AN ABSTRACT OF THE THESIS OF

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Susan M. Haig

Two North American subspecies of Snowy Plovers (*Charadrius alexandrinus*) have been described: the Cuban Snowy Plover (*C. a. tenuirostris*) and the Western Snowy Plover (*C. a. nivosus*). Coastal populations of the Western Snowy Plover are listed as Threatened under the U.S. Endangered Species Act, while populations of the Cuban Snowy Plover in the southeastern U.S. and Puerto Rico show evidence of decline and are being considered for listing. To clarify the relationships among populations, we examined variation in mitochondrial (mtDNA) sequences and at inter-simple sequence repeat (ISSR) loci among 8 populations distributed across North America and the Caribbean. MtDNA d-loop region sequences (322 bp, n = 126) revealed 8 haplotypes, with one haplotype unique to Puerto Rico, and indicated that Puerto Rico is significantly differentiated from all mainland populations. Conversely, neighbor-joining analysis of 16 ISSR loci suggests that Puerto Rico is nested within a cluster of populations from eastern continental North America. Evidence for structure among continental populations was weak among mtDNA haplotypes ($\Phi_{ST} = 0.025$, $p = 0.178$), but analysis of ISSR markers supported subdivision into groups east and west of the Rocky Mountains ($\Phi_{ST} = 0.445$, $p < 0.001$). A significant relationship between genetic distance (pairwise F_{ST}) and geographic distance was observed (Mantel $r = 0.85$, $p < 0.01$). Overall, two management units in continental North America (divided by the Rocky Mountains) and a separate

unit for the Puerto Rican population may be warranted. We recommend that both genetic and demographic considerations be weighed in making policy decisions regarding the status and listing of Snowy Plover population segments.

Population Differentiation among Snowy Plovers (Charadrius alexandrinus) in North America

by

Leah R. Gorman

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Leah R. Gorman, Author

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Population Differentiation among Snowy Plovers (*Charadrius alexandrinus*) in North America

Introduction

Snowy Plovers, or Kentish Plovers (*Charadrius alexandrinus*), are small sequentially polygamous shorebirds with a global distribution that have suffered population declines due to human disturbance, habitat loss, and habitat degradation (Page *et al.* 1995a). At least five subspecies of Kentish Plovers have been described based on morphological variation, although the taxonomy is controversial (Hayman *et al.* 1986). In North America, clarity regarding subspecies definition and the relationship among regional populations is important in determining where conservation efforts should be focused and which populations are listed for protection under the U.S. Endangered Species Act.

There are two subspecies of Snowy Plovers recognized in North America. The Western Snowy Plover (*C. a. nivosus*) is thought to comprise resident populations on the Pacific Coast of the United States, Texas, and Mexico, as well as migratory populations in the Great Basin and Great Plains (American Ornithologists Union 1957; Figure 1). The Cuban Snowy Plover (*C. a. tenuirostris*) is generally thought to encompass resident populations on the United States Gulf Coast east of Louisiana, Greater Antilles, southern Bahama Islands, Yucatan peninsula of Mexico, and islands off Venezuela. Often, *C. a. tenuirostris* is not mentioned in avian taxonomy discussions, and all North American Snowy Plovers are considered *nivosus* (e.g., Hayman *et al.* 1986). No genetic or

morphological examination has been conducted to resolve these issues, aside from describing more or less pale plumage (Conover 1945, AOU 1957, Binford 1989, Page *et al.* 1995a). A third subspecies in the Western Hemisphere, *C. a. occidentalis*, is found in coastal Peru and Chile. This subspecies is distinguished from the North American subspecies by a wider eye stripe and less sexual dimorphism in breeding adults. Together, the three form a group known as the 'Snowy Plover', which has been distinguished from other races of Kentish Plover by the all white lores of their breeding plumage (Hayman *et al.* 1986).

The Western Snowy Plover is one of a few species listed as a distinct population segment under the U.S. Endangered Species Act (Federal Register 1996). Coastal populations of this subspecies are federally listed as Threatened, while others are not (Federal Register 1992). Many of the same pressures that affect populations of the Western Snowy Plover have impacted populations in the southeastern United States (Chase & Gore 1989, Gore 1996, Sprandel *et al.* 1997). As a result, the Cuban Snowy Plover has been listed as threatened by the state of Florida and endangered by the state of Alabama. Moreover, in Puerto Rico, the breeding population only consists of approximately 20 pairs (Lee 1989). Should the U.S. Fish and Wildlife Service proceed with a federal listing of these populations, it will be important to understand whether the Cuban Snowy Plover represents a distinct genetic unit within the species, and should be managed separately from the Western Snowy Plover. Additionally, it will be important to determine the distinctiveness of individual populations of the Snowy Plover in the United States, as threats to the viability of populations vary geographically.

In this study, we describe the population structure and phylogeography of Snowy Plovers in North America by examining variation in the mitochondrial DNA (mtDNA) control region and at inter-simple sequence repeat (ISSR) loci. We analyze the relationship between the geographic distribution of genetic variation and gene flow, genetic drift, and historic processes. We also discuss the implications for defining conservation units and conserving genetic diversity within and among populations.

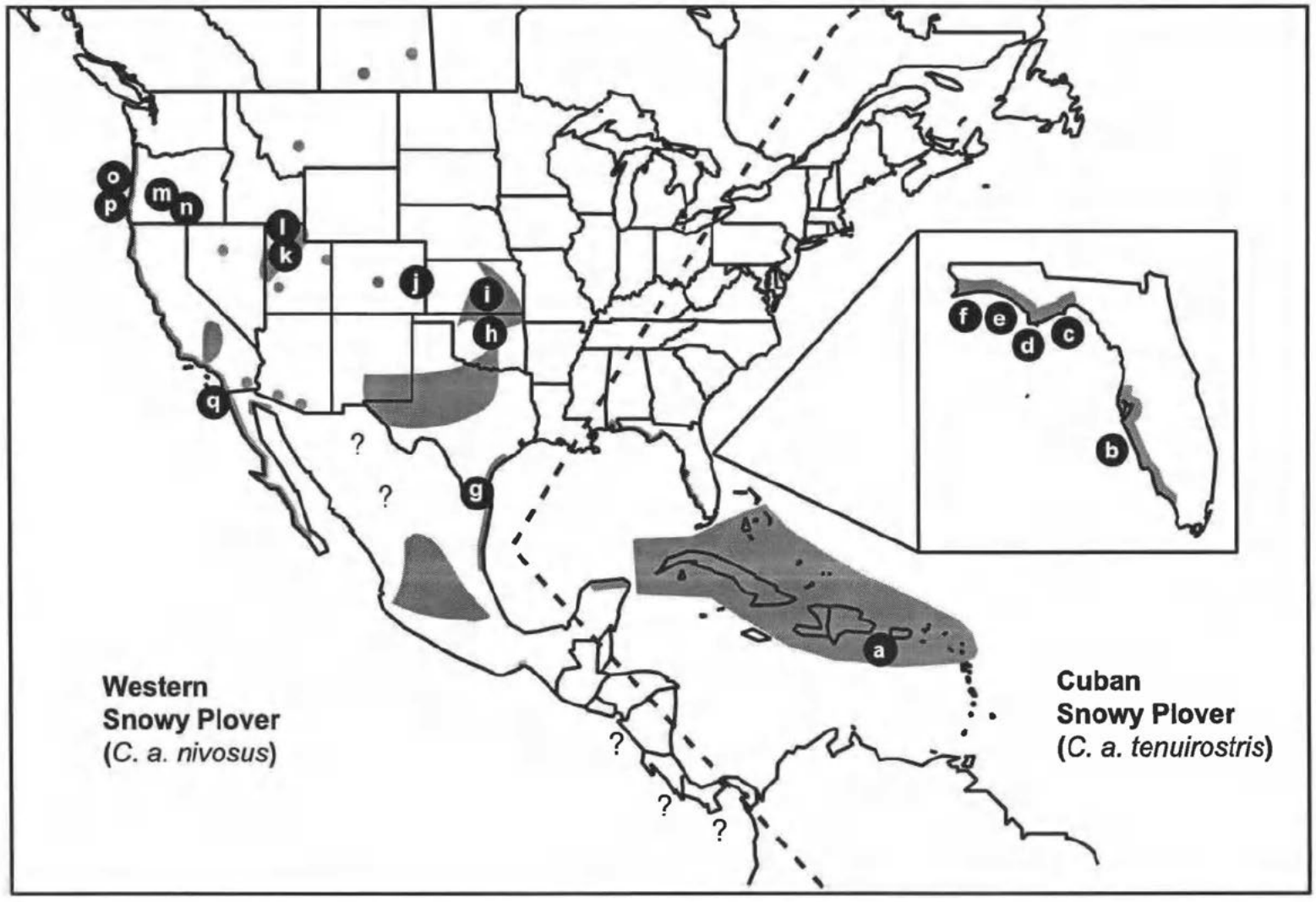
Materials and Methods

Tissue Sampling

Following protocols outlined by the American Ornithologists Union (Oring *et al.* 1988), blood samples were collected from breeding populations in Puerto Rico, Florida, Kansas, Texas, Utah, eastern Oregon, and California (Figure 1). Once Snowy Plover nests were located, standard shorebird nest traps were used to catch adult birds as they returned to their nests (see Hill & Talent 1990). In order to minimize nest desertion, attempts to trap birds were not made unless at least two eggs had been laid. Birds were marked with a U.S. Fish and Wildlife Service aluminum band to insure that individuals were not recaptured. Blood samples were taken from the brachial vein using a 27 gauge needle, collected in a 0.25 ml heparinized capillary tube, and transferred to a 1 mL cryogenic vial containing a buffer solution (100 mM Tris HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS). Samples were stored at room temperature until they could be put into permanent storage at -80°C.

In Oregon and Oklahoma, heart, pectoralis, and embryonic tissue samples were collected from carcasses of individuals that died of natural causes. In addition, membranes were collected from eggs in Colorado. Finally, tissue samples from six individuals of *C. a. occidentalis* from Peru were provided by the Louisiana Museum of Natural Sciences (LMUMZ B-24019, B-24029, B-24030, B-24047, B-24107, B-24157). The dates of collection for the samples from Peru are unknown.

Figure 1. Breeding range of the Snowy Plover in North America, Caribbean, and Bahamas (modified from Page *et al.* 1995a) and sampling site locations. Dashed line indicates currently accepted subspecies boundary between the Western Snowy Plover (*C. a. tenuirostris*) and Cuban Snowy Plover (*C. a. nivosus*). Sites sampled for *C. a. tenuirostris* include: PUERTO RICO (PR): a. Cabo Rojo (n = 8); FLORIDA (FL): b. Cayo Costa State Park (n = 2), c. St. George Island State Park (n = 4), d. Philips Inlet (n = 1), e. Eglin Air Force Base (n = 7), f. Gulf Island National Seashore (n = 5). Sites sampled for *C. a. nivosus* include: TEXAS (TX): g. Lower Rio Grande Valley National Wildlife Refuge (n = 7); EASTERN GREAT PLAINS (GP): h. Great Salt Plains National Wildlife Refuge, Oklahoma (n = 7); i. Quivira National Wildlife Refuge, Kansas (n = 20); COLORADO (CO): j. Arkansas River (n = 7); UTAH (UT): k. Inland Sea Shorebird Reserve (n=12), l. Saltwell Flats (n = 3); EASTERN OREGON (EO): m. Summer Lake (n = 6), n. Lake Abert (n = 2); OREGON COAST (OC): o. Baker Beach, Lane County (n = 1), p. Coos County (n = 11); CALIFORNIA (CA) q. San Diego County (n = 20).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Western
Snowy Plover
(C. a. nivosus)
Cuban
Snowy Plover
(C. a. tenuirostris)

DNA Extraction

High molecular weight DNA was obtained for analysis by a standard phenol/chloroform extraction from blood and tissue samples. Briefly, 10μl of blood or a 1 mm section of tissue was digested in 400 μl of extraction buffer A (50mM Tris, pH 8.0; 10mM EDTA, 200mM NaCl, 2% SDS) and 30 μl Proteinase K (20mg/ml). Samples were vortexed and incubated overnight (~18 h) at 50° C. If blood clots or tissue did not fully disperse, a second aliquot of Proteinase K was added, and samples were incubated for an additional 2 h. Samples were extracted with an equal volume of phenol (saturated with 10mM Tris, pH 8.0) followed by an equal volume of chloroform/isoamyl alcohol (25:1). DNA was precipitated by adding a 1/10 volume of 3M sodium acetate, two volumes of cold 95% ethanol, and pelleted at approximately 15,000 xG for 20 minutes. Pellets were rinsed once with 70% ethanol, dried under vacuum, and resuspended in 30μl sterile water. DNA concentration of samples was quantified with a DyNA Quant 200 fluorometer (Hoefer).

DNA Amplification and Sequencing

The polymerase chain reaction (PCR) was used to amplify a 322 bp fragment of the mitochondrial control region, or d-loop, using the primer pair TS437L and TS778H (Wenink *et al.* 1994). Each reaction was carried out using 100 ng of genomic DNA in a 50 µL volume. Fragments were amplified using a PTC 100 thermal cycler (MJ Research). An initial denaturation of 10 min at 93°C was followed by 44 cycles of 1 min denaturation at 92°C, 1 min annealing at 45°C, and 2 min extension at 72°C, with a single

final extension at 72°C for 10 min. The reactions were purified electrophoretically; bands of approximately 350 bp were extracted from a 1% agarose gel using an ultra-free-mc 0.45 filter (Millipore) and purified over a micron-50 filter (Millipore). Sequence data were generated with an automated sequencer (Applied Biosystems model 373A) located in the Central Services Laboratory at Oregon State University. The sequencer uses a modified Sanger method (Sanger 1977) with fluorescently labeled dideoxy terminators. The region was bidirectionally sequenced to insure sequences of high quality.

PCR Amplification, Screening, and Scoring of ISSR Markers

The ISSR method utilizes a single primer anchored to a simple sequence repeat (SSR), or microsatellite, to amplify the region between adjacent repeats (Gupta *et al.* 1994). Amplifications were carried out in 10X buffer (20 mM Tris-$C_2H_3O_2$, pH 9.0; 10 mM NH_4SO_4 sulfate; 75 mM $KC_2H_3O_2$; 0.05% Tween® 20); 1.5 mM $MgCl_2$; 5 mg/mL acetamide; 100 µM dNTPs; 0.15 µM primer; 50 ng of genomic DNA; fluorescent dUTPs (0.2 mM [R110]dUTP or 0.2 mM [R6G]dUTP or 0.8 mM dUTP [TAMRA]); and 1.9 units of *Taq* polymerase (Promega) per 25 µL reaction. A PTC-100 thermal cycler (MJ Research) was programmed for 34 cycles of the following parameters: denaturation for 30 s at 93°C, annealling for 30 s at 45°C, and elongation for 2 min at 72°C. A single 10 min elongation at 72°C followed the cycles, and reactions were held at 4°C until removed from the thermal cycler. Two methods of electrophoresis were utilized to visualize bands of different sizes. First, we loaded 15 µL of each reaction into a 2% agarose gel stained with EtBr, and electrophoresed for 4 h in TBE (90 mM Tris base; 90 mM Boric Acid; 2 mM

EDTA, pH 8.0). A 1 Kb ladder (GibcoBRL) served as a molecular size standard. Amplification products between 1000 and 1400 bp were scored as present (1) or absent (0) from the agarose gel. Further, PCR products were electrophoresed on an ABI Prism 377 automated sequencer (Applied Biosystems) at the Central Services Laboratory at Oregon State University. Briefly, amplification products were loaded in a 5% LongRanger (6 M Urea) denaturing gel, and run for 5 h with Filter Wheel A, and Genescan 2500 [Rox] as an internal lane standard. Gels were analyzed utilizing GeneScan Analysis 2.1 software (Applied Biosystems). Fragments between 400 and 1000 bp were scored as present (1) or absent (0) using Genotyper software (Applied Biosystems).

Ninety primers from the University of British Columbia Biotechnology Laboratory (UBCBL) primer set #9 were initially screened for PCR amplification on agarose gels. Of these, 44 with distinct, well separated bands were screened for variability using 4 individuals from across the North American range (Cabo Rojo, Puerto Rico; St. George Island, Florida; Lower Rio Grande Valley NWR, Texas; San Diego County, California). We chose 16 bright, reproducible bands from eight primers for scoring in the final analysis. The primers utilized in the final analysis were UBC826: $(AC)_8T$; UBC846: $(CA)_8RT$; UBC848:$(CA)_8RG$; UBC849: $(GT)_8YA$; UBC850: $(GT)_8YC$; UBC857 $(AC)_8YG$; UBC859: $(TG)_8RC$; and UBC886: $VDV(CT)_7$. DNA was amplified from single extractions to minimize variation in DNA quality and concentration. Negative controls were run to prevent scoring artifact bands. Low quality DNA samples were eliminated from the analyses; thus, the set of samples analyzed does

not completely overlap the mtDNA data set, and the Colorado population is not represented in ISSR analyses.

Data Analysis

Mitochondrial DNA Sequences

Snowy Plover sequences were compared to control region sequences from Ruddy Turnstone (*Arenaria interpres*) obtained from Genbank (Accession number L20136) and Piping Plover (*Charadrius melodus*; S. Haig & T. Mullins, unpublished data). Alignments were constructed by hand using the Genetic Data Environment (GDE) software package (Smith *et al.* 1992).

Estimates of nucleotide diversity (π) and haplotype diversity (h) were derived using the equations of Nei (1987). Genetic distance between haplotypes was estimated using the Kimura 2-parameter method (Kimura 1980). We estimated population subdivision via analysis of molecular variance using haplotype frequencies (AMOVA; Excoffier *et al.* 1992). In this analysis, Φ_{ST}, the correlation of random genotypes within a population relative to the species, is analogous to Wright's F_{ST} statistic (Wright 1951). To test significance of the Φ statistics, values were compared to a null distribution obtained by generating 1000 random permutations of the haplotypes among populations. To further test the null hypothesis that the distribution of haplotypes among populations was random, an exact test of population differentiation was conducted (Raymond &

Rousset 1995). All of the analyses listed above were carried out in the program ARLEQUIN 1.1 (Schneider *et al.* 1997).

ISSR Markers

ISSR markers have a dominant or codominant mode of inheritance (e.g., Gupta *et al.* 1994, Wolfe *et al.* 1998), and thus were scored as phenotypes of "present" or "absent". Absence of a band is assumed to indicate the loss of a priming site through divergence, deletion, or chromosomal rearrangement (Wolfe & Liston 1998). Scored loci were assumed to be non-allelic and in Hardy-Weinberg equilibrium.

To correct for bias associated with dominance, band frequencies, percent polymorphic loci (*P*, 95% criteria), and expected heterozygosity (H_e, Nei 1978) were calculated using Lynch and Milligan's (1994) Taylor expansion in TFPGA (Miller 1998b). In addition, the effective number of alleles per locus (A_e) was calculated using the program POPGENE (Yeh *et al.* 1997).

Phylogenetic relationships among populations were examined using the neighbor-joining method (Saitou & Nei 1987). Distance trees were constructed by calculating a matrix of Manhattan distances (Prevosti distance in Wright 1978) of ISSR phenotypes in the program RAPDDIST (Black 1998b). One hundred bootstrap matrices were calculated in RAPDDIST, and used to construct a consensus bootstrap tree with NEIGHBOR and CONSENSE programs in PHYLIP v 3.5C (Felsenstein 1993).

We used non-metric multidimensional scaling, a multivariate approach based on ranked differences between populations, to qualitatively assess differences between

populations (Kruskal 1964, Mather 1976). Distance between populations was estimated using Jaccard's coefficient (Jaccard 1908 in Sneath & Sokal 1973) because of its utility for two-state data (+/-). Final stress (opposite of goodness of fit) was plotted against number of dimensions to determine the minimum number of dimensions that accurately assessed minimum stress; three dimensions were chosen for the final analysis. A plot of number of iterations versus stress determined 30 iterations were sufficient to obtain a stable final solution, and 100 iterations were run in the final analysis. Monte Carlo simulations were conducted to estimate the probability that the final stress could have been determined by chance. All calculations were carried out with PC-ORD software (McCune & Mefford 1999).

Genetic differentiation among population groups suggested by geographic barriers were tested using analysis of molecular variance (AMOVA; Excoffier *et al.* 1992). First, a matrix of squared Euclidean distances between populations was prepared with AMOVA-PREP software (Miller 1998a), where $E = n\,[1 - (\,n_{xy}/n)]$, and n_{xy} is the number of bands shared by two individuals (Excoffier *et al.* 1992). We then calculated within and among group variance components and Φ_{ST} in WINAMOVA (Excoffier 1993). In addition, the level of heterogeneity within these groups was compared to that expected by chance using multi-response permutation procedure (MRPP), carried out on the Euclidean distance matrix in PC-ORD (McCune & Mefford 1999). MRPP is a non-parametric approach, which does not require multivariate normality and homogeneity of variances (Mielke 1984). Pairwise comparisons of genetic differentiation among populations were made with an exact test of population differentiation (Raymond & Rousset 1995), using 1000 permutations in TFPGA (Miller 1998b).

In order to assess the relative historical influence of gene flow and genetic drift on population structure, we compared the relationship between genetic distance and geographic distance to that expected under a stepping-stone model of population structure (Kimura 1953, Malécot 1955, Kimura & Weiss 1964). Pairwise F_{ST} values were calculated as an approximation of genetic distance (Reynolds *et al.* 1983), using a second order Taylor expansion (Lynch & Milligan 1994) in RAPDDIST (Black 1998). Following Hutchinson and Templeton (1999), we constructed scatterplots of pairwise F_{ST} values versus geographic distance to see if genetic distance and scatter increased with geographic distance. Residuals from a linear regression of pairwise F_{ST} values versus geographic distance were plotted against geographic distance. A Mantel test, which allows for lack of independence among points, was used to examine correlation between the geographic distance matrix and matrices of pairwise F_{ST} values and residual values (Hutchinson & Templeton 1999). Significance of the resulting correlation coefficients (r-values) was assessed with a Monte Carlo simulation (1000 iterations) using PC-ORD software (McCune & Mefford 1999). Lynch and Milligan's (1994) F_{ST} statistic, and the associated estimate of gene flow (N_m; Wright 1951), were calculated in the program RAPDFST (Black 1998a).

Results

MtDNA Sequence Data

In the 322 bp of sequence obtained from 126 individuals, we observed 6 variable nucleotide sites comprising 8 haplotypes (Table 1). Of these 8 substitutions, 7 were transitions and 1 was a transversion. No haplotypes were separated from other haplotypes by more than a single base pair change, as indicated in a hand-generated parsimony network (Figure 2).

Haplotypes varied greatly in their degree of geographic dispersion (Figure 3). The most common haplotypes, C and D, were quite widespread and were identified in all populations in the continental United States. These haplotypes accounted for 83.3% of individuals. Haplotype C was also present in two individuals from Peru. In contrast, haplotype A was unique to Puerto Rico and was the only haplotype represented in that population. Haplotypes B and G were present at low frequencies in the Texas and the Great Plains populations. Haplotype H was also present in one individual from Kansas and one from Colorado. Haplotype F was unique to California, but appeared only in a single individual. The final haplotype was identified in three individuals from Peru and one from Eastern Oregon.

Overall, nucleotide diversity for mtDNA sequences was low within all populations, averaging 0.003, and ranging from 0 to 0.003 (Table 2). Haplotype diversity, on the other hand, ranged from 0 in Puerto Rico and Colorado populations to 0.714 in Texas, with an overall value of 0.617 ± 0.031 (SE) for continental North

Table 1. Variable nucleotide sites from the aligned 322 bp of the mitochondrial control region for North American, Puerto Rican, and Peruvian Snowy Plover populations. Sampling locations are identified in Figure 1.

	Sequence Position						Sampling Location										
Haplotype	456	554	572	637	736	757	PR	FL	TX	GP	CO	UT	CA	EO	OC	PE	Total
A	C	T	G	A	C	T	8	-	-	-	-	-	-	-	-	-	8
B	C	T	G	G	C	T	-	-	1	1	-	-	-	-	-	-	2
C	C	T	G	G	C	C	-	3	4	10	2	7	6	3	4	2	41
D	T	T	G	G	C	C	-	14	1	12	4	8	13	4	8	-	64
E	C	T	G	G	T	C	-	-	-	-	-	-	-	1	-	3	4
F	T	T	C	G	C	C	-	-	-	-	-	-	1	-	-	-	1
G	T	T	G	A	C	C	-	-	1	3	-	-	-	-	-	-	4
H	T	C	G	G	C	C	-	-	-	1	1	-	-	-	-	-	2
Total							8	17	7	27	7	15	20	8	12	5	

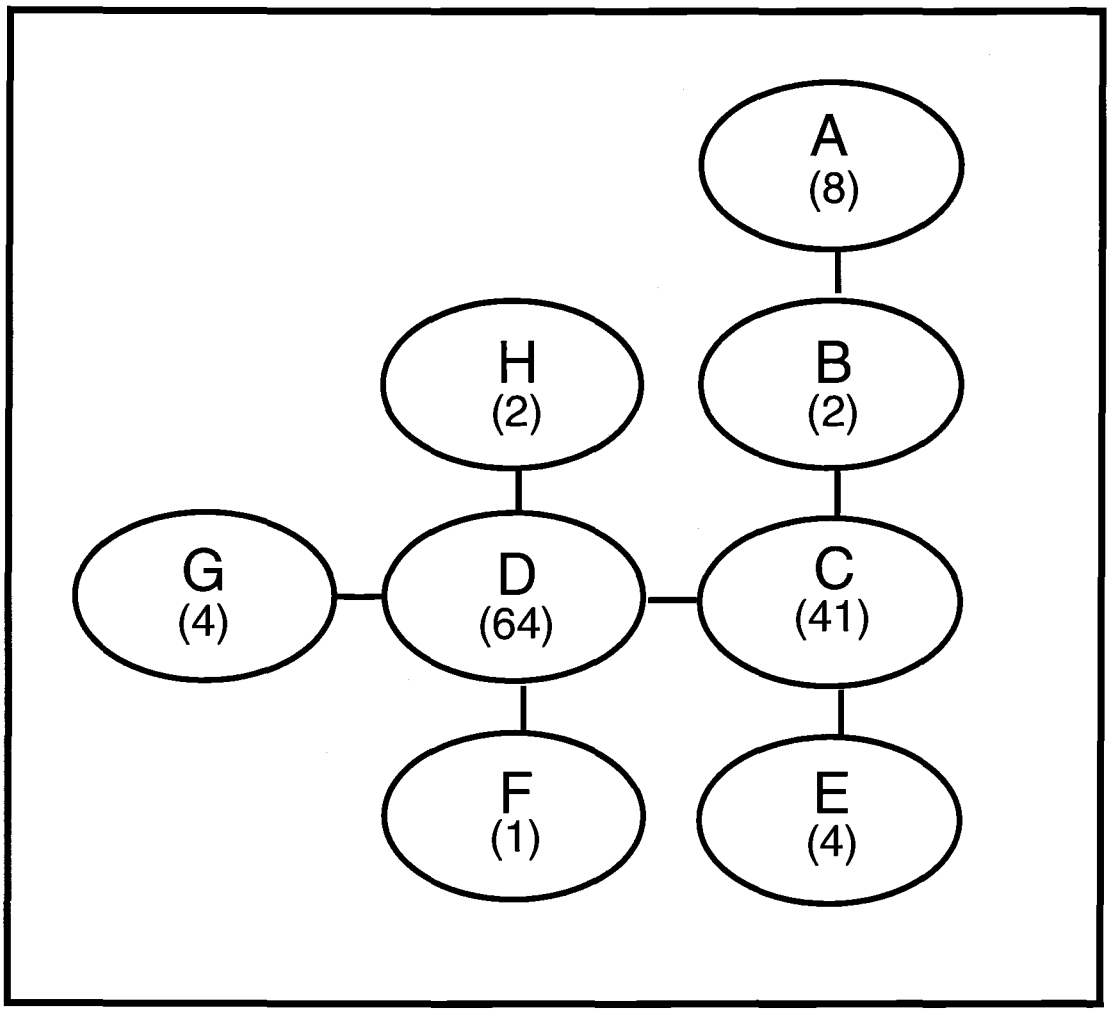

Figure 2. Minimum spanning network reflecting the evolutionary relationships of 8 maternal haplotypes of Snowy Plover. Haplotypes are indicated by the letters given in Figure 1. Lines between haplotypes represent single base pair changes.

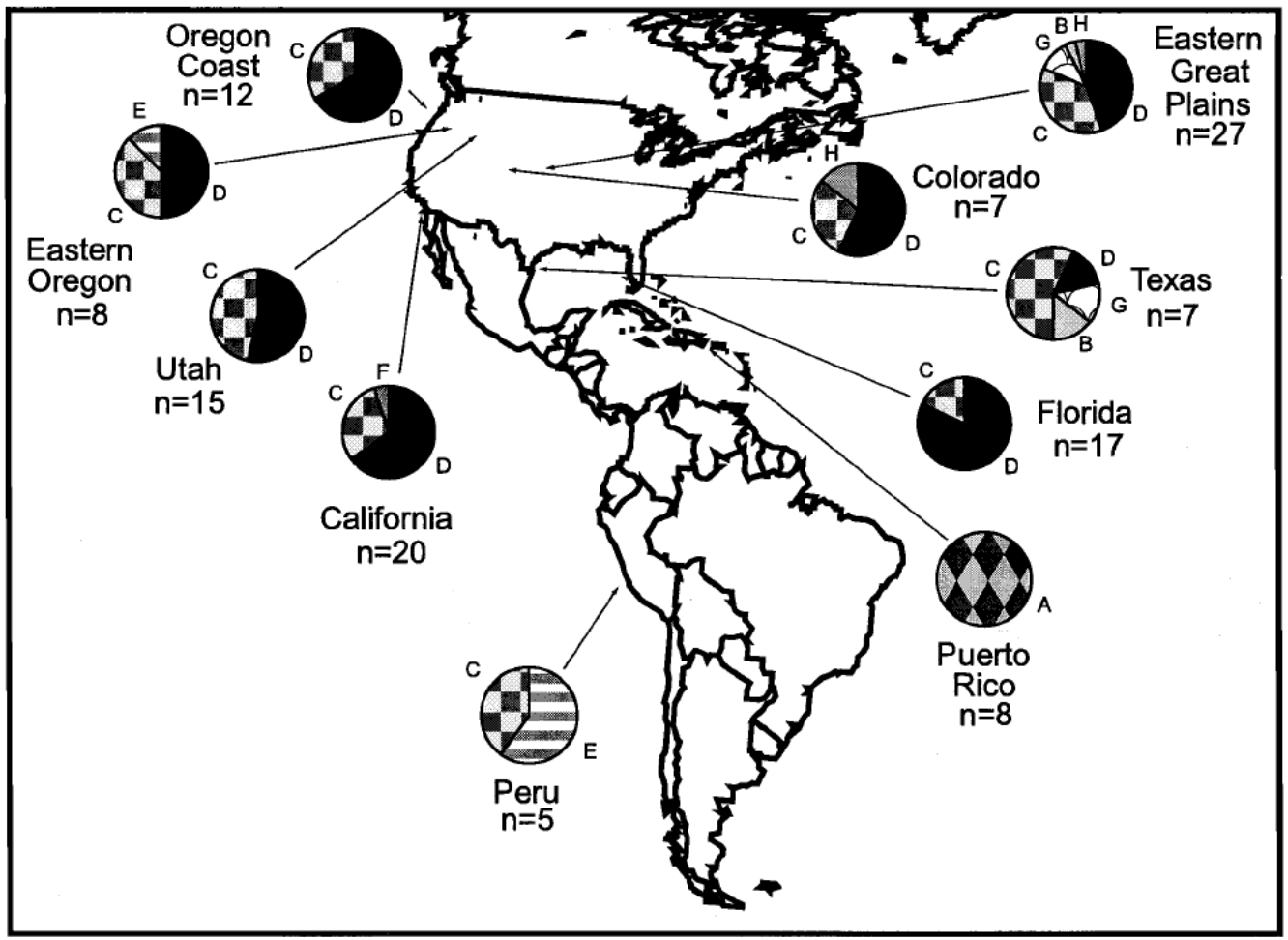

Figure 3. Distribution of Snowy Plover haplotypes. Pie charts represent haplotype frequencies.

Table 2. Haplotype (h) and nucleotide (π) diversity for North American and Puerto Rican Snowy Plovers based on mtDNA control region sequence.

Region	Haplotype diversity (h) ± SE	Nucleotide diversity (π) ± SE
Puerto Rico	0.000	0.000
Florida	0.309 ± 0.122	0.001 ± 0.001
Texas	0.714 ± 0.181	0.003 ± 0.003
Eastern Great Plains	0.675 ± 0.056	0.003 ± 0.002
Colorado	0.667 ± 0.160	0.002 ± 0.002
Utah	0.533 ± 0.052	0.002 ± 0.002
Eastern Oregon	0.679 ± 0.122	0.003 ± 0.003
Oregon Coast	0.485 ± 0.106	0.002 ± 0.002
California	0.511 ± 0.091	0.002 ± 0.002
Overall	0.617± 0.031	0.003 ± 0.002

America and Puerto Rico. Haplotype diversity was lower in Florida than in western populations, which all reflected haplotype diversity values between 0.485 and 0.679 (Table 2).

Analysis of molecular variance (AMOVA) revealed that the majority of variance in haplotypic diversity was attributable to the within population component, regardless of which populations were considered (Table 3). When only continental populations were considered, analysis of molecular variance provided no evidence of structure (Φ_{ST} = 0.025, p = 0.178). However, when all North American populations were considered (including Puerto Rico), significant differentiation between populations was observed, indicating that population structure was due to differentiation between Puerto Rico and mainland populations (Table 3). Hierarchical analysis revealed that birds with different migratory patterns (east and west of the Rocky Mountains) were not structured with respect to mtDNA haplotypes.

The exact test of population differentiation revealed low probability of non-differentiation between the Puerto Rican population and all continental populations (Table 4). Among eastern populations, Texas appears to be significantly differentiated from Florida. Texas is also significantly differentiated from California and coastal Oregon. There was no support for differentiation of Florida from any population west of the Great Plains, nor evidence that the Great Plains population was differentiated from any other continental population at mtDNA haplotypes. Furthermore, the exact test provided no support for population differentiation in the western United States. Results indicate that the probability of non-differentiation between all pairs of western populations (coastal Oregon, Eastern Oregon, California, and Utah) is greater than 0.45,

Table 3. Analysis of molecular variance for Snowy Plover populations based on mtDNA sequence variation and 16 variable ISSR loci. Sampling locations identified in Figure 1.

	MtDNA				ISSR			
	df	% var	Φ	p	df	% var	Φ	p
Nested analysis								
Among groups	1	-3.31	Φ_{CT} = -0.03	p = 0.543	1	32.43	Φ_{CT} = 0.32	p < 0.001
Among populations within groups	7	19.89	Φ_{SC} = 0.19	p < 0.001	6	12.09	Φ_{SC} = 0.18	p < 0.001
Within populations	112	83.42	Φ_{ST} = 0.17	p < 0.001	95	55.48	Φ_{ST} = 0.45	p < 0.001
Eastern group (PR+FL+TX+GP)								
Among populations	4	32.77	Φ_{ST} = 0.33	p < 0.001	3	21.41	Φ_{ST} = 0.21	p < 0.001
Within populations	61	67.23			52	78.59		
Western group (OC+EO+CA+UT)								
Among populations	3	- 4.73	Φ_{ST} = - 0.05	p = 0.756	3	13.48	Φ_{ST} = 0.14	p < 0.001
Within populations	51	104.73			43	86.52		
All populations								
Among populations	8	17.73	Φ_{ST} = 0.18	p < 0.001	7	35.83	Φ_{ST} = 0.36	p < 0.001
Within populations	112	82.27			95	64.17		

Table 4. Probability of non-differentiation between populations based on mtDNA sequence variation (below diagonal) and 16 variable ISSR loci (above diagonal).

	Puerto Rico	Florida	Texas	Eastern Great Plains	Colorado	Utah	Eastern Oregon	Oregon Coast	California
Puerto Rico	-	0.002	0.003	0	-	< 0.001	< 0.001	< 0.001	< 0.001
Florida	< 0.001	-	0.868	0.006	-	< 0.001	< 0.001	< 0.001	< 0.001
Texas	< 0.001	0.003	-	0.983	-	< 0.001	0.001	< 0.001	< 0.001
Eastern Great Plains	< 0.001	0.125	0.384	-	-	< 0.001	< 0.001	< 0.001	< 0.001
Colorado	< 0.001	0.645	0.243	0.643	-	-	-	-	-
Utah	< 0.001	0.129	0.064	0.774	0.323	-	0.077	0.004	0.078
Eastern Oregon	< 0.001	0.659	0.278	0.659	1.000	0.584	-	0.997	0.646
Oregon Coast	< 0.001	0.396	0.038	0.795	0.569	0.696	0.623	-	0.843
California	< 0.001	0.588	0.032	0.288	0.535	0.593	0.461	1.000	-

and the probability of non-differentiation between Oregon and California populations is 1.0.

ISSR Markers

Screening for genetic variability resulted in 16 scorable ISSR loci (Table 5), ranging in size from 450 to 1400 bp (Table 5). Across all populations, the effective number of alleles was 1.45 ± 0.30, the percentage of polymorphic loci was 87.5, and level of expected heterozygosity was 0.28 (Table 6). Puerto Rico had the lowest genetic diversity of all populations, while western populations had the highest diversity.

Neighbor-joining analysis revealed a cluster of populations east of the Rocky Mountains (Figure 4). In contrast to mitochondrial analysis, Puerto Rico was not isolated from continental populations. In fact, Puerto Rico fell within a cluster of eastern continental populations, and grouped most closely with Florida, although bootstrap support for the Florida - Puerto Rico relationship was weak. Populations from Texas and the Great Plains clustered with Florida and Puerto Rico, which suggests that the *nivosus* subspecies may be a paraphyletic group.

Non-metric multidimensional scaling of 16 loci further supported differences between populations east and west of the Rocky Mountains. Two dimensions (axes) represented most of the variance among individuals, while the third accounted for the remainder (axis 1: $R^2 = 0.15$, axis 2: $R^2 = 0.26$, axis 3: $R^2 = 0.08$, cumulative $R^2 = 0.48$). Additional dimensions did not significantly improve the model. Monte Carlo simulations indicated that the probability of obtaining a lower final stress by chance was

Table 5. Estimates of ISSR dominant marker allele frequencies (+) using Lynch and Milligan's (1994) Taylor expansion for 8 Snowy Plover breeding areas across North America and the Caribbean (see Figure 1 for locations).

	Frequency (+)							
ISSR locus	PR	FL	TX	GP	UT	EO	OC	CA
UBC 826 - 490	0.0000	0.1807	0.0000	0.0440	0.0000	0.0000	0.0625	0.0000
UBC 846 - 450	0.0625	0.0000	0.0000	0.1386	1.0000	0.5767	0.6030	0.7251
UBC 846 - 630	0.0625	0.0278	0.0000	0.0217	0.7707	0.3294	0.2019	0.5031
UBC 846 - 810	0.0000	0.0000	0.0000	0.0217	0.3103	0.0000	0.0625	0.1572
UBC 846 - 1300	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0625	0.0000
UBC 848 - 570	0.4754	0.2879	0.0000	0.0901	0.0000	0.4405	0.3713	0.1235
UBC 848 - 780	0.3713	0.1807	0.0000	0.1140	0.2643	0.2338	0.0625	0.0597
UBC 849 - 550	0.0000	0.0000	0.0625	0.2741	0.5374	1.0000	0.6030	0.4479
UBC 850 - 750	1.0000	0.5169	0.4754	0.4414	0.3103	0.4405	0.6030	0.1924
UBC 850 - 780	0.4754	0.1807	0.1294	0.1386	0.0000	0.0000	0.0625	0.0294
UBC 857- 640	0.1294	0.3695	0.6030	0.5719	0.7077	1.0000	1.0000	0.6370
UBC 859 - 610	0.0000	0.0564	0.0625	0.0901	0.3595	0.3294	0.6030	0.4479
UBC 859 - 730	0.0000	0.0000	0.0000	0.0667	0.2643	0.5767	1.0000	1.0000
UBC 886 - 650	0.0000	0.1807	0.0625	0.0901	0.1801	0.0000	0.0000	0.0000
UBC 886 - 800	0.0000	0.0278	0.0625	0.1640	0.0679	0.0000	0.0625	0.0294
UBC 886 - 1400	0.0625	0.5169	0.6030	0.2741	0.1801	0.5767	0.2817	0.3083

Table 6. Genetic variability within populations of Snowy Plovers averaged over 16 ISSR loci.

Population (n)	Effective # Alleles/ Locus (A_e)	% Polymorphic Loci (P_{95})	Expected Heterozygostity (H_E)
Puerto Rico (8)	1.23 ± 0.38	43.75	0.13
Florida (18)	1.34 ± 0.36	56.25	0.20
Texas (8)	1.22 ± 0.35	50.00	0.14
Eastern Great Plains (23)	1.34 ± 0.31	75.00	0.22
Utah (15)	1.43 ± 0.35	68.75	0.26
Eastern Oregon (7)	1.43 ± 0.45	50.00	0.23
Oregon Coast (8)	1.39 ± 0.37	81.25	0.25
California (17)	1.41 ± 0.40	62.50	0.24
All populations	1.45 ± 0.30	87.50	0.28

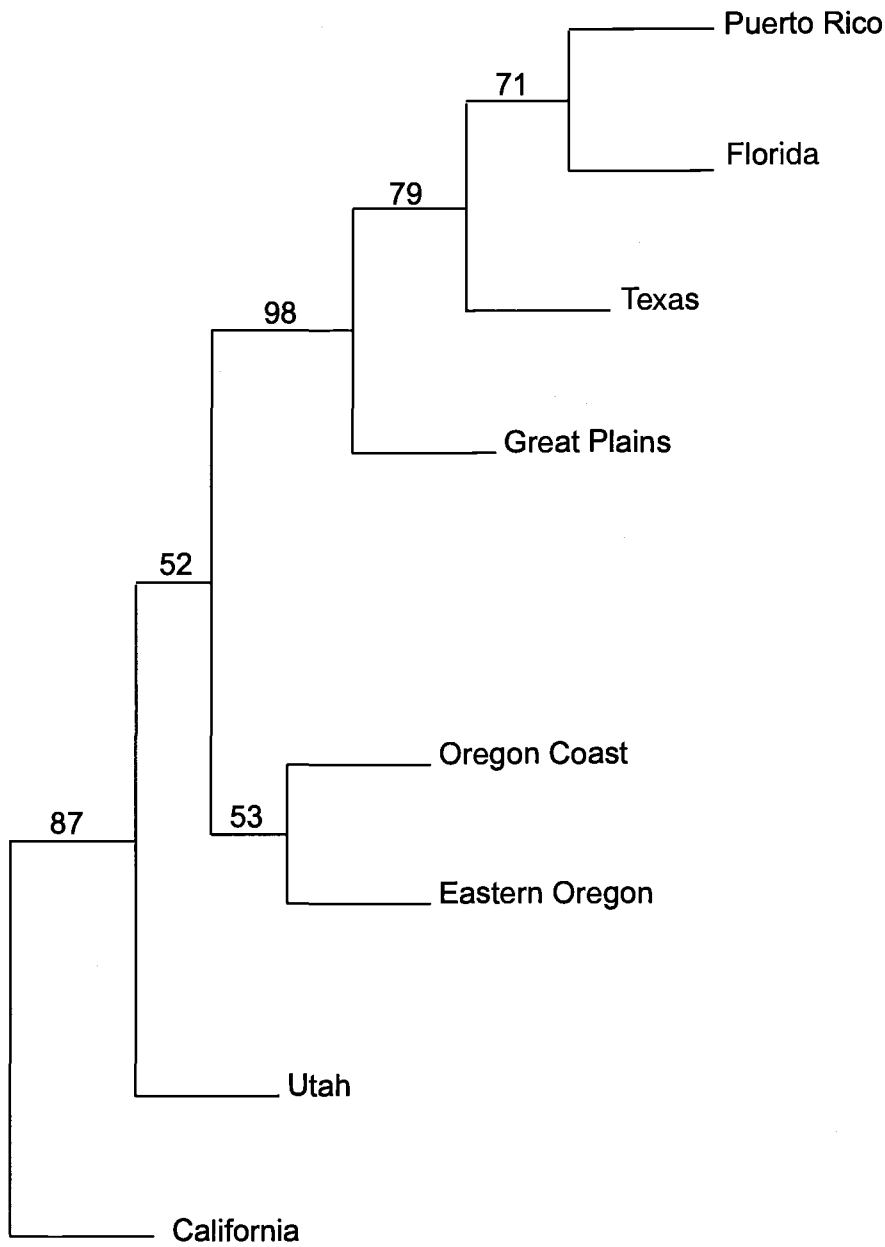

Figure 4. Neighbor-joining tree of North American and Caribbean Snowy Plover populations based on Manhattan distances of 16 variable ISSR loci. Numbers above the branches indicate percent bootstrap support. Sample sizes for individual populations are listed in Table 6.

very low (final stress = 15.95, $p < 0.01$). A plot of the final solution reveals clustering of populations east and west of the Rocky Mountains, with a good deal of overlap among individuals within these two groups (Figure 5). Of the populations east of the Rocky Mountains, the Great Plains population displayed the greatest overlap with western populations.

Analysis of molecular variance (AMOVA) of ISSR markers provided further support for differences between eastern and western groups. When nested analyses were conducted, a greater proportion of the variation was attributable to groups (east and west of the Rocky Mountains) than among populations within groups, or within groups (Table 3). The eastern group appeared to be more highly structured than the western group, with a greater proportion of the variance attributable to the among population component. Likewise, multi-response permutation procedures resulted in significant within group agreement among all populations ($R = 0.33$, $p < 0.05$) and for the eastern and western groups ($R = 0.22$; $p < 0.05$).

Differentiation among all pairs of populations was compared with an exact test of population differentiation (Table 4). As with mtDNA results, Puerto Rico had a high probability of differentiation from all continental populations. Results provide evidence that Florida is differentiated from the Great Plains; however, Texas was not significantly differentiated from either Florida or the Great Plains. All populations in the eastern group (Puerto Rico, Florida, Texas, Great Plains) appear to be significantly differentiated from the western group (California, coastal Oregon, and eastern Oregon, and Utah). Members of the western group were not significantly differentiated from one another.

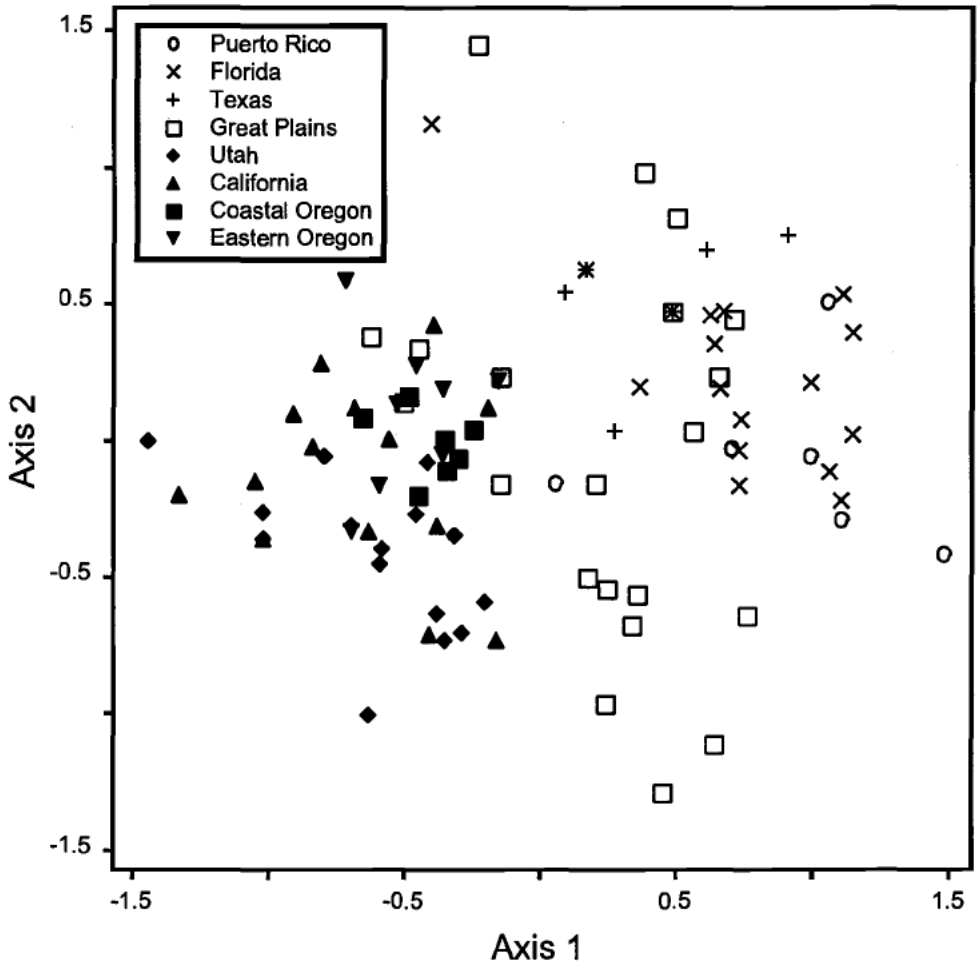

Figure 5. Non-metric multidimensional scaling based on Jaccard's distances for Snowy Plovers (n=104) using 16 variable ISSR loci.

Utah, however, had a much lower probability of differentiation from other western populations.

The correlation between pairwise F_{ST} values and geographic distance was examined to illustrate the relative influence of genetic drift and gene flow on population structure. The null hypothesis of equilibrium can be rejected if the relationship between geographic distance and genetic distance is not significant, or if a scatterplot of geographic distance does not reveal a positive relationship between genetic and geographic distance and a pattern of increasing variance (Hutchinson & Templeton 1999). Comparison of pairwise F_{ST} values and geographic distance reveals a positive linear relationship, starting near the origin, as predicted under an equilibrium model (Figure 6). A Mantel test revealed a highly significant relationship between pairwise F_{ST} and geographic distance ($r = 0.85$; $p < 0.01$). However, we did not observe a significant positive correlation between the residuals and geographic distance ($r = 0.00$; $p = 0.47$), as expected under equilibrium conditions.

As equilibrium conditions were not met, it was inappropriate to estimate gene flow across all populations. Assuming equilibrium conditions within groups, the eastern group ($F_{ST} = 0.30 \pm 0.24$; $N_m = 0.6$) appears to be more highly structured and has lower levels of gene flow than the western group ($F_{ST} = 0.16 \pm 0.13$; $N_m = 1.3$). However, if Puerto Rico is excluded from the eastern group, gene flow between populations is higher east of the Rocky Mountains ($F_{ST} = 0.06 \pm 0.07$; $N_m = 3.9$). Gene flow between the eastern and western groups appears to be limited ($F_{ST} = 0.25 \pm 0.22$; $N_m = 0.7$).

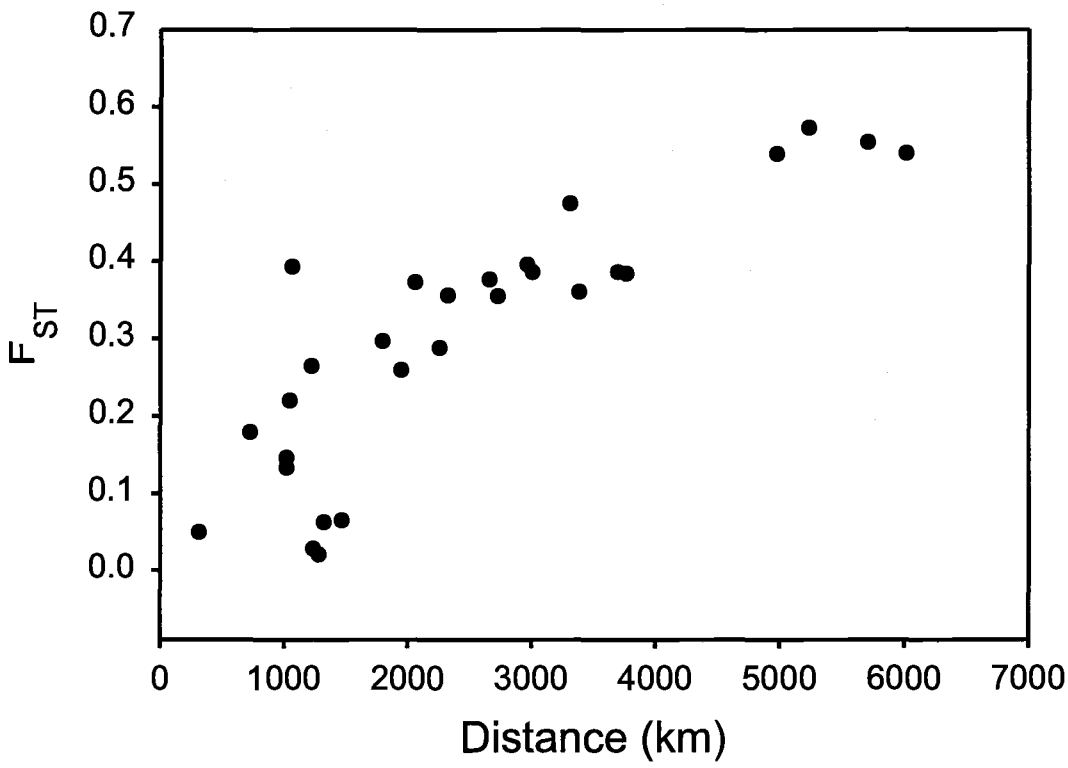

Figure 6. Scatterplot of F_{ST} estimates based on 16 variable ISSR loci (Lynch and Milligan 1994) versus geographic distance for all pairwise comparisons of populations.

Discussion

Genetic Variation within Populations

Few studies have estimated within population genetic variation in shorebirds (Baker & Strauch 1988, Haig & Oring 1988a, Baker 1992, Haig *et al.* 1997). Allozyme analyses revealed low variability within populations of Piping Plovers (Haig & Oring 1988a). Detailed phylogeographic analyses have been completed on only one shorebird species, the Dunlin (*Calidris alpina*; Wenink et al. 1993, 1994, 1996; Wenink and Baker 1996). Interestingly, estimates of within population haplotypic diversity in Dunlin for Region II of the mtDNA control region were similar to those of Snowy Plover populations (Dunlin: h = 0.00 - 0.72). When data from cytochrome b and control Region I were considered, however, higher levels of within population diversity were observed, ranging from 0.63 to 1.00 (Wenink 1994). This illustrates the need to exercise caution when interpreting estimates of genetic diversity based on a single region, as different domains of the control region appear to have different mutation rates (Baker & Marshall 1997). Overall, our estimates of haplotypic and nucleotide diversity are low, but within the range observed among other avian species (Gill *et al.* 1993, Seutin *et al.* 1994, Gill & Slikas 1992).

Populations of Snowy Plover varied in degree of genetic diversity, with the Puerto Rico samples exhibiting the lowest level at both mtDNA and ISSR markers. Given the small population size of the resident Puerto Rican population (n = 40; Lee 1989), a local population bottleneck may have led to a reduction in genetic variability. Low variability

may also be attributable to founder effect, given the insularity of the Puerto Rican population. Comparison of the Puerto Rican population with other populations in the Caribbean will be necessary in order to distinguish between these hypotheses. Within the continental United States, patterns of genetic diversity at ISSR loci were not concordant with measures of haplotypic diversity. The Texas population had the highest mtDNA haplotypic diversity, while western populations had the highest diversity at ISSR loci.

Genetic Structure and Phylogeographic Patterns

MtDNA structure among North American and Caribbean Snowy Plovers is due primarily to differentiation of Puerto Rican Snowy Plovers from continental populations. While ISSR markers reveal significant differentiation between Puerto Rico and mainland populations, Puerto Rico appears to be paraphyletic with populations in the Eastern United States. Thus, lack of overlap in haplotype composition between Puerto Rico and continental populations is consistent with a recent population bottleneck, versus a prolonged period of evolutionary separation from mainland populations.

ISSR analyses support further subdivision of continental populations of Snowy Plovers in North America into two groups: east and west of the Rocky Mountains. While we observed no statistical differences in mtDNA haplotype composition between groups, three haplotypes common to populations in the central United States were not observed west of the Rocky Mountains. Groups east and west of the Rocky Mountains are concordant with known migratory patterns: eastern birds have been observed to winter

along the coast of the Gulf of Mexico, while western populations winter along the Pacific coast and the Gulf of California (Page *et al.* 1995b; W. Conway, pers. comm.).

Within the eastern United States, pairwise comparisons of populations suggest some structure to populations. However, the results are not consistent between mtDNA and ISSR markers, except that Texas and Great Plains populations do not appear to be differentiated. Qualitative examination of mtDNA haplotype distribution reveals two haplotypes identified exclusively among Texas, Great Plains, and Colorado populations. Presence of these shared haplotypes might reflect either a pattern of gene flow between resident Texas and migratory Great Plains birds or a common population history. For example, the Great Plains breeding population could represent a historic breeding range expansion of the Texas population. Theory on the evolution of migratory behavior in birds predicts that partial migration is a stage in the evolution of fully migratory populations (e.g., Cox 1985).

Lack of differentiation between coastal Oregon, California, and Eastern Oregon is not unexpected because birds have been observed dispersing between breeding sites in the region (Stenzel *et al.* 1994). Within a single breeding season, female birds from the central California coast were observed as far south as San Diego County (615 km) and inland at Summer and Abert Lakes, Oregon, 660 km north in the Great Basin. Between seasons, movements as far north as Washington (1140 km) have been documented (Stenzel *et al.* 1994). The low magnitude of structure observed among western populations might also reflect high rates of natal dispersal. Rates of natal philopatry have not been examined for North American Snowy Plovers, but anecdotal evidence suggests

that dispersal of hundreds of kilometers from the natal site is not uncommon (Page *et al.* 1995a).

At ISSR loci, the Great Salt Lake population has a much lower probability of non-differentiation from birds at other Great Basin sites or Pacific coastal sites, although the populations are not differentiated at mtDNA alleles. One might predict differentiation between these populations because movements between the Great Salt Lake and other Great Basin sites have not been documented despite banding efforts, and high breeding site fidelity at Great Salt Lake sites has been reported (Paton & Edwards 1996). In addition, birds from the Great Salt Lake apparently do not mix as extensively on wintering grounds with coastal California birds as birds from the Western Great Basin (Page *et al.* 1995b). While mixing of populations on the wintering grounds is not as reliable an indicator of gene flow as natal philopatry and breeding site fidelity (Oring and Lank 1984), in this case, the breeding range overlaps the wintering range. Because pair bond formation occurs on the breeding grounds and may occur up to two months before egg laying (Warriner *et al.* 1986, Chase & Gore 1989), wintering together might increase the likelihood of pair bond formation and decrease the likelihood of a bird to migrate.

Overall, mtDNA markers revealed less structure within continental North America than ISSR makers. Lack of concordance between mtDNA and ISSR markers might reflect inadequate time since separation of populations for mtDNA differentiation to become apparent (Neigel & Avise 1986). The relatively low variation observed consistently across all populations supports this hypothesis. An alternative hypothesis might be that differences between mtDNA and nuclear genes reflect differential dispersal patterns between males and females. For organisms with sex-biased dispersal patterns,

maternally inherited mtDNA may not accurately represent population structure for biparentally transmitted genes or the species as a whole (Avise *et al.* 1992, Melnick & Hoelzer 1992, Bowen *et al.* 1992). Shorebirds, on average, have higher rates of female than male dispersal, although this varies depending on the mating system (Oring *et al.* 1983, Oring & Lank 1984, Gratto *et al.* 1985, Haig & Oring 1988b, Colwell & Oring 1988, Reed & Oring 1993). The Snowy Plover mating system is sequentially polygamous and females have lower breeding site fidelity than males (Page *et al.* 1983, Warriner *et al.* 1986, Stenzel *et al.* 1994, Paton 1994).

Usually, when female dispersal is greater than male dispersal, we would expect to see weak population structure in both mtDNA haplotypes and autosomal genes, as female mediated gene flow homogenizes both. Therefore, differences between male and female dispersal alone do not adequately explain the observed pattern in Snowy Plovers. Because the effective population size for mtDNA is generally smaller than for nuclear genes, mtDNA markers are expected to sort more rapidly, and often reveal structure not observed at nuclear genes (Neigel & Avise 1986). However, the effective population size for mitochondrial genes may not always be smaller than autosomal genes if the assumption of random mating is not met (Chesser & Baker 1996, Piertney *et al.* 2000). In Snowy Plovers, deviations from the assumption of random mating, including sex-biased dispersal rates and a polygamous mating system, may explain greater structure at ISSR loci.

Given the putative barriers to gene flow and complex dispersal patterns of Snowy Plovers, it is not surprising that the null hypothesis of equilibrium was rejected at ISSR loci. Snowy Plovers, as partial migrants, may not fit the assumptions of the stepping

stone model that dispersal is limited to the closest populations. Although we found a highly significant relationship between geographic distance and genetic distance, the scatter of points did not reflect a pattern of increasing variance expected under equilibrium (Hutchinson & Templeton 1999). From the observed pattern, it is difficult to distinguish between a hypothesis of isolation by distance and a more complex structure where individual regions are influenced differentially by gene flow and genetic drift. MtDNA evidence for the isolation of the Puerto Rican population and the likelihood of the Rocky Mountains as a barrier suggest that the latter might be the case.

Lack of equilibrium between gene flow and genetic drift indicates that it would not be appropriate to estimate gene flow across all populations (Hutchinson & Templeton 1999). If we assume equilibrium within regional groups, comparison of relative estimates reveals greater gene flow among western populations than among eastern populations only when Puerto Rico is included in the eastern group. Migration of a few individuals per generation has been shown to be adequate to prevent populations from becoming fixed for different alleles (Wright 1951). Our estimates suggest that gene flow is higher than this threshold for continental populations of Snowy Plovers.

Relationships among Subspecies

Our results call for a reexamination of subspecies boundaries for Snowy Plovers. At mtDNA alleles, the Florida population appears to be much more closely related to other continental populations of *C. a. nivosus* than to the Puerto Rican population of *C. a. tenuirostris*. Conover (1945) similarly found no morphological differences between birds

from Florida and California, but lacked specimens to draw conclusions about the identity of Caribbean populations. At ISSR loci, the Puerto Rican population is nested within a group of populations from the eastern United States. However, this group also does not correspond to previously defined subspecies, as Great Plains and Texas birds appear to be more closely related to eastern than western populations.

This study provides evidence of overlap in haplotype composition of the South American subspecies *(C. a. occidentalis)* and the Western Snowy Plover (*C. a. nivosus).* Haplotype C, one of the more common North American haplotypes, was also observed in two of five individuals from Peru. In addition, a single copy of haplotype E was detected in eastern Oregon, which was otherwise found only in the Peruvian population. The observed pattern might reflect incomplete lineage sorting in populations that diverged relatively recently on an evolutionary time scale. Additional sampling of *C. a. occidentalis* would be required to detect statistical differences in allele frequencies between the two subspecies. Overall, an examination of the global population structure of Kentish Plovers would be prudent before drawing conclusions about subspecies boundaries.

Conservation Unit Designation

Preservation of biological diversity below the species level has become increasingly recognized as a priority in endangered species conservation. One approach to this problem has been the identification of intraspecific conservation units, which rely to a varying degree on genetic criteria (e.g., evolutionary significant unit, management

unit, distinct population segment; Ryder 1986; Waples 1991, 1995; Moritz 1994, Moritz *et al.* 1995). Based on differences at ISSR loci, populations of Snowy Plovers east and west of the Rocky Mountains could be considered separate management units according to Moritz's (1994) criteria, requiring differences in allele frequencies at nuclear loci. Because the populations do not exhibit reciprocal monophyly at mtDNA alleles, these groups could not be considered separate evolutionary significant units (ESU; Moritz 1994; Moritz et. al. 1995). However, both ISSR and mtNDA data suggest a separate management unit (MU) for the Puerto Rican population may also be warranted. While the Puerto Rican population appears to be closely related to other eastern populations, Puerto Rico has significant differences in mtDNA and nuclear gene frequencies from all mainland populations. Additional sampling of other Caribbean populations will clarify whether a separate or overlapping management unit should be considered for the eastern United States and Caribbean.

Within management units, distinguishing whether populations are separate genetic and demographic units is important because threats to the viability of these breeding populations differ. Coastal and inland populations of Snowy Plovers in the western United States are currently being managed separately; coastal populations are protected as a Distinct Population Segment under the U.S. Endangered Species Act, while inland populations are not listed. Our study provides no evidence of genetic differentiation between coastal and inland populations. Likewise, decisions must be made about the status and listing of the resident Florida population (n = 400 individuals; Chase & Gore 1989), the resident coastal Texas population (n = 500 individuals; Page *et al.* 1995a), and the migratory Great Plains population (n = 1900-2150 individuals; Page *et al.* 1995a).

Considered together, Texas and Great Plains populations contain unique mtDNA lineages within North American Snowy Plovers which should be recognized to conserve genetic diversity. Nonetheless, evidence for genetic differentiation among populations in the eastern continental United States is inadequate to support designation of additional management units, according to Moritz's criteria.

In making policy decisions about whether to manage populations separately, genetic data need to be evaluated in light of demographic considerations. Evidence of differentiation at mtDNA alleles provides a strong indication of demographic autonomy (Avise 1995). However, in some cases levels of gene flow may be high enough to prevent genetic differentiation when populations are demographically independent (Avise 1995, Taylor & Dizon 1996). In the context of conservation, a population experiencing declines may have enough dispersal to homogenize populations genetically, but inadequate recruitment to prevent extirpation (Taylor & Dizon 1996, 1999). If the policy goal is to halt a decline over an extensive part of a species range, a conservative approach may be to manage populations in smaller units based on demographic considerations.

Finally, according to the U.S. Fish and Wildlife Service policy, the Eastern United States, the Western United States, and the Puerto Rican populations could all be considered Distinct Population Segments due to the genetic discontinuity between populations, corresponding to the three management units described above (Federal Register 1996). However, additional groups may also be considered Distinct Population Segments, as the policy allows for other criteria to be used to judge distinctiveness of populations (Federal Register 1996). We recommend that both genetic and demographic

considerations be weighed in making policy decisions regarding the status and listing of Snowy Plover population segments.

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