36	0.712	0.539	0.847	0.635	0.534	0.624	0.520	0.523	0.389	0.871	0.330	0.518	0.585	0.628	0.783	0.318	0.495	–

The mitochondrial cline's centre was coincident with the nuclear cline's centre estimated from the full dataset, and the width of the mitochondrial cline was concordant with four of the nuclear loci (Table 3).

Linkage disequilibrium, hybrid class and introgression

Both the consensus correlation coefficient and the number of locus pairs in linkage disequilibrium peaked at the estimated location of the hybrid zone (Fig. 5A, B). The number of locus pairs in linkage disequilibrium and consensus correlation coefficients were higher south of the hybrid zone (closer to Isla Pajaros) than north of the hybrid zone. All average standardized estimates of linkage disequilibrium were significantly positive, as was the overall estimate of standardized average linkage disequilibrium (Table 4).

All morphological hybrids possessed control region haplotypes that grouped with Peruvian booby haplotypes in the gene tree, indicating that each hybrid's mother was a Peruvian booby (Fig. 2; discussed in Taylor et al. 2010b). There was no evidence of mitochondrial introgression in that no other boobies possessed heterospecific control region haplotypes (Fig. 2).

Structure correctly assigned 89% of simulated individuals (111/125), including 100% of simulated pure Peruvian or blue-footed boobies, and 72–76% of simulated backcross hybrids (Table 2). Newhybrids correctly assigned 82% of simulated individuals (103/125) (Table 2). Newhybrids incorrectly assigned simulated pure Peruvian and blue-footed boobies and F1 hybrids more frequently than Structure, but correctly assigned simulated backcross hybrids with a higher frequency than did Structure (Table 2). Although the real data were analyzed further with Newhybrids, results were interpreted with caution through comparison with results from Structure, and the hybrid class of an individual was not inferred past F1.

Results from Structure for the full dataset indicated that eight individuals had <0.9 probability of assignment to either of the two genetic populations (Fig. 3). Of these, five were known hybrids (Taylor et al. 2010b) and three were identified as pure parentals by morphology, indicating that introgression is very low and that backcross hybrids exist but were infrequent (0.9%) among the 321 birds included in this study. Although the Newhybrids power test suggested that the data set was not powerful enough for reliable detection of hybrids, results from Newhybrids for the real dataset were congruent with results from Structure. Newhybrids indicated that the five known hybrids were likely F1 hybrids ($q_i > 0.5$) and that one other individual had a high posterior probability of being an F1 hybrid (Fig. 6). This individual was classified as a backcross hybrid by Structure according to the threshold q values used here. The two other backcross hybrids detected by Structure were also classified as hybrids (F1 or backcross) by Newhybrids; however, support for these hybrids fell below the q threshold and we interpret these results with caution.

Discussion

We used 19 putatively neutral genetic markers to evaluate interspecific differentiation between blue-footed and Peruvian boobies, to characterize changes in species specific allele frequencies across the geographic range of both species, to assess linkage disequilibrium, and to determine hybrid class of intermediate individuals and frequency of hybrids within the hybrid zone. Blue-footed and Peruvian boobies

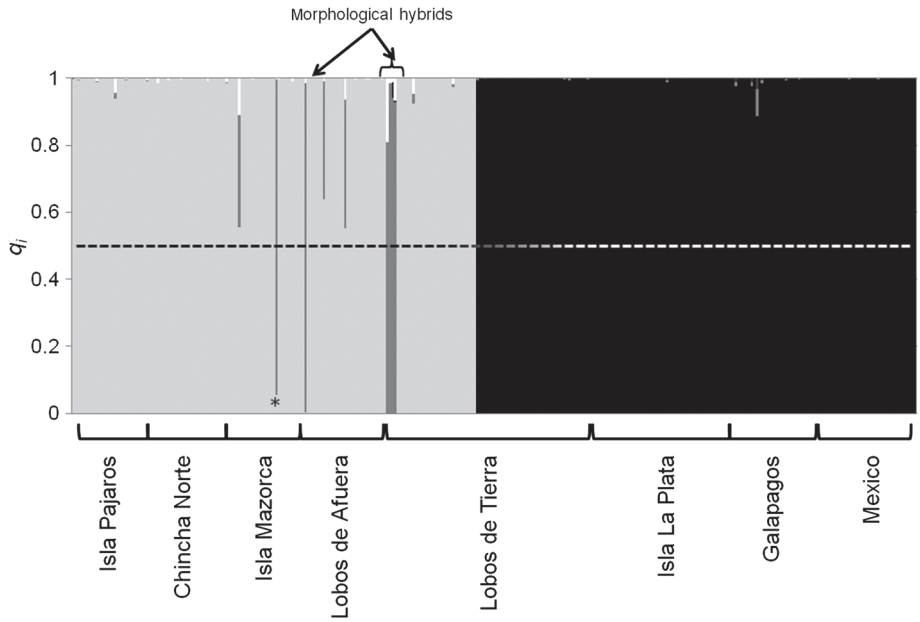

Figure 6. Output from Newhybrids with five genotype frequency classes. Posterior probability of being a blue-footed booby displayed in black, of being a Peruvian booby displayed in light grey, of being an F1 displayed in medium grey, of being a Peruvian booby backcross hybrid displayed in white, and of being a blue-footed booby backcross hybrid displayed in dark grey. Dashed line indicates $q_i = 0.5$ threshold above which the classification of an individual was accepted. *indicates individual identified as pure parental by morphology, but as a possible hybrid by Newhybrids.

were well differentiated at neutral genetic markers, linkage disequilibrium was present and peaked in the region of sympatry, hybrids occurred infrequently within the hybrid zone and the majority of hybrids were likely F1 individuals, and species specific alleles generally displayed clinal geographic variation characteristic of a bimodal hybrid zone, a hybrid zone where parental species are common but hybrids are rare (Jiggins and Mallet 2000).

Introgression and hybrid class

Across the region of sympatry, introgression was low at nuclear microsatellite loci and absent for the mitochondrial control region (Fig. 2, 3, 6); however, introgression was more prevalent south of the hybrid zone than north of the hybrid zone (Fig. 4A, B, 5A, B). The additional nuclear microsatellite data analyzed here indicated that the morphologically intermedate individuals reported in Taylor et al. (2010b) as possible F1 hybrids are most likely F1 hybrids. Other than the five morphologically intermediate individuals, only three individuals were assigned as possible backcross hybrids from molecular data. Two of these individuals were morphologically Peruvian boobies sampled on Lobos de Afuera and Isla Mazorca, and one was morphologically a blue-footed booby sampled in the Galapagos.

According to Structure these individuals possessed nuclear microsatellite genotypes characteristic of backcross hybrids of the species that they were identified as by morphology, and a control region haplotype appropriate to their morphology. Given the low power of the dataset to classify backcross hybrids reliably (Results), we interpret these results with caution. A more conservative interpretation of the data, that these individuals are not hybrids, nonetheless remains congruent with our suggestion that hybridization and subsequent introgression are rare in the region of sympatry between blue-footed and Peruvian boobies. Although Structure did not misidentify simulated F1s as parentals, it did misidentify back-crosses (16/100) as parentals, so introgression may be underestimated in the Structure analysis. Indeed, cline analysis highlighted a lack of cline concordance that may be indicative of introgression (see below).

Linkage disequilibrium

Deviations from linkage equilibrium can occur within hybrid zones if parental gene complexes are not broken down by recombination and segregation in hybrid individuals (Harrison 1993). If hybridization is rare, or hybrids experience low fitness, recombination and segregation occur infrequently and linkage disequilibrium should be high. High levels of linkage disequilibrium within a hybrid zone are indicative of little introgression (parental gene complexes are maintained); while an absence of linkage disequilibrium indicates free recombination of the parental genomes (Harrison 1993). The high level of linkage disequilibrium we detected within the region of sympatry between blue-footed and Peruvian boobies indicates that parental gene complexes are being maintained in the face of ongoing, rare hybridization. Additionally, linkage disequilibrium was higher south of the hybrid zone than north of the hybrid zone. This may have been caused by rare long distance migrant blue-footed boobies carrying 'pure' gene complexes across the hybrid zone. Interestingly, a blue-footed booby was recorded on Isla Pajaros by Simeone et al. (2002).

Cline analyses and introgression

Variation in both nuclear and mitochondrial DNA described a stepped cline across the sampling transect with little evidence of introgression from blue-footed to Peruvian boobies but some evidence of introgression from Peruvian boobies to blue-footed boobies (Fig. 4). The centres of the clines were coincident and could be constrained to a single location, a position 2790 km north of Isla Pajaros and 50 km north of Lobos de Tierra, the northernmost island where both blue-footed and Peruvian boobies breed.

Cline widths were not equivalent across loci. Estimated cline widths ranged from 50 km to 3300 km using the centre constrained full piecewise cline model. The absence of concordance can result from any of at least three factors including stochastic processes, homoplasy, or differential selection and introgression. If variable loci had a higher number of shared alleles due to homoplasy, the compound allele method could result in wide clines for highly variable loci. Given that the number of alleles or haplotypes and cline width were not correlated (Supplementary material Appendix 1, Fig. A2), homoplasy is probably not responsible for the absence of cline concordance. An absence of concordance could also indicate that the loci are experiencing different levels of selection and/or mutation and introgression, which requires further investigation.

Prezygotic isolation

Observations during field work and from other studies (Ayala 2006, Figueroa and Stucchi 2008, Taylor et al. 2010b) indicate that mating on islands where blue-footed/Peruvian boobies are sympatric is nonrandom. Under a random mating scenario hybrids would be expected to make up 23% of the population on Lobos de Afuera and 9% of the population on Lobos de Tierra given the population sizes on each island (Introduction). On both islands hybrids exist at much lower frequencies than those expected under a random mating scenario: one hybrid was detected on Lobos de Afuera, four were detected on Lobos de Tierra, and, aside from the morphologically intermediate individuals, only three individuals within the current sample may be hybrids (Fig. 3, 6).

Nonrandom mating within a hybrid zone, often the result of sexual selection, acts to stabilize the zone by increasing premating isolation (Barton and Hewitt 1985). Sexual selection appears to be an important generator of biodiversity within the Sulidae, which is known for elaborate colouration of bare parts and feet and often elaborate courtship displays (Nelson 1978). Blue-footed and Peruvian boobies differ greatly in several aspects of their behaviour and morphology, potentially the result of sexual selection (Nelson 1978). Blue-footed booby courtship behaviours (sky-pointing, jabbing, parading etc., Nelson 1978) are essentially an exaggeration of those exhibited by Peruvian boobies (Nelson 1978), which may promote reproductive isolation. Additionally, bare part colouration, particularly

foot colouration, is important for signaling quality during courtship and pair bond maintenance in blue-footed boobies at least (Torres and Velando 2005, Velando et al. 2006), and the ability of sexual selection to drive changes in mate recognition may explain the extensive premating isolation we observed. The extent of overlap of phenotypic traits between these species has not been evaluated quantitatively: these data are necessary to effectively evaluate mechanisms involved in mate choice.

Interspecific mating

As reported in Taylor et al. (2010b), interspecific mating between blue-footed and Peruvian boobies appears to occur preferentially between female Peruvian boobies and male blue-footed boobies. Unidirectional hybridization can result from a number of factors including biased sex ratios, unequal numbers at colonies, and mate choice (Wirtz 1999).

Female-biased sex ratios in Peruvian boobies could explain the observed mating pattern. If female Peruvian boobies are more common than male Peruvian boobies, unpaired female Peruvian boobies may choose to mate with heterospecific (blue-footed booby) males, resulting in F1 hybrids with Peruvian booby mitochondrial haplotypes. Data on sex ratios within Peruvian booby colonies are not available; however, many seabird colonies have male-biased sex ratios (Wirtz 1999). A male biased sex ratio in Peruvian boobies would generate the opposite pattern to what we reported.

Unequal numbers of each species within a colony could also explain the observed mating pattern. The sexual selection hypothesis (Wirtz 1999) predicts that the rarer of the parental species will most often be female, and subsequently the source of mtDNA. If females of the rare species in mixed populations experience a high search cost when looking for conspecific mates they may become less discriminating and mate with heterospecific males (Wirtz 1999). If Peruvian boobies were generally the rarer species at mixed colonies, the sexual selection hypothesis would explain the pattern of heterospecific paring we recorded; however, hybridization is not more common where Peruvian boobies are rare (Lobos de Tierra) than where the species exist at similar numbers (Lobos de Afuera). As such, the cost of searching for a conspecific mate does not appear to be driving the observed unidirectional mate choice between female Peruvian boobies and male blue-footed boobies.

We propose that the most likely reason for unidirectional mate choice when hybridization occurs in this system is an underlying female preference for exaggerated traits in some individuals. Blue-footed boobies exhibit a more exaggerated sexual display than Peruvian boobies, and possess more brightly coloured feet (Nelson 1978). If these behaviours and foot colouration are the product of sexual selection it would seem unlikely that a female blue-footed booby would choose to mate with a male Peruvian booby, whose display and behaviour could parallel that of a very low quality male blue-footed booby. On the other hand, some female Peruvian boobies may choose to mate with male blue-footed boobies: their elaborate displays and colouration may act as supernormal stimuli for some female Peruvian boobies.

Patterns of mate choice in other species pairs where one species exhibits more aggressive or exaggerated sexual displays or colours also tend to be unidirectional (Wirtz 1999). Examples include the mallard *Anas platyrhynchos* and black duck *A. rubripes* system, where male mallards exhibit higher levels of sexual aggression during courtship than male black ducks and are preferentially selected as mates (D'Eon et al. 1994), and from the common *Uria aalge* and thick-billed murre *U. lomia* system, where common murre males are more sexually aggressive and are chosen by female thick-billed murres in the majority of recorded heterospecific pairings (Taylor et al. 2011c).

Postzygotic isolation

Although premating isolation appears strong on islands where blue-footed and Peruvian boobies are sympatric, reproductively active hybrid individuals (exhibiting courting behaviours, or paired to another individual) were recorded during each scientific visit to these two islands, and they have frequently been documented with eggs and chicks (Ayala 2006, Figueroa and Stucchi 2008, Taylor et al. 2010b). The low level of introgression and high level of linkage disequilibrium in this system indicates that the reproductive success of hybrids must be limited and/or fitness of F2 hybrids and backcrosses must be low: i.e. there is some level of postzygotic isolation. The extent of hybrid breakdown cannot yet be examined, however, because backcrossed individuals are rare and the system is not amenable to captive breeding. The possibility that hybrids are formed regularly but have low fitness is interesting and warrants a brief discussion.

Given the apparent importance of foot colour in premating isolation between blue-footed and Peruvian boobies, foot colour may also play a role in isolating hybrids. F1 hybrids between blue-footed and Peruvian boobies possess mottled blue-grey feet (Taylor et al. 2010b), and so may appear to be of low quality to potential blue-footed booby mates.

Hybrids may also experience environmental selection. The centre of the blue-footed/Peruvian booby hybrid zone is located in northern Peru at the terminus of the Humboldt Current upwelling system, an ecosystem characterized by high productivity where the Humboldt Current meets the coastal shelf along South America (Fig. 7, Duffy 1987, Pennington et al. 2006). Environmental conditions within the zone of upwelling, to which Peruvian boobies are endemic, are very different than those experienced by blue-footed boobies throughout most of their range (Nelson 1978, Duffy 1987): e.g. the average water temperature is consistently lower, and air temperatures on the islands can fluctuate considerably (Duffy 1987). Thus, blue-footed and Peruvian boobies may be adapted to different marine environments. Blue footed boobies possess smaller amounts of contour feather down than Peruvian boobies, especially on their head feathers, which could reduce the ability of blue-footed boobies to withstand the cold environments within the Humboldt Current upwelling system (Taylor et al. unpubl.). Hybrid individuals may possess combinations of parental characteristics that also make them poorly adapted to environmental extremes.

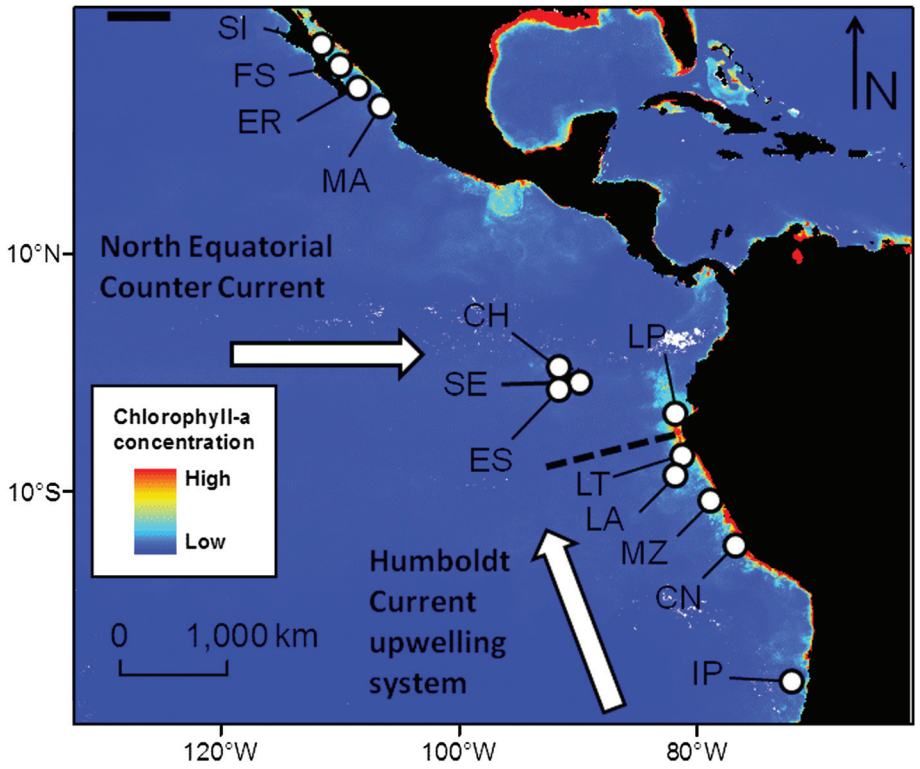

Figure 7. Eastern Tropical Pacific Aqua MODIS Chlorophyll-a (monthly average for June 2010). Dashed line indicates position of ecotonal change between Humboldt Current upwelling system and the North Equatorial Counter Current. White arrows indicate dominant oceanic currents. Colony codes as in Table 1; colonies not colored according to species.

The islands on which blue-footed boobies breed within the hybrid zone can experience colder temperatures than are typical of blue-footed booby nesting habitat, and blue-footed boobies appear to select nest sites that attain higher temperatures than do Peruvian boobies (Duffy 1987). Examination of adult and nestling tolerance to heat and wind chill are important avenues of future research given that both species may be constrained by their thermal tolerance when choosing nesting sites (Vogt 1942, Duffy 1987). Of particular interest would be studies of hybrid thermal tolerance and nest site selection.

Summary

Like a number of other well-studied avian hybrid zones, the blue-footed/Peruvian booby hybrid zone is bimodal (Jiggins and Mallet 2000) and narrow, and there is evidence for both prezygotic and postzygotic isolation. The nature of the fitness disadvantage experienced by hybrids is unknown and requires further examination using a genomic approach paired with studies of the physiology of both species (Duffy 1987, Hewitt 1988, Jiggins and Mallet 2000). Our results provide evidence that blue-footed and Peruvian boobies may be nearing the end of the speciation process: they diverged from their common ancestor recently yet reproductive barriers seem well established and the species are well differentiated. Given that studies of speciation with gene flow commonly use recently diverged species that continue to experience restricted gene flow via rare hybridization, blue-footed and Peruvian boobies may be excellent candidates for such an examination.

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Supplementary material (Appendix J5660 at <www.oikosoffice.lu.se/appendix>). Appendix 1.