

# Genetic Variation among Subspecies of Least Tern (*Sterna antillarum*): Implications for Conservation

JOANNA B. WHITTIER<sup>1,3</sup>, DAVID M. LESLIE, JR.<sup>2</sup> AND RONALD A. VAN DEN BUSSCHE<sup>1</sup>

<sup>1</sup>Oklahoma Cooperative Fish and Wildlife Research Unit, Department of Zoology  
Oklahoma State University, Stillwater, OK 74078 USA

<sup>2</sup>U.S. Geological Survey, Oklahoma Cooperative Fish and Wildlife Research Unit  
Oklahoma State University, Stillwater, OK 74078 USA

<sup>3</sup>Present Address: Kansas Cooperative Fish and Wildlife Research Unit, Division of Biology  
Kansas State University, Manhattan, KS 66506 USA  
Internet: whittier@ksu.edu

**Abstract.**—DNA sequence variation from two nuclear introns and part of the mitochondrial cytochrome-*b* gene were used to evaluate population structure among three subspecies of Least Tern that nest in the United States (California [*Sterna antillarum browni*], Interior [*S. a. athalassos*], Eastern [*S. a. antillarum*]). Sequence variation was highest for nuclear intron XI (*Gadp*) within the glyceraldehyde-3-phosphate dehydrogenase gene. The second nuclear intron was fixed for the same allele in all subspecies. Fixation indices,  $F_{ST}$  and  $M_{ST}$ , for *Gadp* indicated genetic divergence between California and Interior subspecies. Estimates of nuclear gene flow were <4 individuals/generation, except between the Interior and Eastern subspecies (4 individuals/generation). Genetic indices for mitochondrial DNA did not differ among subspecies, and gene flows (reflecting female dispersal) ranged from 10 to 83 individuals/generation. Reservations are expressed about the validity of the current subspecific divisions and further research is required, including their taxonomic relationship to the Little Tern (*Sterna albifrons*). Received 17 October 2004, accepted 7 July 2005.

**Key words.**—Least Tern, *Sterna antillarum*, population structure, genetic variation, mitochondria, nuclear introns.

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Multiple taxonomic subdivisions have been proposed for the Least Tern (*Sterna antillarum*) since its initial description (Lesson 1847; Mearns 1916; van Rossem and Hachisuka 1937; Brodtkorb 1940; Burleigh and Lowery 1942; Fig. 1). Originally classified as a Little Tern (*Sterna albifrons*), the Least Tern was given its own specific epithet in 1976 (Massey 1976). The American Ornithologists' Union (AOU) recognizes three subspecies: California, *S. a. browni*; Eastern, *S. a. antillarum*; and Interior, *S. a. athalassos* (AOU 1957). The United States Fish and Wildlife Service (USFWS), who oversees endangered species management, does not recognize any subspecies for Least Terns due to taxonomic uncertainties, instead USFWS considers the California, Interior, and Eastern Least Tern to be distinct geographic variants of which the California and Interior populations have been designated federally endangered as a result of population declines related to habitat loss (USFWS 1980, 1985).

Taxonomic status within Least Terns has been debated for several years (Thompson

*et al.* 1992; Patten and Erickson 1996; Massey 1998). Criticisms of original descriptions for subspecies focused on inadequacies of qualitative and variable characteristics, such as feather color and number of black flight feathers, used to describe type specimens (Massey 1976; Thompson *et al.* 1992; Patten and Erickson 1996). In separate studies, Burleigh and Lowery (1942), Massey (1976), and Thompson *et al.* (1992) could not differentiate Eastern and California subspecies using morphology, behavior, or vocalizations (Table 1). Allozyme electrophoresis also failed to differentiate between Eastern and Interior Least Terns (Thompson *et al.* 1992), but all specimens came from Texas, where the two subspecies may overlap, and sample size for the Interior population was small ( $N = 4$ ). Furthermore, identification is complicated by potential interbreeding between Eastern and Interior Least Terns (e.g., one Least Tern banded as a juvenile in Texas was found nesting at Quivara National Wildlife Refuge, Kansas; Boyd and Thompson 1985). The only study that appeared to definitively

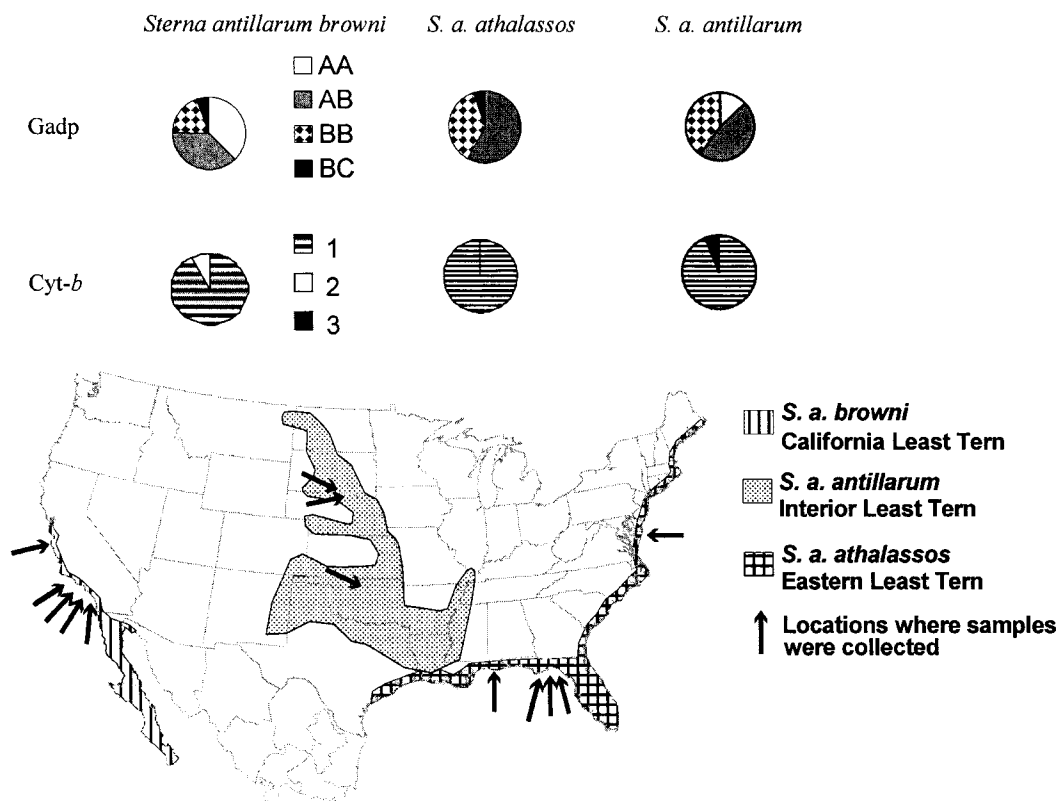


Figure 1. Breeding ranges for subspecies of Least Tern (*Sterna antillarum*) based on distribution presented in U.S. Fish and Wildlife Service (1990). Genotype frequencies for the nuclear intron, *Gdp* (4 genotypes), and haplotype frequencies for cytochrome-*b* (3 haplotypes) are shown for each of the three subspecies examined in this study.

distinguish all three subspecies was a spectrophotometric examination of feather color by Johnson *et al.* (1998). Johnson *et al.* (1998) separated the subspecies using body feathers from the hind neck and center of back of adults collected in early summer. However, alternative scenarios unrelated to subspecies could explain the differences that Johnson *et al.* (1998) detected in feather color. Most of the specimens likely formed their body feathers during the Definitive Prealternate molt (which occurs February-April in Least Terns; Massey and Atwood 1978; Thompson *et al.* 1997a) prior to or during the northward migration. The eumelanin that creates gray hues, such as those in Least Tern feathers, can be influenced by environment or food sources (Welty and Baptista 1988) and our understanding of winter distribution is very limited. It is conceivable that differences in feather color observed by Johnson *et al.*

(1998) were a function of wintering site and not an inherited feature.

Our understanding of metapopulation dynamics among Least Terns breeding in the United States is limited. Philopatry estimates are 5-82% with about 95% of banded chicks, that returned to breed, nesting  $\leq 75$  km of their natal colony (Massey *et al.* 1992; Boyd 1993; Lingle 1993; Renken and Smith 1995). Only one instance of dispersal between Interior and Eastern Least Terns has been documented (Boyd and Thompson 1985). Attempts to examine how the three subspecies interact on wintering grounds have been difficult due in part to uncertainty of wintering locations (Thompson *et al.* 1997a).

Our objectives were to examine taxonomic status and relative level of interaction among Least Terns breeding in the United States using molecular genetic techniques. DNA sequences from two nuclear introns

**Table 1. Review of taxonomic studies of Least Terns that evaluated whether or not subspecies (California, Interior, and Eastern) of Least Tern could be differentiated.**

	Pairwise comparisons of subspecies			Technique
	California-Interior	California-Eastern	Interior-Eastern	
Johnson 1998	Yes	Yes	Yes	Colorimetrics
Thompson <i>et al.</i> 1992	No	No	No	Morphology
Thompson <i>et al.</i> 1992			No	Protein analysis
Massey 1976		No		Vocalizations
Burleigh and Lowry 1942	Yes	No	Yes	Morphology
This study	Yes	No	No	Nuclear
This study	No	No	No	Mitochondrial

(non-coding sections of nuclear genes that are not subject to selective pressures) and a section of mitochondrial cytochrome-*b* were examined for the three breeding populations. The use of mitochondrial and nuclear DNA can provide a more complete assessment of population structure due to differences in mutation rates, selection pressure, and heritability. Mitochondrial DNA lacks the efficient repair mechanisms of nuclear DNA, and therefore, mutations are more likely to be maintained. In addition, mtDNA is maternally inherited so the number of individuals contributing to the gene pool is less than for nuclear DNA, which means fewer generations are required for divergence to occur (Hartl and Clark 1989). Although rate of divergence tends to be slower for nuclear DNA, a benefit is that several evolutionarily independent sites can be examined. These genomic sites were selected because of their ability to detect population substructure within other migratory avian species (Birt-Friesen *et al.* 1992; Friesen *et al.* 1996a; Friesen *et al.* 1997). Although the control region of the mitochondria commonly is used to assess population substructure, this region tends to be heteroplasmic (variable within individuals) in Charadriiformes (Kidd and Friesen 1998). Our data were compared with other studies of Least Tern population structure based on morphology, behavior, and allozyme analysis. Genetic diversity was compared with intraspecific studies of other Charadriiformes.

#### METHODS

Several cooperators collected blood or tissue samples from various sites within each subspecies breeding

range of Least Terns in the United States (Fig. 1). For the Eastern breeding population, samples were collected from three sites in Georgia (N = 6), one in Mississippi (N = 12), and one in Massachusetts (N = 10). Interior samples were collected from one site in Oklahoma (N = 38), one in Missouri (N = 2), and two in South Dakota (N = 16). California samples were collected from seven sites in four counties: Alameda (N = 3), Orange (N = 10), San Diego (N = 11), and Ventura (N = 2). Because breeding ranges of Interior and Eastern subspecies may overlap (Fig. 1), no samples were taken from Texas to avoid problems with misclassification.

Genomic DNA was extracted from tissue and blood samples using standard protocols (Longmire *et al.* 1997). We used intron primers for  $\alpha$ -enolase (*Enol*) and a section of intron XI within glyceraldehyde-3-phosphate dehydrogenase (*Gadp*) that revealed genetic variation in Marbled Murrelets (Friesen *et al.* 1997). The *Gadp* primers developed by Friesen *et al.* (1997) did not amplify clean product in all tern samples so we developed the *Gadp*<sub>LT</sub> primer [5'-CCAGGTTGCCAATGTGGGGTGATA-3'] from Least Tern sequence data. The thermal profile for *Enol* primers described by Friesen *et al.* (1997) was modified by doubling initial denaturation time and increasing final extension time to 15 min. The *Gadp* thermal profile, using the new primer, began with 3 min at 94°C, followed by 35 cycles of denaturation (94°C, 45 s), annealing (54°C, 45 s), and polymerase extension (72°C, 45 s) with a final extension of 15 min at 72°C. Amplifications were conducted using standard protocols with the MgCl<sub>2</sub> concentration set at 2.5 mM (Friesen *et al.* 1997). Part of the *cyt-b* gene was amplified using primers L15136 and H15498 developed by Krajewski *et al.* (1992).

Number of alleles for each amplified region was determined using single-stranded conformation polymorphism analysis (SSCP; Orita *et al.* 1989). Autoradiography was used to identify variants. Variants were sequenced from individuals that were homozygous or heterozygous with a rare allele.

Sequencing was conducted using an automated sequencer (ABI Prism® 377 DNA Sequencer, Applied Biosystems, Foster City, California, USA). These samples were electrophoresed through a 5% non-denaturing polyacrylamide gel (acrylamide:bis-acrylamide = 49:1) containing 10% glycerol for 24 h at ambient temperature and 300 volts. Sequences were verified by comparing complementary strands and sequencing two individuals for each allele. Rare alleles that occurred in single individuals were sequenced at least twice using PCR product from separate amplifications.

Sequences from Least Terns were aligned with corresponding sequences from Marbled Murrelet (GenBank accession number AF306887) and Common Guillemot (accession number U37307) using ClustalX (version 1.81, Thompson *et al.* 1997b) to verify amplification of the correct regions. Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium using the exact probability test of Haldane (1954) with complete enumeration (Louis and Dempster 1987) in GENEPOP genetics analysis software (version 3.2a, Raymond and Rousset 1995a). Allele frequencies were tested for differences between subspecies using a Markov chain (GENEPOP version 3.2a, Raymond and Rousset 1995a). Estimates of F-statistics were calculated using a weighted ANOVA (Weir and Cockerham 1984) with haplotype frequencies (GENEPOP version 3.2a, Raymond and Rousset 1995a). Because F-statistics may underestimate population divergence by not including sequence divergence (Friesen *et al.* 1996a), population genetic structure also was investigated using AMOVA in the computer program Arlequin (version 2.000, Schneider *et al.* 2000) to incorporate information on variation between haplotype sequences (Excoffier *et al.* 1992). That test calculated F-statistic analogs ( $M$ -statistics) using proportion of distances and tested significance of the variance components using a randomization approach (Excoffier *et al.* 1992). Gene flow was calculated from the  $M_{ST}$ -values (Wright 1965; Birky *et al.* 1983). This calculation of gene flow is an estimate of the number of migrants per generation using the amount of replacement of local genes by immigrant genes (Wright 1965).

## RESULTS

*Enol* primers amplified 332 base pairs (bp; accession number AF495809) consisting of 80 bp of the bordering exons and 257 bp of Intron VIII. Examination of SSCP gels indicated that all 53 Least Terns (17 California, 22 Interior, 14 Eastern) examined were fixed for the same allele (Table 2). Sequence data from four individuals verified the presence of a single allele. Because Least Terns were monomorphic at this amplified region, no tests of population structure were relevant.

*Gadp* primers amplified 289 bp. Three alleles (accession numbers AF495806-AF495808) were found among 50 Least Terns (16 California, 19 Interior, and 15 Eastern; Table 2). Those differed by 16 polymorphic sites consisting of one to two substitutions and 13 indels in 289 bp. The rarest allele was found in single individuals from the California population and the Interior population (Fig. 1). Genotypic frequencies for the three breeding populations were in Hardy-Weinberg equilibrium (exact probability test;  $P > 0.15$ ) despite the absence of the

homologous state for a common allele in the Interior breeding population (Fig. 1). This apparent incongruity may be an artifact of sample size. Genotypic distribution was identical across populations (modified  $G$ -test [Goudet *et al.* 1996];  $\chi^2 = 4.24$ ,  $df = 2$ ,  $P = 0.12$ ). Allelic distribution also was the same across populations (unbiased probability test [Raymond and Rousset 1995b];  $\chi^2 = 5.06$ ,  $df = 2$ ,  $P = 0.08$ ).

Genotypic variation ( $F_{ST}$ ) and sequence variation ( $M_{ST}$ ) among breeding populations indicated a significant level of geographic differentiation (AMOVA,  $F_{ST} = 0.06$ ,  $M_{ST} = 0.06$ ,  $df = 2$ ,  $P < 0.001$  for both; Table 3). Pairwise comparisons for both variation indices indicated that the California and Interior breeding populations differed from each other (permutation test,  $P < 0.05$ ), but neither differed from the Eastern population ( $P = n.s.$  Table 4). Overall, 6% of the total nucleotide variation was distributed among the breeding populations. Gene flow was highest (4 individuals/generation) between Interior and Eastern Least Terns and lowest (1.9 individuals/generation) between Interior and California terns (Table 4).

A 362-bp segment of the *cyt-b* gene was obtained for 51 individuals (18 California, 21 Interior, 12 Eastern). Three haplotypes

**Table 2. Haplotype frequencies for two nuclear introns (*Enol* and *Gadp*) and part of the cytochrome-*b* (*cyt-b*) gene in three subspecies of Least Tern. Three haplotypes were found in one intron and the *cyt-b* gene; second intron was monomorphic.**

Haplotype	Subspecies		
	California	Interior	Eastern
<i>Enol</i>			
N	17	22	14
Enol-A	1.00	1.00	1.00
<i>Gadp</i>			
N	16	19	15
Gadp-A	0.56	0.29	0.37
Gadp-B	0.41	0.68	0.63
Gadp-C	0.03	0.03	0.00
<i>Cyt-b</i>			
N	18	21	12
Cytb-1	0.92	1.00	0.94
Cytb-2	0.08	0.00	0.00
Cytb-3	0.00	0.00	0.06

**Table 3.** Estimates of population differentiation in Least Tern using a nuclear intron (*Gadp*) and part of the cytochrome-*b* (*cyt-b*) gene. AMOVA analysis (Arlequin, version 2.000; Schneider *et al.* 2000) detected differences between subspecies for the *Gadp* intron at both the genotypic ( $F_{ST}$ ) and the nucleotide ( $M_{ST}$ ) measurements.

	<i>Gadp</i>	<i>Cyt-b</i>
$F_{ST}$	0.0556 <sup>a</sup>	0.000
$F_{IS}$	-0.0641	na <sup>b</sup>
$F_{IT}$	-0.0049	na
$M_{ST}$	0.0622 <sup>a</sup>	0.0169
$M_{IS}$	-0.1045	na
$M_{IT}$	-0.0357	na

<sup>a</sup>P < 0.0001.

<sup>b</sup>na = not applicable. uias8.

were found that differed from each other by one substitution. The Interior population was monomorphic for the most common haplotype (accession number AF95810), while a single individual in each coastal population possessed a unique haplotype (Table 2; accession numbers AF495811 and AF495812). Haplotype distribution did not differ across subspecies (unbiased probability test [Raymond and Rousset 1995b]; P = n.s.). Analyses of haplotypic and sequence variation indicated no difference among the three breeding populations (AMOVA,  $F_{ST}$  = 0.00,  $M_{ST}$  = 0.02, df = 2, P = n.s. for both; Table 3). Gene flow was highest (82.8 individuals/generation) between California and Eastern Least Terns and lowest (9.7 individu-

**Table 4.** Population pairwise  $F_{ST}$  and  $M_{ST}$ -values for *cyt-b* (bottom left of each matrix) and *Gadp* intron (upper right of each matrix) for subspecies of Least Tern in North America. Pairwise comparisons of the nuclear intron revealed that the California subspecies differed from the Interior although neither subspecies differed from the Eastern. Estimated gene flow (number of females per generation) was calculated from  $M_{ST}$ -values (second number in the  $M_{ST}$ -matrix).

	California	Interior	Eastern
$F_{ST}$ -values			
California		0.111 <sup>a</sup>	0.054
Interior	0.000		0.0
Eastern	0.000	0.000	
$M_{ST}$ -values			
California		0.119 <sup>a</sup> /1.9	0.068/3.4
Interior	0.009/55.1		0.0/4
Eastern	0.006/82.8	0.049/9.7	

<sup>a</sup>P < 0.05.

als/generation) between Interior and Eastern Least Terns (Table 4). Gene flow estimates based on mitochondrial markers reflect dispersal by females.

## DISCUSSION

Two main findings emerged from our examination of Least Tern genetics. At both the nuclear and mitochondrial level, little diversity existed relative to number of haplotypes or nuclear variation. Second, genetic differences were found between two subspecies based on nuclear data that was not supported by mitochondrial data.

The minimal variation we observed in both nuclear and mitochondrial DNA of Least Terns may be suggestive of recent expansion, a population bottleneck (Halley and Hoelzel 1996), or an inherent trait. Although post-glacial expansion of Least Terns and/or population declines of this endangered species could have contributed to the observed lack of genetic diversity, several studies of other avian species and other Charadriiformes demonstrated a similarly low level of diversity (Thompson *et al.* 1992; Friesen *et al.* 1996b; Friesen *et al.* 1997). The levels of genetic variation observed for Least Terns tend toward the low end of variation observed for Laridae with similar life histories but are not unusually low (see Crochet and Desmarais 2000). The general lack of genomic diversity in Charadriiformes may indicate that the minimal variation observed for Least Tern is an inherent trait, not the result of either population expansion or bottleneck.

Analyses of nuclear and mtDNA sequences indicated that Eastern Least Terns did not differ from California or Interior Least Terns. These data concurred with findings of several researchers who examined variation among Least Terns (Table 1). However, conclusions from nuclear and mtDNA analyses were contradictory for differences between the California and Interior Least Terns. Results from analysis of nuclear DNA suggested that Interior and California Least Terns differed from each other and exhibited minimal rates of gene flow while mtDNA suggested no difference and frequent gene flow. A

pattern of significant variation between populations based on nuclear DNA that is not supported by mtDNA suggests that 1) females disperse while males return to nest near their natal colony or 2) incomplete lineage sorting (Weckstein *et al.* 2001).

While female-biased dispersal has been documented for many bird species (Greenwood 1980, Johnson and Gaines 1990), the sex of dispersing or non-dispersing Least Terns is rarely reported, which is expected due to the lack of dimorphic traits between sexes (Thompson *et al.* 1997a). Of the 765 band encounter records for Least Terns in the U.S. Bird Banding Laboratory database on August 2003, only six included sex of the individual (five females, one male), and none of those individuals moved between breeding populations. Boyd and Thompson (1985) speculated that the tern they documented moving from Texas to Kansas to nest was female, based on morphologic and behavioral characteristics. Although direct observations are lacking to explain the apparent incongruity in subspecific differentiation, the combined information from band return data from Least Terns and genetic indices from this study indicate female-biased dispersal.

Band-return data suggest that Least Tern populations conform to the stepping-stone model of distribution (Kimura 1953) because dispersal of first-time breeders and adults was greatest to nearby colonies (Atwood and Massey 1988; Massey *et al.* 1992; Boyd 1993; Lingle 1993; Renken and Smith 1995). Crow and Aoki (1982) estimated that for stepping-stone dispersers, 2-4 migrants/generation would be sufficient to prevent population divergence due to genetic drift. Female dispersal rates among subspecies (>9 migrants/generation; Table 4), as reflected by mtDNA gene flow estimates, indicate adequate gene flow to prevent divergence. It should be noted that the single reported instance of long-distance dispersal between Least Tern subspecies (Boyd and Thompson 1985) may reflect difficulties in relocating long-distance dispersers, not rarity of occurrence. This has been noted in other species such as the Kittiwake (*Rissa tridactyla*), which has a bimodal dispersal pattern with many

individuals moving > 400 km from their natal colony making detection difficult (Coulson and De Mevergnies 1992). If long-distance dispersal occurs commonly in Least Terns, the stepping-stone model would not apply, but fewer dispersers would be necessary to prevent divergence.

Based on nuclear gene flow, which reflects both male and female dispersal, genetic material is being exchanged between Eastern and California terns and between Eastern and Interior terns at sufficient rates (3.4 migrants/generation; Table 4) to prevent divergence, but the lower rate of genetic exchange between Interior and California terns (1.9 migrants/generation; Table 4) may not be adequate to prevent divergence. Sporadic pulses of gene flow related to population fluctuations could prevent divergence (Hanski and Gilpin 1997; Avise 2000). Additional support for gene flow between the Eastern and Interior subspecies comes from the combined evidence of apparent lack of a geographical barrier between the two breeding ranges, dispersal of an individual from a coastal colony to an interior colony, and tendency for short-distance dispersal of first-time breeders and adults.

Crochet (2000) demonstrated that under assumptions of no sex-biased dispersal and even sex-ratios,  $F_{ST}$ -values for mtDNA should be higher than for nuclear DNA, which was not the case for our study. No research has documented uneven sex-ratios for Least Terns (Thompson *et al.* 1997a) so these data suggest a sex-biased dispersal. Although no direct demographic data could be found to support the conclusion of female-biased dispersal, the trend toward male-biased philopatry and female-biased dispersal has been reported in several Laridae (see reviews in Ryder 1993; Verbeek 1993; Moskoff and Bevier 2002); therefore, the mtDNA gene-flow estimates may be evidence that this occurs in Least Terns.

The other possible explanation for incongruent findings between the nuclear and mtDNA analyses is recency of radiation or a greater divergence rate for the nuclear intron than *cyt-b* resulting in incomplete linkage sorting. This phenomenon is suggested

by the existence of multiple shared alleles among the subspecies (Takahashi *et al.* 2001). In general, mtDNA is presumed to diverge faster than nuclear DNA because it is haploid and maternally inherited, has a smaller effective population size, and lacks corrective processes observed in the nuclear genome (Ballard and Whitlock 2004). A commonly cited estimate of mtDNA sequence divergence is 2% divergence between pairs of lineages/MY, which is about ten fold faster than for nuclear DNA (Avice 2000; Ballard and Whitlock 2004). Therefore, lineage sorting should occur more rapidly in the mtDNA genome than the nuclear genome (Pacheco *et al.* 2002). Pacheco *et al.* (2002) noted that the mutation rate for the *Gadp* intron has not been estimated, but Prychitko and Moore (1997) determined that another nuclear intron experienced one-quarter the divergence rate of *cyt-b*. Given those data, we would have expected to see divergence within the nuclear intron to occur more slowly than for *cyt-b*, which was not the case.

Only two definitive measurements appear to distinguish all three subspecies of Least Terns in North America: breeding location (albeit vague where Interior and Eastern subspecies likely overlap) and feather colorimetry (Johnson *et al.* 1998), which may be potentially confounded by environmental conditions during feather development. Because Least Tern subspecies cannot yet be differentiated consistently based on genetics, morphology, or behavior (Massey 1976; Thompson *et al.* 1992), we are dubious of their current subspecific classification. While our study suggests little genetic differentiation, future research using additional genetic markers (i.e. microsatellites) is needed to fully elucidate the subspecific status of Least Terns in North America. In addition, genetic markers should be used to reevaluate the relatively recent separation of Least Tern and the European Little Tern (*Sterna albifrons*) into distinct species because this separation was based on minor variations in call (Massey 1976; Massey 1998) of a taxa with a nearly global distribution (Olsen and Larson 1995). Further, similarly low patterns of philopatry of Little Terns and Least Terns

(Oro *et al.* 2004) may suggest similar patterns of genetic differentiation.

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