

HYBRIDIZATION FROM POSSIBLE SEXUAL MIS-IMPRINTING: MOLECULAR CHARACTERIZATION OF HYBRIDIZATION BETWEEN BROWN *SULA LEUCOGASTER* AND BLUE-FOOTED BOOBIES *S. NEBOUXII*

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SUMMARY

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Hybridization occurs commonly between avian taxa but apparently less frequently between seabird species. The causes and consequences of hybridization between seabirds have been explored in a number of recent papers, highlighting that hybridization may be an important aspect of seabird evolution in some groups. Hybridization has been reported between three pairs of booby species; however, only one of these has been investigated in any depth. We report the first molecular investigation of hybridization between Blue-footed *Sula nebouxii* and Brown *S. leucogaster* boobies. We used a fragment of the mitochondrial control region and a panel of microsatellites and introns to robustly classify two aberrant individuals collected in the Gulf of California as F1 hybrids. Hybridization between these species may be restricted to colonies where both species breed in close proximity and could be the result of sexual imprinting on the wrong parental species.

Keywords: Blue-footed Booby, Brown Booby, hybridization, *Sula* spp.

INTRODUCTION

Approximately 9.2% of avian taxa are known to hybridize, a larger proportion than most vertebrate groups. However, rates of hybridization vary widely across avian orders: ducks and geese (Anseriformes) hybridize widely and frequently, while hybridization between swift species (Apodiformes) has never been recorded (Grant & Grant 1992). Seabirds, excluding the white-headed gull complex (Charadriiformes), are assumed to experience limited hybridization (Schreiber & Burger 2002). The potential for hybridization in seabirds may be lower than for other avian taxa because of the colonial nature of most species, elaborate courtship and thus stringent sexual selection, and/or the long-term pair bonds that many seabirds exhibit (Schreiber & Burger 2002).

Recent seabird population genetic studies incorporating advances in molecular methods for inferring the ancestry of individuals with aberrant plumage have reported higher levels of hybridization in some seabird groups than previously thought (Reinhardt *et al.* 1997; Gay *et al.* 2007, 2008; Taylor *et al.* 2010a, 2012a,b). These and other studies are also beginning to provide insight into the causes and evolutionary consequences of hybridization in this diverse group. For some species of seabirds, exaggerated courtship behaviours may facilitate directional hybridization between females

of one species and the elaborate males of another species (Randler 2002; Taylor *et al.* 2012a, b). This pattern has also been observed in other bird species (D'Eon *et al.* 1994) and fish (Wirtz 1999). For others, imprinting on the incorrect species may cause subsequent hybridization in mixed colonies of closely related species (Castillo-Guerrero *et al.* 2005).

The phenotypic results of hybridization are most evident when parent species have distinctive and divergent plumage and/or bare-part colouration. Booby species, as tropical and subtropical sulids, have distinctive foot colour ranging from azure blue to bright red; irises with colours including red, yellow and blue; and distinct plumage patterns (Nelson 1978). Reports of hybridization between boobies are relatively rare; this is not surprising given the importance of bare-part colouration in sexual signalling in this group (Pierotti 1987; Torres & Velando 2005; Velando *et al.* 2006). Individuals should not choose a phenotypically divergent mate if these signals are the product of sexual selection.

Based on observations of birds that possessed aberrant plumage in mixed colonies, hybridization between booby species has been reported between Brown *Sula leucogaster* and Masked *S. dactylatra* boobies (McCarthy 2006), Blue-footed *S. nebouxii* and Peruvian *S. variegata* boobies (Ayala 2006; Figueroa & Stucchi 2008; Taylor *et al.*

al. 2010a, 2012a), and Blue-footed and Brown Boobies (Castillo-Guerrero & Mellink 2005). But aberrant plumage is not necessarily indicative of hybridization (Baião *et al.* 2007; Baião & Parker 2012), so further investigation of individuals with aberrant plumage using genetic data is necessary before hybridization between species pairs can be confirmed.

Thus far, one hybridizing pair of booby species has been examined using molecular markers. Taylor *et al.* (2010a, 2012a) generated multilocus genotypes for aberrant individuals from mixed Blue-footed and Peruvian Booby colonies to investigate hybrid status. They confirmed that hybridization takes place regularly on two islands where Blue-footed and Peruvian Boobies are sympatric. The exaggerated displays of male Blue-footed Boobies may be attractive to some female Peruvian Boobies, leading to hybridization, and backcrossing events appear to follow the same pattern (i.e., backcrossing of female hybrids to male Blue-footed Boobies). However, selection against hybrids appears high in this system and mating is strongly assortative (Taylor *et al.* 2012a).

Here we extend genetic analysis of aberrant boobies to two potential Blue-footed/Brown Booby hybrids reported from the Gulf of California (one previously reported, Castillo-Guerrero & Mellink 2005; and one newly reported; Fig. 1a). We use a 500 base pair (bp) fragment of the mitochondrial control region, six microsatellite loci, and five nuclear introns to classify these aberrant individuals and subsequently explore the causes and potential consequences of hybridization between Brown and Blue-footed Boobies.

STUDY AREA AND METHODS

Sample collection and DNA extraction

Blood, feather or tissue samples were collected from 30 Brown and 15 Blue-footed Boobies from the Eastern Tropical Pacific (Fig. 1b, 2, Table 1). All samples were taken from breeding adults or chicks at nests. The presumed parents of sampled chicks were not sampled, and only one chick was sampled per nest. Samples were also collected from two individuals with aberrant plumage that were

suspected to be Brown Booby/Blue-footed Booby hybrids (Fig. 1). Both putative hybrids were sampled in mixed Brown/Blue-footed Booby colonies, one at Isla San Pedro Mártir collected by B. Tershy in 1992 and one at Farallón de San Ignacio collected by A. Castillo-Guerrero in 2007 (Fig. 3). DNA was extracted from all samples using either a standard phenol-chloroform procedure (Sambrook & Russel 2001) or a DNeasy Tissue Kit (Qiagen, Mississauga, Ontario). The sex of both putative hybrids was determined molecularly using the methods of Fridolfsson and Ellegren (1999).

Laboratory methods: mitochondrial DNA

Approximately 500 bp of the mitochondrial control region were PCR-amplified and sequenced for both putative hybrids using the protocols of Morris-Pocock *et al.* (2010). The same fragment of the control region was previously sequenced for all Brown and Blue-footed Booby individuals included in this study (Morris-Pocock *et al.* 2010; Taylor *et al.* 2011). These sequences as well as the putative hybrid sequences were aligned using ClustalW (Thompson *et al.* 1994) as implemented in BioEdit (Version 7.0.5.3; Hall 1999). Due to the difficulty of aligning the hyper-variable domain I of the control region between Brown and Blue-footed Boobies, control region sequences were pruned to include domain II variation only (~230 bp), and all subsequent mitochondrial DNA analyses were performed using this fragment.

Laboratory methods: microsatellites and nuclear introns

All samples were genotyped at six microsatellite loci developed for boobies (Sn2b-83, Sv2a-53, Sv2a-47, Sn2b-68, Sv2a-26, Sv2b-138; Taylor *et al.* 2010b). Brown and Blue-footed Boobies have previously been genotyped at a subset of four or five of these loci, respectively (Morris-Pocock *et al.* 2011; Taylor *et al.* 2011). All sample/locus combinations (including those from both putative hybrids) not previously genotyped were genotyped using the methods outlined in Taylor *et al.* (2010b). All raw microsatellite sizing data, including those previously published, were assembled together for allele-calling to eliminate potential inconsistencies between species.

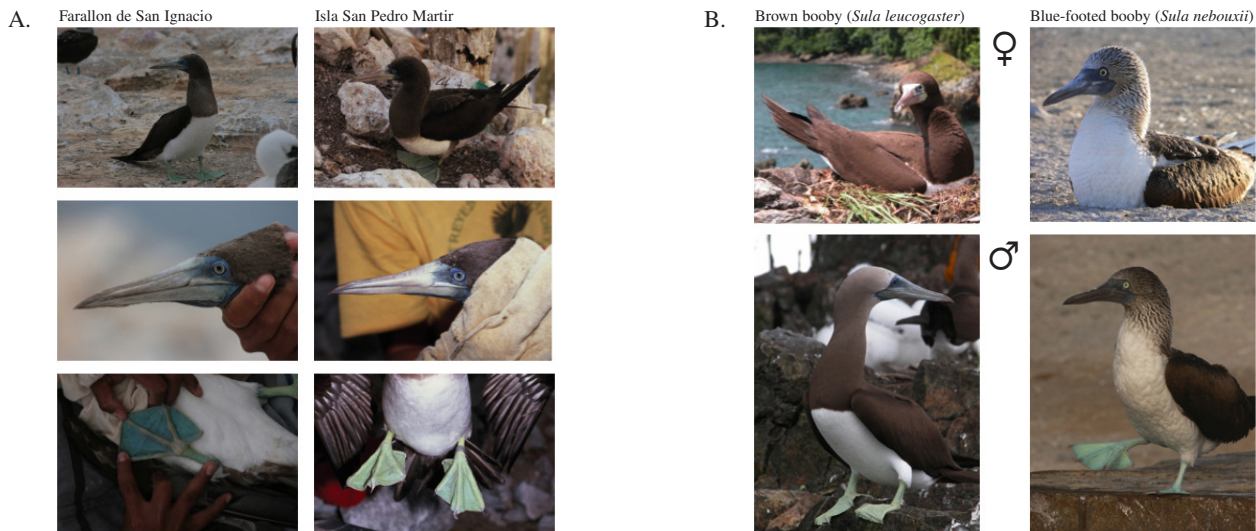


Fig. 1. A. Putative hybrids identified from aberrant morphology in Castillo-Guerrero *et al.* (2005). Photographs by M. Guevara (Farallón de San Ignacio) and Bernie Tershy (San Pedro Mártir). **B.** Adult Blue-footed and Brown Boobies for comparison to hybrids. Photographs by Giacomo Dell’Omo (Blue-footed Booby) and Felipe Estela (Brown Booby).

Five nuclear introns were also sequenced for all samples: triosephosphate isomerase intron 4 (TIM), ornithine decarboxylase introns 6 and 7 (OD), β -fibrinogen intron 5 (FIB), δ -crystallin intron 7 (CRYST), and α -enolase intron 8 (ENOL; see Appendix 1 available on the Web site for primer sequences and sources). OD, FIB and ENOL were previously sequenced for all of the Brown Boobies included in the present study (Morris-Pocock *et al.* 2011) and were amplified and sequenced in the putative hybrids and Blue-footed Boobies in the current study using the protocols of Morris-Pocock *et al.* (2011). TIM and CRYST were amplified and sequenced in all individuals using the protocols outlined in Patterson *et al.* (2011). All chromatograms were manually inspected, and heterozygotes were identified by the presence of two peaks of approximately equal

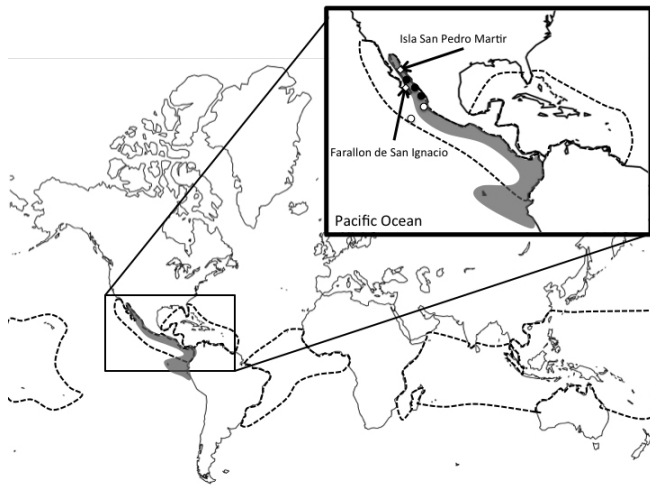


Fig. 2. Global distribution of Brown (dashed outline) and Blue-footed (shaded grey area) Boobies. Inset shows region of overlap, including islands within the Gulf of California where hybrid samples were collected. Colonies where both species occur are indicated by white diamonds; Blue-footed booby colonies, by black circles; and Brown booby colonies, by white circles.

TABLE 1
Sampling site locations and the number of individuals analyzed

Colony	Latitude	Longitude	Sample Size		
			Brown Booby	Blue-footed Booby	Putative hybrids
Clipperton	10°18'N	109°13'W	4		
Piedra Blanca	21°25'N	106°28'W	6		
Isla San Pedro Mártir	28°24'N	112°16'W	6		1
Farallón de San Ignacio	25°26'N	109°22'W	14	5	1
El Rancho	25°08'N	108°14'W		4	
Isla San Ildefonso	26°62'N	111°43'W		5	
Islas Marietas	20°42'N	105°35'W		1	

height at one or more nucleotide sites. Sequences from each intron were aligned using ClustalW, as above. For heterozygotes with more than one polymorphic site, haplotypes were phased using the default settings of the software PHASE (Version 2.1; Stephens *et al.* 2001). All of the introns were tested for intralocus recombination using the four-gamete test (Hudson & Kaplan 1985).

Data analysis

Mitochondrial control region sequences were collapsed into haplotypes, and a Bayesian gene tree of the haplotypes was estimated using MrBayes (Version 3.1.2, Ronquist & Huelsenbeck 2003). We used the best-fit nucleotide substitution model as determined by Akaike's information criterion in jModelTest (Guindon & Gascuel 2003, Posada 2008), and parameters of the substitution model were allowed to vary. Each analysis used one cold chain and three incrementally heated chains to explore parameter space, and was run for 1.0×10^7 generations (sampling trees every 100 generations and discarding the first 25% of sampled trees as burnin). Convergence was monitored by ensuring that the standard deviation of split frequencies between two simultaneous runs was lower than 0.01, by plotting the trends of all parameters, and by running all analyses three times from different starting seeds.

Deviations from Hardy-Weinberg and gametic equilibrium were tested using Arlequin (Version 3.1; Excoffier *et al.* 2005). Statistical parsimony networks were estimated for haplotypes of each intron locus using TCS (Version 1.2.1, Clement *et al.* 2000). Insertion/deletion (indel) polymorphisms were treated as a fifth character, and default settings were used for all other parameters.

Two methods were used to assess the status of the two putative hybrids: *structure* (Version 2.3.1, Pritchard *et al.* 2000) and NewHybrids (Version 1.1 beta, Anderson & Thompson 2002).

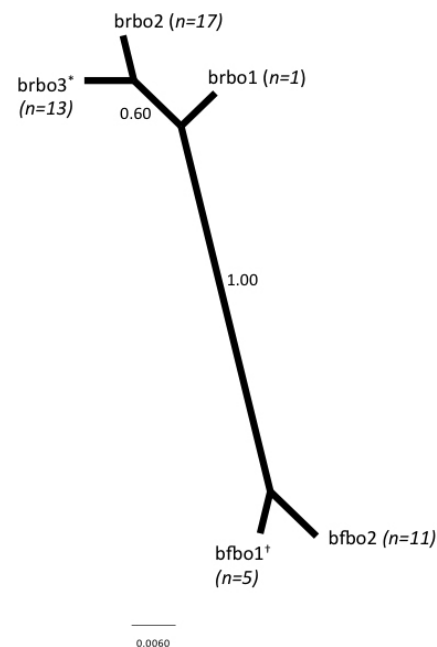


Fig. 3. Unrooted Bayesian phylogenetic tree for mitochondrial control region (domain II) haplotypes. Branch support is indicated with posterior probabilities. * hybrid from Farallón de San Ignacio. † hybrid from San Pedro Mártir.

structure was used to cluster individuals into genetic populations based on their microsatellite and intron genotypes. Twenty *structure* runs were performed for each value of K between one and four using the admixture model with allele frequencies correlated among populations. Species of origin (i.e., Brown or Blue-footed Booby) was not used as prior information in this analysis. Each run consisted of an initial burnin of 10000 generations, followed by 100000 generations. The best fit value of K was determined using the methods of Pritchard *et al.* (2000) and Evanno *et al.* (2005). After determining the best-fit value of K , the 20 individual runs at that value were averaged into a final result using the "Full Search" option in CLUMPP (Version 1.1.2; Jakobsson & Rosenberg 2007). The same thresholds for individual hybrid or parental status were used as in Taylor *et al.* (2012a): an individual was classified as a pure Brown Booby if the estimated membership coefficient $q \leq 0.1$, as a pure Blue-footed Booby if $q \geq 0.9$, as an F1 hybrid if $0.7 \geq q \geq 0.3$, as a Brown Booby backcross if $0.3 \geq q \geq 0.1$, or as a Blue-footed Booby backcross if $0.9 \geq q \geq 0.7$.

To test the power of the microsatellite and intron data for determining the hybrid category of the putative hybrids, the programs HybridLab (Version 1.0, Nielsen *et al.* 2006) and NewHybrids were used as described in Taylor *et al.* (2012a). Multilocus genotypes for 30 Blue-footed Boobies, 30 Brown Boobies, 5 F1 hybrids, 5 Blue-footed Booby backcross hybrids, and 5 Brown Booby backcross hybrids were simulated from existing data using HybridLab. Simulated data were subsequently analyzed in NewHybrids and an individual was recorded as belonging to a certain hybrid class if its probability of belonging to a category $q_i > 0.5$ (Aboim *et al.* 2010; Anderson & Thompson 2002). No F2 hybrids were simulated for this power test because of the probable rarity of this hybrid class in nature.

The real multilocus genotypes (5 microsatellite loci and 6 introns) for all 47 individuals were subsequently analyzed using NewHybrids to determine the hybrid class of morphological hybrids. NewHybrids was run as described in Taylor *et al.* (2012a), using five genotype frequency classes. As suggested by Anderson and Thompson (2002), the F2 genotype frequency class was removed from the analysis given the probable rarity of this hybrid class in nature. The same limits for determining individual classification were used as during power analysis.

RESULTS

Molecular sexing revealed that both putative hybrids were male. Five mitochondrial control region haplotypes were found in our sample of boobies, and none were shared between species. Three and two haplotypes were unique to Brown and Blue-footed Boobies, respectively. These haplotypes differed by eight fixed nucleotide substitutions and formed separate, strongly supported clades on the Bayesian gene tree (posterior probability = 1.00; Fig. 3). The putative hybrid from Farallón de San Ignacio had a haplotype that was shared with 13 Brown Boobies (brbo3 on Fig. 4), and the putative hybrid from San Pedro Mártir had a haplotype that was shared with five Blue-footed Boobies (bfbo1 on Fig. 3).

No microsatellite or intron loci deviated from Hardy-Weinberg or gametic equilibrium. For microsatellite loci, an average of 6.5 alleles/locus and 4.7 alleles/locus were found for Brown and Blue-footed Boobies, respectively (Appendix 2, available on the Web site). For most microsatellites, the majority of alleles were

private to one species or the other. One locus (Sn2b-83) had allele size ranges diagnostic of Brown versus Blue-footed Booby. Both putative hybrids had one Brown and one Blue-footed Booby allele at this locus.

No intron loci showed evidence of intralocus recombination. For intron loci, averages of 1.8 alleles/locus and 1.6 alleles/locus were found for Brown and Blue-footed Boobies, respectively (Appendix 2). For all intron loci except ENOL, no haplotypes were shared among species, and Brown and Blue-footed Booby haplotypes differed by one to four fixed differences (Fig. 4). In each case, both putative hybrids had one Blue-footed and one Brown Booby haplotype (Fig. 4).

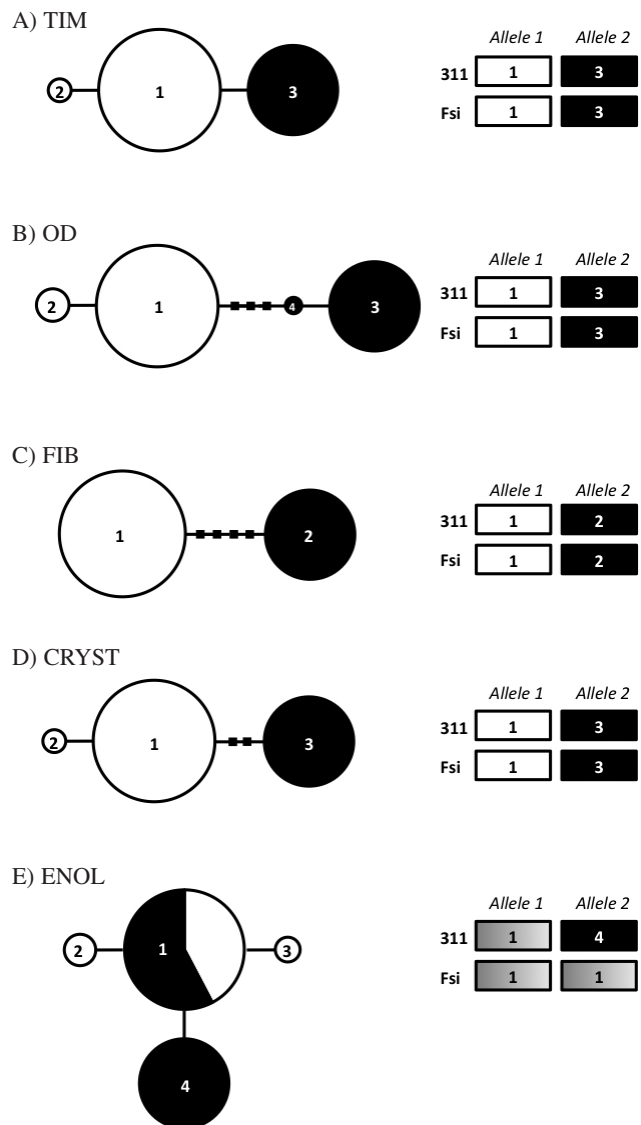


Fig. 4. Statistical parsimony networks of haplotypes from the five nuclear introns. Black and white circles represent haplotypes found only in Blue-footed Boobies or Brown Boobies, respectively. ENOL haplotype 1 was found in both Brown and Blue-footed Boobies and is coded grey to reflect this. Sizes of circles are proportional to the number of individuals that possessed a given haplotype, and small black squares represent inferred missing haplotypes. The genotype of each hybrid is given beside each parsimony network.

The methods of Pritchard *et al.* (2000) and Evanno *et al.* (2005) both suggested that $K = 2$ was the best model as determined by *structure* ($\text{Pr}[K = 2] = 1.00$; $\Delta K = 2$). All Brown Booby individuals were assigned to one cluster with probability > 0.98 , while Blue-footed Boobies were assigned to the other cluster with probability > 0.99 (Fig. 5a). Both putative hybrids appeared to have mixed ancestry (Isla Farallón de San Ignacio: 53% Brown Booby, 47% Blue-footed Booby; Isla San Pedro Mártir: 51% Brown Booby, 49% Blue-footed Booby; Fig. 5a). NewHybrids correctly assigned 100% of simulated individuals (75/75), and indicated that the two putative hybrids were likely F1 hybrids ($q_i > 0.5$) (Fig. 5b).

DISCUSSION

Our results confirm that the two boobies with aberrant plumage are the product of hybridization between Blue-footed and Brown Boobies. This is the second genetic confirmation of hybridization between two sulid species. Both hybrids are male, as predicted by Haldane's Rule (Haldane 1922), and are most likely F1 individuals. The (maternally inherited) mtDNA haplotypes of the two hybrids suggest that hybridization is not unidirectional between Brown and Blue-footed Boobies; i.e., the hybrid from Isla San Pedro Mártir is the offspring of a female Blue-footed Booby and male Brown Booby, while the hybrid from Isla Farallón de San Ignacio is the offspring of a female Brown Booby and male Blue-footed Booby. We did not detect any evidence of introgression with our set of neutral markers. This pattern of bidirectional hybridization is unlike

that observed between Blue-footed and Peruvian Boobies, for which all 5 individuals with aberrant plumage were the offspring of female Peruvian Boobies and male Blue-footed Boobies (Taylor *et al.* 2010a, 2012a).

Although the sample of hybrids is small, these results follow Haldane's Rule. As the heterogametic sex, female avian hybrids should experience a more severe reduction in viability and/or fertility than males (Haldane 1922). Blue-footed and Brown Boobies are not sister species: divergence between Brown Boobies and the clade that includes Masked, Nazca (*S. granti*), Blue-footed and Peruvian Boobies probably occurred between 1.6 and 2.1 million years ago (Patterson *et al.* 2011). Thus, genetic incompatibilities probably exist between the species, resulting in more strict adherence to Haldane's Rule than was observed in Blue-footed / Peruvian Booby hybrids (Taylor *et al.* 2012a), which diverged between 0.8 and 1.1 million years ago (Patterson *et al.* 2011).

Of particular interest is the fact that hybridization between these species is bidirectional. Unidirectional hybridization tends to occur when female choice guides pair formation, especially when one species exhibits a more elaborate courtship display or is more aggressive (Wirtz 1999). In such systems supernormal stimuli exhibited by males and chosen by females and/or more aggressive behaviour by males of one species cause hybridization to occur in a predictable direction (Wirtz 1999). Interestingly, on Isla San Pedro Mártir and elsewhere in eastern Pacific, the sex ratio of adult Brown Boobies is female-biased, so more females fail to mate in a given year than males, and the sex ratio of Blue-footed Boobies is slightly male-biased. Thus, if a supernormal-based stimulus was causing hybridization, one would expect female Brown Boobies to regularly mate with male Blue-footed Boobies. We did not detect introgression, and hybridization appears rare. As such, hybridization between Brown and Blue-footed Boobies in the eastern Pacific does not appear to be the result of directional female choice for more elaborate courtship displays.

Instead, hybridization in this system may, as suggested by Castillo-Guerrero *et al.* (2005), be the result of sexual imprinting on the wrong species. This explanation is consistent with the facts that hybridization is bidirectional and, to our knowledge, has only been found on islands where mixed nesting colonies occur (Castillo-Guerrero *et al.* 2005). Mixed colonies of Brown and Blue-footed Boobies in the eastern Pacific facilitate cross-species adoption for a number of reasons. Nests of the two species are regularly within 50 cm of each other and are sometimes directly above and below each other, which would increase the likelihood that eggs and/or very young chicks could roll or drop into neighbouring nests; this is exacerbated by frequent siblicide. Additionally, it appears that parents do not recognize their very young chicks and will adopt readily (B.T. pers. obs.).

Hybridization resulting from sexual imprinting on the wrong parental species is not uncommon in birds. It has received considerable attention in the literature and can have important evolutionary consequences (Grant & Grant 1997; Irwin & Price 2001; Slagsvold *et al.* 2002). Generally, barriers to gene flow after egg-laying are weaker than those during courtship in birds (Grant & Grant 1997). Therefore, when courtship barriers fail due to sexual imprinting or some other factor, introgressive hybridization may occur between even distantly related bird species (Grant & Grant 1992, 1997; Grant *et al.* 2005).

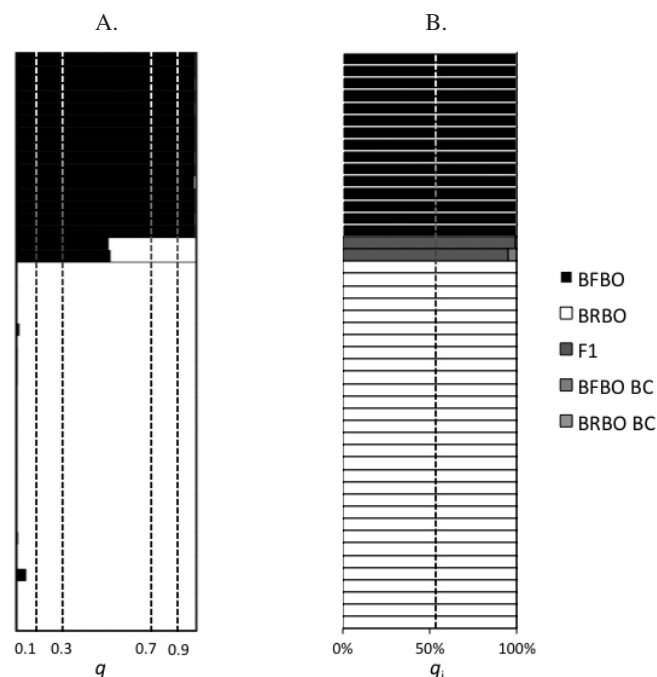


Fig. 5. A. Probability of assignment to each of two genetic populations for individual Blue-footed (in black) and Brown (in white) Boobies as inferred by *structure*. q = the probability of assignment to the genetic population displayed in black. Dashed lines indicate threshold q values used to classify individuals (see text). **B.** Output from NewHybrids with five genotype frequency classes. Shading indicates posterior probability of belonging to each of five genotype frequency classes. Dashed line indicates $q_i = 0.5$ threshold above which the classification of an individual was accepted.

In summary, hybridization between Brown and Blue-footed Boobies appears to occur at least occasionally, possibly as the result of sexual imprinting on the wrong parental species. Though we currently lack data to explore fully the potential evolutionary impacts of hybridization between these species (i.e., extent of genomic introgression), we found no evidence that hybrids backcross successfully. Whether this is due to hybrid infertility or prezygotic isolating mechanisms is unknown and warrants further investigation.

Exploring the causes of hybridization in avian systems where sexual selection has resulted in elaborate courtship displays and colouration (e.g., the foot colour of Blue-footed Boobies) is important for understanding the process of speciation in birds (Irwin & Price 2001). If rare hybridization due to sexual imprinting results in subsequent introgression, the resulting genetic exchange can have significant impacts on the stability of species barriers and the evolutionary potential of a taxon (Grant & Grant 1997). Further investigations of hybridization between seabirds should be aware of this and, whenever possible, incorporate genetic analyses.

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