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## Molecular Support for Species Status of the Nazca Booby (*Sula granti*)

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**ABSTRACT.**—Pitman and Jehl (1998) recently argued that Masked Boobies (formerly *Sula dactylatra granti*) breeding on the Nazca Plate in the eastern Pacific Ocean are morphologically and ecologically distinct from other Masked Boobies and may represent a full species. The American Ornithologists' Union subsequently elevated that subspecies to a full species: the Nazca Booby (*S. granti*). To evaluate that change in classification, we compared sequence variation in the mitochondrial cytochrome-*b* gene among 75 Nazca Boobies and 37 Masked Boobies representing three subspecies from the central and eastern Pacific and Atlantic oceans. Results indicated strong differentiation of cytochrome-*b* variation among taxa. Sequences constituted three distinct groups: Nazca Boobies, Masked Boobies from the central and eastern Pacific (*S. d. personata* and *S. d. californica*), and Masked Boobies (*S. d. dactylatra*) from the Caribbean and Atlantic. Those three groups probably diverged within a very short period, 400,000–500,000 years ago. Our results support the proposal that *S. granti* represents a distinct species.

**RESUMEN.**—Pitman et Jehl (1998) ont récemment soutenu que les Fous masqués (anciennement *Sula dactylatra granti*) nichant sur la plaque de Nazca dans l'est de l'Océan Pacifique sont morphologiquement et écologiquement distincts des autres Fous masqués et pourraient représenter une espèce à part entière. Par conséquent, l'Union des Ornithologistes Américains a élevé cette sous-espèce au statut d'espèce: le Fou de Nazca (*S. granti*). Pour évaluer ce changement dans la classification, nous avons comparé la variation de séquences dans le gène mitochondrial cytochrome-*b* parmi 75 Fous de Nazca et 37 Fous masqués représentant trois sous-espèces du centre et de l'est des Océans Pacifique et Atlantique. Les résultats indiquaient une forte différenciation de variation du cytochrome-*b* parmi les taxa. Les séquences constituaient trois groupes distincts: les Fou de Nazca, les Fous masqués du centre et de l'est du Pacifique (*S. d. personata* et *S. d. californica*), et les Fous masqués (*S. d. dactylatra*) des Caraïbes et de l'Atlantique. Ces trois groupes ont probablement divergé au cours d'une courte période (i.e. il y a 400,000–500,000 ans). Nos résultats supportent la proposition que *S. granti* représente une espèce distincte.

Masked Boobies (*Sula dactylatra*; Pelecaniformes: Sulidae) are large, plunge-diving seabirds that breed on oceanic islands throughout the tropics. Historically they were divided into six subspecies on the basis of morphology and geographic distribution (Nelson 1978). Three of those traditional subspecies breed in the central and eastern Pacific Ocean: *Sula d. granti* nests in the Galápagos Islands and on a few other islands in the far eastern tropical Pacific; *S. d. californica* nests on islands off western Mexico and Clipperton Island in the eastern Pacific north of *S. d. granti*; and *S. d. personata* nests primarily north and west of *S. d. granti*. The other three subspecies breed in the central and western Indian Ocean (*S. d. melanops*), northwestern Australia (*S. d. bedouti*), and the Caribbean and Atlantic Ocean (*S. d. dactylatra*). A seventh subspecies (*S. d. fullagari*), distinguished primarily by eye color, also has been recognized on islands off eastern Australia (O'Brien and Davies 1990).

Recently, *S. d. granti* was the subject of a taxonomic reassessment by Pitman and Jehl (1998), who argued that individuals of that subspecies differ markedly in morphology and ecology from other Pacific subspecies: they have orange bills instead of yellow; they are generally smaller with longer wings; they have a distinct juvenal plumage; they have a different foraging distribution; and they tend to nest on rocky volcanic islands with rugged topography as opposed to flat atolls. In addition, *S. d. granti* individuals eat predominantly sardines (Anderson 1989, D. J. Anderson unpubl. data; but see Murphy 1936), whereas Masked Boobies elsewhere in the Pacific eat predominantly flying fish (Nelson 1978, Harrison 1983). Most importantly, orange- and yellow-billed "masked" boobies pair assortatively on islands where they coexist; for example, on Clipperton Island 150 orange-billed boobies nest sympatrically but assortatively with over 60,000 yellow-billed birds. On the basis of those data, Pitman and Jehl (1998) argued that *S. d. granti* satisfies the requirements of both a morphological species and a biological species, and recommended that it be recognized as a distinct species, *Sula granti*, with the common name "Nazca Booby." The American Ornithologists' Union (AOU 2000) has since recognized the Nazca Booby as a full species. However, Pitman and Jehl invited a molecular evaluation of genetic relationships among the "masked" boobies. In this article, we compare variation in a neutral molecular marker—the mitochondrial cyto-

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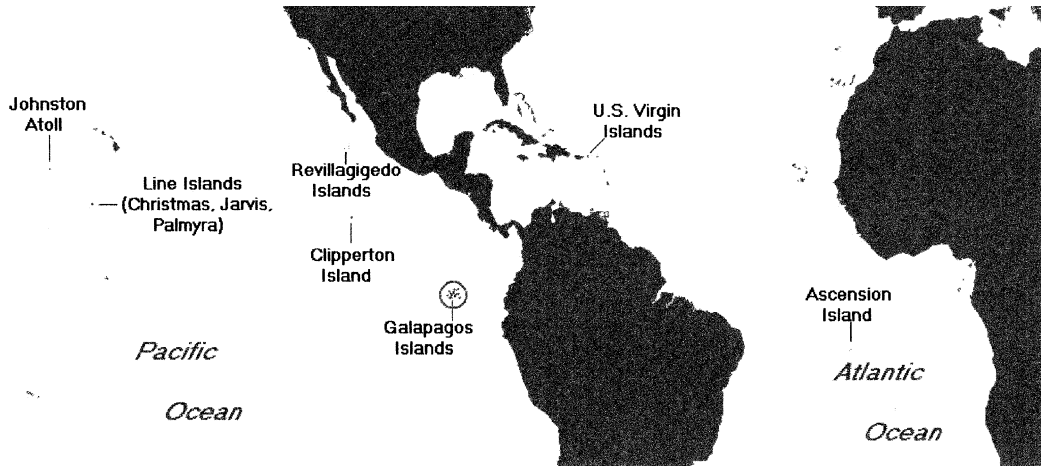


FIG. 1. Locations of sampling sites for Masked Boobies. Pitman and Jehl (1998) and D. Roberson (unpubl. data) give detailed descriptions of breeding and at-sea distributions of the different “masked” booby forms in the Pacific and Caribbean.

chrome-*b* gene—among Nazca Boobies and three subspecies of Masked Boobies to evaluate further whether *S. granti* merits recognition under either of the two most common species definitions: the biological (Mayr 1963) and phylogenetic (Cracraft 1989) species concepts.

**Methods.**—Blood samples, feathers, embryos, or unfertilized eggs were collected from 75 Nazca Boobies breeding on Española (Galápagos Islands), and 37 Masked Boobies representing two Pacific subspecies (*S. d. californica* and *S. d. personata*) and the Caribbean and Atlantic subspecies (*S. d. dactylatra*; Fig.

1; Table 1). Blood samples also were collected from two orange-billed individuals (presumably Nazca Boobies) from Clipperton Island. DNA was purified from samples by protease digestion, phenol extraction, and isopropanol precipitation (Kocher et al. 1989, Friesen et al. 1997). A 450 bp fragment of the mitochondrial cytochrome-*b* gene was amplified using primers CytbL15063 and CytbH15506 (T. P. Birt unpubl. data); previous analyses confirmed that those primers amplify the target locus rather than a nonfunctional nuclear (paralogous) copy in sulids (Friesen and Anderson 1997). Samples were screened

TABLE 1. Frequencies of cytochrome-*b* haplotypes among Nazca and Masked boobies.

Taxon and site	Haplotype							Total	
	A	B	B4	C	D	E	F		G
<b><i>Sula dactylatra personata</i></b>									
Johnston Atoll	0	4	1	0	0	0	0	0	5
Line Islands <sup>a</sup>	2	16	0	0	0	0	0	0	18
<b><i>S. d. californica</i></b>									
Revillagigedos <sup>b</sup>	0	2	0	0	0	0	0	0	2
Clipperton Island	0	2	0	0	0	0	0	0	2
<b><i>S. d. dactylatra</i></b>									
Caribbean Islands <sup>c</sup>	0	0	0	4	1	0	0	0	5
Ascension Island	0	0	0	1	4	0	0	0	5
<b><i>S. granti</i></b>									
Galápagos Islands <sup>d</sup>	0	0	0	0	0	0	74	1	75
Clipperton Island	0	0	0	0	0	1	1	0	2
<b>Total</b>	<b>2</b>	<b>24</b>	<b>1</b>	<b>5</b>	<b>5</b>	<b>1</b>	<b>75</b>	<b>1</b>	<b>114</b>

<sup>a</sup> Palmyra, Jarvis, and Christmas islands.

<sup>b</sup> San Benedicto Island.

<sup>c</sup> U.S. Virgin Islands.

<sup>d</sup> Daphne, Española, Caldwell, and Genovesa islands.

for variation using single-stranded conformational polymorphisms (SSCPs), which provide an efficient and sensitive tool for detecting sequence variation (Hayashi 1991, Lessa and Applebaum 1993, Friesen et al. 1996a). At least one representative of each haplotype then was sequenced directly using either ThermoSequenase Radiolabeled Terminator<sup>™</sup> manual cycle sequencing kits (Amersham Biosciences, Piscataway, New Jersey) according to the manufacturer's protocol, or an ABI Prism<sup>™</sup> 373 automated Sequencing System (Mobix, McMaster University, Hamilton, Ontario). For two representatives of haplotypes B, C, and F (common haplotypes within each of three major clades on the gene tree; see below), an additional 357 bp of the cytochrome-*b* gene were sequenced for comparison with other species of sulids (Friesen and Anderson 1997; Genbank accession numbers U90000–U90009).

Phylogenetic relationships among mitochondrial haplotypes of Nazca and masked boobies were inferred in two ways: (1) by parsimony analysis using PAUP\* (Swofford 1998) with support for clades inferred by bootstrapping with 1,000 replications (Felsenstein 1985); and (2) by maximum-likelihood analysis using PAUP\*, on the basis of empirical nucleotide frequencies, a transition:transversion ratio of 2, and the default settings, and with support for clades inferred from bootstrapping with 1,000 replications. In both cases, midpoint rooting was specified. Relationships among haplotypes B, C, and F and sequences of other sulids also were inferred using each of those methods, with sequence from a Pelagic Cormorant (*Phalacrocorax pelagicus*) used for rooting.

Due to small numbers, samples of orange-billed boobies from Clipperton Island (presumably *S. granti*) and *S. d. californica* were excluded from population-level analyses. Genetic differentiation among species and subspecies was indexed using  $\Phi_{ST}$ , estimated by analysis of molecular variance using ANOVA (Excoffier et al. 1992, Michalakis and Excoffier 1996). The statistical significance of estimates of  $\Phi_{ST}$  was determined by randomization using 1,000 permutations of the data. Gene flow ( $N_m$ ; females per generation) was estimated from  $\Phi_{ST}$  assuming  $N_m = 1/2 ([1/\Phi_{ST}] - 1)$ ; Crow and Aoki 1982). Use of  $F_{ST}$  analogues such as  $\Phi_{ST}$  for estimating gene flow involves several assumptions, including equal population sizes and symmetrical gene flow (Beerli 1998), which may not hold for seabirds (Congdon et al. 2000); therefore gene flow also was estimated using a maximum-likelihood approach based on coalescent theory (Beerli and Felsenstein 1999). Specifically, magnitudes and directions of gene flow were estimated from cytochrome-*b* sequence variation using MIGRATE (Beerli 1997); the default settings of MIGRATE were used except that the numbers of genealogies sampled for the short and long chains were

TABLE 2. Variable sites within a 450 bp fragment of the cytochrome-*b* gene among Nazca and Masked boobies. Numbers refer to positions of sites relative to the 3' end of the light strand primer (Friesen and Anderson 1997). Two substitutions at first codon positions, both of which result in amino acid replacements, are in bold.

	Site
	112222223344
	47591345561602
	55926438976959
A	TAGTCGCAGCAACC
B	. . . . . <b>A</b> . . . . .
B4	. . . . T . . . <b>A</b> . . . . .
C	. . A . . ATT <b>ATG</b> . . .
D	. . A . . ATG <b>ATG</b> . . .
E	. G . C . AT <b>G</b> A . . GTA
F	. G . C . AT <b>G</b> A . . TA
G	CG . C . AT <b>G</b> A . . TA

increased to 100,000 and 1,000,000, respectively, to avoid local maxima on the likelihood surface.

Genetic distances between taxa were estimated as  $\delta = \pi_{xy} - 0.5(\pi_x + \pi_y)$ , where  $\pi_{xy}$  is mean pairwise sequence divergence between populations *x* and *y*, and  $\pi_x$  and  $\pi_y$  are mean pairwise sequence divergence within taxa *x* and *y*, respectively (Wilson et al. 1985). Time since divergence of taxa was estimated as  $t = \delta/r$ , where *r* is sequence divergence rate. Friesen and Anderson (1997) estimated a divergence rate for cytochrome *b* for sulids of 2.8%  $\text{my}^{-1}$ , but we also calculated *t* assuming the more typical rate of 2%  $\text{my}^{-1}$  (Fleischer et al. 1998, Nunn et al. 1996). Relationships among taxa were inferred by UPGMA cluster analysis (Sneath and Sokal 1973) of  $\delta$  values; distances were tested for significance using one-tailed Student's *t*-tests for a difference from zero.

**Results.**—Eight cytochrome-*b* haplotypes, described by 14 variable sites, were found among the 114 samples (Tables 1 and 2). The nucleotide sequence of haplotype F was identical to that described by Friesen and Anderson (1997) for two "Masked" Boobies (actually Nazca Boobies) from the Galápagos Islands (Genbank accession number U90004). Haplotypes differed from each other by one to nine substitutions, two of which involved transversions and two of which involved first position substitutions and resulted in amino acid replacements (Table 2).

Phylogenetic analyses suggested that our samples included three monophyletic lineages: (1) haplotypes A, B, and B4; (2) haplotypes C and D; and (3) haplotypes E through G (Fig. 2A). All three groups received strong bootstrap support. Haplotypes within the same group differed by only 0.2–0.4%, whereas haplotypes from different groups differed by 1.3–2.0%. In addition, haplotypes A through D

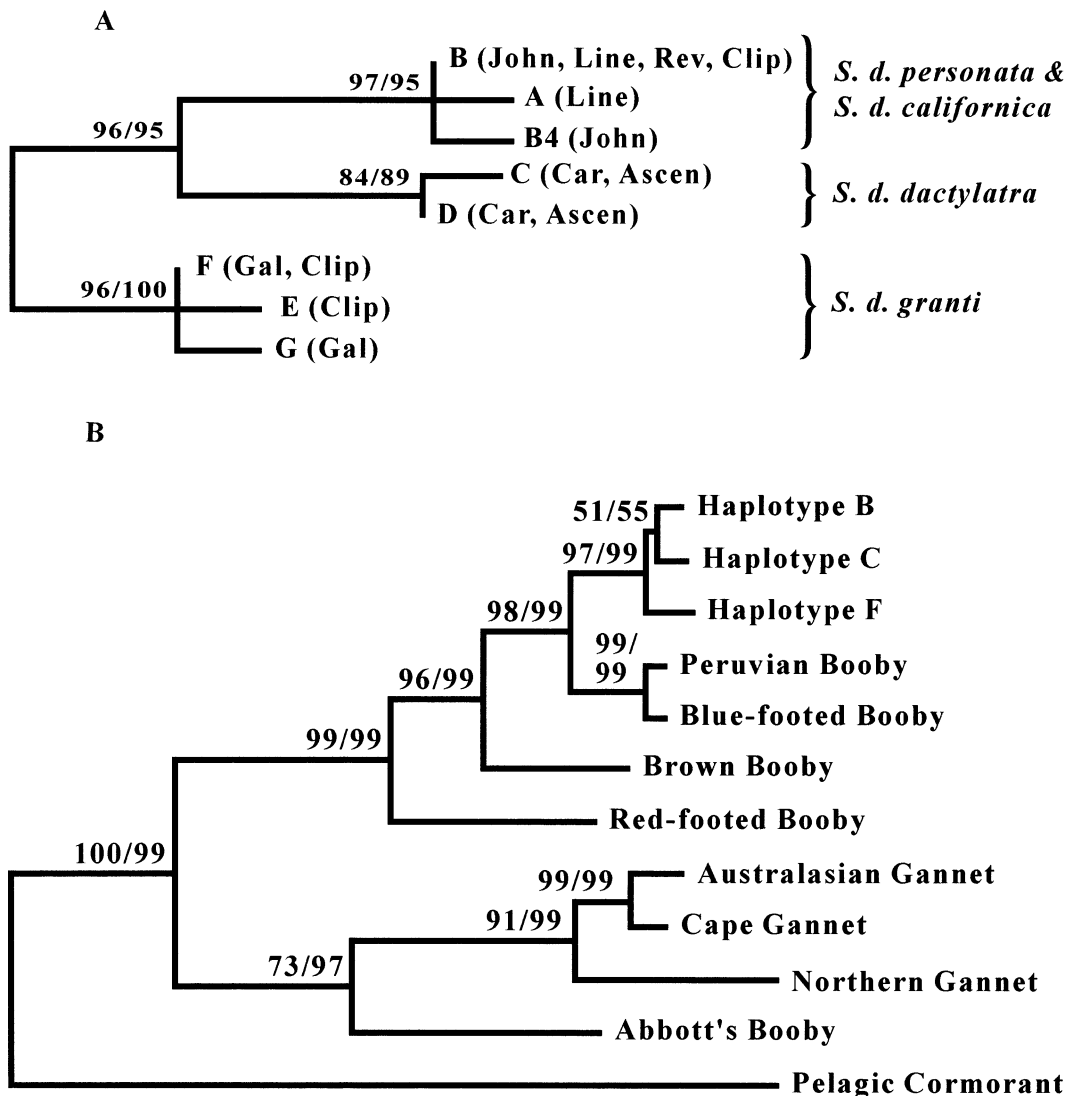


FIG. 2. Maximum-likelihood trees for cytochrome-*b* haplotypes of (A) Nazca and Masked boobies (based on 450 bp sequence), and (B) the Sulidae (based on 807 bp sequence). Both methods of phylogenetic analysis gave the same topologies; numbers at branches indicate support from bootstrap analyses of the parsimony trees (first number) and maximum-likelihood trees (second number). Line = Line Islands; John = Johnston Island; Rev = Revillagigedos; Clip = Clipperton Island; Car = Caribbean Islands; Ascen = Ascension Island; Galap = Galápagos Islands; Brown Booby = *Sula leucogaster*; Red-footed Booby = *S. sula*; Australasian Gannet = *Morus serrator*; Cape Gannet = *M. capensis*; Northern Gannet = *M. bassanus*; Abbott's Booby = *Papasula abbotti*. All other scientific names are given in the text.

formed a monophyletic group receiving strong bootstrap support.

Within the cytochrome-*b* gene tree for the Sulidae, haplotypes B, C, and F formed a strongly supported monophyletic clade. That clade was most closely related to sequences of the Blue-footed (*S. neboxii*) and Peruvian (*S. variegata*) boobies, as in Friesen and Anderson (1997; Fig. 2B). Haplotypes C (from *S. d.*

*dactylatra*) and B (from *S. d. personata* and *S. d. californica*) appeared to be more closely related to each other than either was to haplotype F (from *S. granti*), although bootstrap support was weak.

The geographic distribution of haplotypes was strongly structured: haplotypes A, B, and B4 were found only in Masked Boobies from the central and eastern Pacific (*S. d. personata* and *S. d. californica*);

TABLE 3. Estimates of the proportion of variation distributed among populations ( $\Phi_{ST}$ ; above diagonal, upper number), gene flow estimated from  $\Phi_{ST}$  ( $N_f m$  in females per generation; above diagonal, lower number), mean percentage sequence divergence within populations ( $\pi_x$ , percentage  $\pm$  SE; diagonal), mean percentage sequence divergence between populations ( $\pi_{xy}$ , percentage  $\pm$  SE; below diagonal, upper number), and corrected percentage sequence divergence between populations ( $\delta$ , percentage  $\pm$  SE; below diagonal, lower number) between Nazca Boobies and two subspecies of masked boobies.

	<i>Sula d. personata</i>	<i>S. d. dactylatra</i>	<i>S. granti</i>
<i>S. d. personata</i>	0.37 $\pm$ 0.05	0.94**	0.99**
<i>S. d. dactylatra</i>	1.35 $\pm$ 0.004	0.03	0.01
<i>S. granti</i>	1.13 $\pm$ 0.044*	0.12 $\pm$ 0.02	0.99**
	1.59 $\pm$ 0.002	1.67 $\pm$ 0.004	0.01
	1.34 $\pm$ 0.042*	1.53 $\pm$ 0.020*	0.17 $\pm$ 0.01

\*  $P < 0.01$ .

\*\*  $P < 0.001$ .

haplotypes C and D were found only in Masked Boobies from the Caribbean and Atlantic (all *S. d. dactylatra*); and haplotypes E, F, and G were found only in Nazca Boobies and in orange-billed boobies from Clipperton Island (Table 1). The frequencies of haplotypes C and D differed between samples from the Caribbean and Atlantic, but sample sizes were too small to test for statistical significance. Haplotype B was the most common haplotype of both *S. d. personata* and *S. d. californica*, suggesting that mitochondrial DNA (mtDNA) of those two subspecies may not be differentiated; however, more samples are needed from *S. d. californica* for a more rigorous analysis of that possibility. Otherwise, no haplotypes were shared among taxa. The proportion of sequence variation distributed among taxa approached one and was significantly different from zero ( $\Phi_{ST} = 0.98$ ,  $P < 0.001$ ). Estimates of  $\Phi_{ST}$  for pairwise comparisons of taxa also differed significantly from zero (Table 3). Estimates of gene flow derived from  $\Phi_{ST}$  (Table 3) were  $< 0.1$  females per generation for all comparisons, suggesting that essentially no female-mediated gene flow occurs among *S. granti*, *S. d. personata*, and *S. d. dactylatra*. Results from MIGRATE also suggest that female-mediated gene flow among those taxa is highly restricted (all estimates were  $< 0.1$  females per generation; results not shown); however, the likelihood surfaces were too flat for derivation of confidence intervals on estimates.

Estimates of  $\pi_{xy}$  were substantially higher than  $\pi_x$ , and estimates of  $\delta$  were significantly greater than zero for all comparisons (all  $P > 0.10$ ). UPGMA analysis of  $\delta$  estimates grouped *S. d. personata* and *S. d. dactylatra* together to the exclusion of *S. granti* with strong support (all  $P < 0.05$ ). Estimates of  $\delta$  suggested that *S. granti* diverged from the other taxa  $\sim 0.5$  my ago (assuming  $r = 2.8\%$   $\text{my}^{-1}$ ; 0.7 my ago if  $r$  is  $2\%$   $\text{my}^{-1}$ ), and that *S. d. dactylatra* diverged from *S. d. personata*  $\sim 0.4$  my ago (assuming  $r = 2.8\%$   $\text{my}^{-1}$ ; 0.6 my ago if  $r = 2\%$   $\text{my}^{-1}$ ).

**Discussion.**—Results of the present study indicate that variation in mtDNA of Nazca and Masked boobies is strongly structured geographically. Cytochrome-*b* sequences from *S. granti*, *S. d. personata*, and *S. d. dactylatra* revealed essentially no female-mediated gene flow (Table 3), were reciprocally monophyletic on the gene tree (Fig. 2A), had genetic distances ( $\delta$  estimates) significantly greater than zero (Table 3), and had almost all sequence variation distributed among populations ( $\Phi_{ST} = 0.98$ ; Table 3). Estimates of  $\Phi_{ST}$  based on cytochrome-*b* sequences for other species of birds are typically much lower than those found in the present study (e.g. 0.10 for the Atlantic population of Common Murres (*Uria aalge*), 0.02 for the Atlantic population of Thick-billed Murres (*U. lomvia*; Friesen et al. 1996b). Estimates of  $\Phi_{ST}$  rival values obtained in studies in which two or more reproductively isolated but morphologically similar species were initially treated as a single group. For example,  $\Phi_{ST}$  based on cytochrome-*b* variation in Marbled Murrelets (*Brachyramphus marmoratus*) was 0.97 when all North Pacific samples were included, but dropped to 0.02 when Asian samples were removed (Friesen et al. 1996a); Asian Marbled Murrelets are now recognized by the AOU as a distinct species (the Long-billed Murrelet [*B. perdix*]). Furthermore, although sample sizes were small, cytochrome-*b* sequences of yellow- and orange-billed boobies on Clipperton Island were distinct from each other and similar to those of boobies with corresponding bill colors elsewhere in the Pacific (Table 1). Thus, our data, combined with Pitman and Jehl's (1998), indicate that *S. granti* satisfies the requirements of the two most common species concepts—biological and phylogenetic—and support its recognition as a valid species (AOU 2000).

Although we sampled only 10 Masked Boobies from the Caribbean and Atlantic, cytochrome-*b* sequences of those birds were as distinct from those in the Pacific as sequences of *S. d. personata* were from *S. granti*. Masked Boobies from the Atlantic (*S. d. dactylatra*) appear to differ from those in the Pacific in size and male leg color (Murphy 1936, Nelson 1978, Anderson 1993). Thus the possibility that *S. d.*

*dactylatra* represents a separate species requires further exploration. Although our sample size for *S. d. californica* was small, only one phylogeographic lineage was found among Masked Boobies from the central and eastern Pacific: *S. d. californica* samples from the islands off western Mexico were indistinguishable from *S. d. personata* samples from the central Pacific. Separate subspecific classification of Masked Boobies from western Mexico was viewed with suspicion by Nelson (1978) and was explicitly rejected by Pitman and Jehl (1998) on morphological grounds (D. Roberson unpubl. data). Both of those treatments advocated subsuming *S. d. californica* within *S. d. personata*, whereas an obvious phenotypic marker (bill color) separates both of those subspecies from *S. granti*. On the basis of our genetic results and the detailed morphological results of Pitman and Jehl (1998), Masked Boobies from western Mexico may not differ genetically from those elsewhere in the Pacific. However, a global genetic analysis of Masked Boobies, using both the mitochondrial control region and nuclear markers, is required before definitive taxonomic recommendations can be made regarding *S. d. dactylatra* and *S. d. personata* (T. E. Steeves unpubl. data).

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