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Subspecies Status and Population Genetic Structure in Piping Plover (*Charadrius melodus*)

Mark P. Miller
U.S. Geological Survey, mpmiller@usgs.gov

Susan M. Haig
U.S. Geological Survey, Susan_Haig@usgs.gov

Cheri L. Gratto-Trevor
Prairie and Northern Wildlife Research Centre

Thomas D. Mullins
U.S. Geological Survey, tom_mullins@usgs.gov

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SUBSPECIES STATUS AND POPULATION GENETIC STRUCTURE IN PIPING PLOVER (*CHARADRIUS MELODUS*)

MARK P. MILLER,[1,3] SUSAN M. HAIG,[1] CHERI L. GRATTO-TREVOR,[2] AND THOMAS D. MULLINS[1]

[1]*U.S. Geological Survey Forest and Rangeland Ecosystem Science Center, 3200 SW Jefferson Way, Corvallis, Oregon 97331, USA; and*
[2]*Prairie and Northern Wildlife Research Centre, Environment Canada, 115 Perimeter Road, Saskatoon, Saskatchewan S7N 0X4, Canada*

ABSTRACT.—Piping Plover (*Charadrius melodus*) is a migratory shorebird that is listed as endangered in Canada and the U.S. Great Lakes and as threatened throughout the rest of its breeding and winter range. We undertook a comprehensive molecular-genetic investigation to (1) address subspecific taxonomy, (2) characterize population genetic structure, and (3) infer past bottlenecks and demographic processes in this species. Analyses included individuals from 23 U.S. states and Canadian provinces and were based on mitochondrial DNA sequences (580 base pairs, $n = 245$) and 8 nuclear microsatellite loci ($n = 229$). Our findings provide support for separate Atlantic and Interior subspecies (*C. m. melodus* and *C. m. circumcinctus*, respectively). Birds from the Great Lakes region were allied with the Interior subspecies and should be referred to as *C. m. circumcinctus*. Population genetic analyses illustrated stronger genetic structure among Atlantic than among Interior birds, which may reflect reduced natal- and breeding-site fidelity of Interior individuals. Furthermore, analyses suggested that Interior birds previously experienced genetic bottlenecks, whereas there was no evidence of such patterns in the Atlantic subspecies. We interpret these results in light of 25 years of range-wide census data. Overall, differences between Interior and Atlantic Piping Plovers may reflect differences in spatiotemporal stability of nesting habitat between regions. *Received 1 April 2009, accepted 20 August 2009.*

Key words: bottleneck, *Charadrius melodus*, genetic structure, Piping Plover, population expansion, subspecies.

Estatus de las Subespecies y Estructura Genética Poblacional en *Charadrius melodus*

RESUMEN.—*Charadrius melodus* es un ave playera migratoria que se considera en peligro en Canadá y en los grandes lagos de los Estados Unidos, y amenazada en el resto de su distribución reproductiva y de invierno. Realizamos una investigación exhaustiva de genética molecular para (1) evaluar la taxonomía subespecífica, (2) caracterizar la estructura poblacional y (3) inferir cuellos de botella del pasado y procesos demográficos en esta especie. Los análisis incluyeron individuos de 23 estados de los Estados Unidos y provincias de Canadá y se basaron en secuencias de ADN mitocondrial (580 pares de bases, $n = 245$) y 8 loci microstatélites nucleares ($n = 229$). Nuestros resultados apoyan la existencia de subespecies separadas en el Atlántico y el interior (*C. m. melodus* y *C. m. circumcinctus*, respectivamente). Las aves de la región de los grandes lagos estuvieron asociadas con la subespecie del interior y deberían llamarse como *C. m. circumcinctus*. Los análisis de genética poblacional ilustraron la existencia de estructura genética más marcada entre las aves del Atlántico que entre las del interior, lo que podría reflejar una fidelidad reducida a los sitios natales y de reproducción por parte de los individuos del interior. Además, los análisis sugirieron que las aves del interior sufrieron cuellos de botella genéticos previamente, mientras que no existió evidencia de patrones de este tipo en la subespecie del Atlántico. Interpretamos esos resultados a la luz de datos de censos tomados a escala de toda la distribución de la especie durante 25 años. En general, las diferencias entre las aves del interior y del Atlántico podrían reflejar diferencias entre regiones en la estabilidad del hábitat de anidación en el espacio y el tiempo.

THE PIPING PLOVER (*Charadrius melodus*) has long been a species of conservation concern throughout its range (Fig. 1). In Canada, two subspecies are recognized: *C. m. melodus* in the Atlantic Canada region and *C. m. circumcinctus* in Ontario and Prairie Canada. Both subspecies are listed as endangered under the Species at Risk Act (Department of Justice Canada 2002). In the United States, Piping Plovers are listed under the Endangered Species Act (ESA) as endangered in the Great Lakes watershed and as threatened in the rest of its breeding and winter range (U.S. Fish and Wildlife Service [USFWS] 1985). The USFWS has approved separate recovery plans for populations breeding on the Atlantic Coast (USFWS 1988a, 1996), Great Lakes (USFWS 2003), and Northern Great Plains (USFWS 1988b). Primary threats include nest and chick disturbance stemming from habitat degradation

[3]E-mail: mpmiller@usgs.gov

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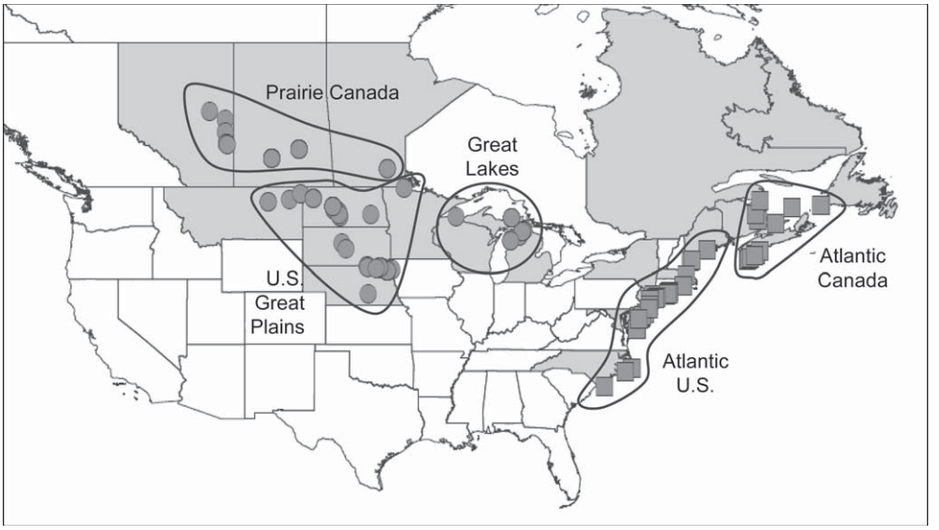


Fig. 1. Collection locations of Piping Plovers analyzed in the present study. Shaded U.S. states or Canadian provinces highlight general geographic regions. Symbols reflect specific collection locations (circles = Interior group, squares = Atlantic group). See Table 1 for regional designations and sample-size information.

associated with human land use and development practices. Predation also has been suggested as a pertinent threat (Cuthbert and Roche 2008). Complete species censuses over the past 25 years have documented range expansions, contractions, and local extirpations as well as areas where numbers have increased or decreased (Haig and Oring 1985, Haig and Plissner 1993, Plissner and Haig 2000, Haig et al. 2005, Elliott-Smith et al. 2009).

Despite continued conservation concerns, there has not been a modern molecular-genetic study to address higher-level taxonomic issues or elucidate population patterns and processes. A formal evaluation of subspecific taxonomic status is a primary need. *Charadrius m. melodus* (hereafter "Atlantic" subspecies) are thought to breed in the Atlantic coastal region of North America, whereas *C. m. circumcinctus* (hereafter "Interior" subspecies) have been described as breeding in the continent's interior (American Ornithologists' Union [AOU] 1957, Elliott-Smith and Haig 2004). Subspecific identification of Great Lakes birds has not been resolved, although an early allozyme study described them as belonging to the Interior subspecies (Haig and Oring 1988b). From a recovery perspective, clarifying the taxonomy of Piping Plovers may be important for informing listing and status reviews, establishing management strategies, and prioritizing funding under the ESA or the Species at Risk Act.

An understanding of taxonomy and population structure on multiple geographic and temporal scales provides critical details of a species' status and changes in status that are almost impossible to obtain without taking a molecular approach. The genetic affinity of populations is routinely used to determine the degree of vulnerability of species at risk or of population segments within species. Here, we used mitochondrial DNA (mtDNA) sequence analyses to examine taxonomic issues and provide a historical perspective on population structure in Piping Plover. We also analyzed variable microsatellite markers to provide a more recent assessment of population structure. Our analyses focused on two levels. At the primary level, we separately quantified patterns within Interior and Atlantic birds (i.e., putative subspecies). This partitioning of individuals was later substantiated by data generated in the present study (see below). Subsequent analyses also were performed at the secondary level, where data were further subdivided to reflect samples from Prairie Canada, U.S. Great Plains, Great Lakes (all Interior birds), Atlantic Canada, and Atlantic United States (all Atlantic birds) (Table 1). Although likely not pertinent from an organismal perspective, the latter set of analyses reflected geographic regions encompassed by separate U.S. and Canadian recovery plans that have been outlined for Piping Plover and, therefore, may provide a basis for informing resource managers regarding species status in their local area of charge. Taken together, these analyses provide a comprehensive assessment of population genetics for the species and serve as a basis for comparison with other measures of population status such as censuses and other demographic parameters.

Methods

Sample collection.—Following protocols outlined by the AOU (Gaunt and Oring 1997), tissue samples were collected from breeding populations throughout the Piping Plover's North American breeding range (Fig. 1). No known close relatives (parents, offspring, siblings, etc.) were included in analyses, and all samples were collected during the breeding season before birds immigrated from other sites. Specimen collection dates are provided in Appendix 1. DNA extractions were performed as described in Haig et al. (2004).

Mitochondrial sequences.—Piping Plover specific primers PPL-26L (CCCCATACTAAATTCTTAGTATGTTTGC) and PPL-657H (CACGGACGAAAATGATGATATATAGC) were designed to generate a bidirectional DNA sequence of ~650 base pairs (bp) in domains I and II of the control region. Polymerase chain reactions (20 μL volume) were used with the following concentrations: 10 mM

TABLE 1. Collection locations and sample sizes for genetic analyses of Piping Plovers.

Locations	Sample size (mitochondrial)	Sample size (microsatellite)
Interior	96	92
Prairie Canada	29	27
Alberta	7	6
Saskatchewan	19	18
Manitoba	3	3
U.S. Great Plains	50	51
Montana	4	5
North Dakota	20	20
South Dakota	19	20
Nebraska	6	5
Minnesota	1	1
Great Lakes	17	14
Wisconsin	1	1
Michigan	16	13
Atlantic	149	137
Atlantic Canada	69	67
Quebec	20	20
Newfoundland	2	1
New Brunswick	6	6
Prince Edward Island	20	20
Nova Scotia	21	20
Atlantic United States	80	70
Maine	6	6
Masachusetts	1	2
Delaware	2	2
Maryland	17	16
Rhode Island	3	2
New York	20	19
New Jersey	22	17
North Carolina	9	6
Total	245	229

Tris-HCl at pH 8.3; 50 mM KCl; 0.001% gelatin; 3.5 mM $MgCl_2$; 100 μM for each of the dNTPs; 0.2 μM of each primer; 100 ng of template; and 1.5 U AmpliTaq Gold Polymerase (Perkin Elmer, Waltham, Massachusetts). The following parameters were used for amplifications: 12 min denaturation at 93°C, followed by 35 cycles of 30 s at 93°C, annealing at 50°C for 30 s, and elongation at 72°C for 1 min. A final 10-min period of elongation at 72°C followed the last cycle. Successful reactions were cleaned by centrifugation dialysis using Microcon 30,000 MW cutoff filters (Amicon Bioseparations, Bedford, Massachusetts). Bidirectional DNA sequence was generated with flanking primers PPL-26L and PPL-657H. Sequencing with internal primers TS-437L (Wenink et al. 1994) and PPL-493H (GGTCTTGAAGCTAGTAACGTAGGA) was also used to facilitate reads through a problematic poly-C region within the control region. Sequences were generated using ABI Prism Big Dye Terminator Cycle Sequencing chemistry on an ABI 3100 capillary DNA automated sequencer (Applied Biosystems, Foster City, California). Ambiguities were resolved by comparing light- and heavy-strand sequences or from overlap of different fragments. The final alignment contained 580 bp of data from 245 individuals sampled in 23 U.S. states and Canadian provinces (Table 1 and Fig. 1).

Microsatellites.—Microsatellite primer sequences were obtained from several sources. Primers CALEX-8, -13, -35, and -37 (developed for Snowy Plover [*C. alexandrinus*]) were described in Küpper et al. (2007). Microsatellite locus C-201 was obtained from ISSR-suppression-PCR clone libraries (Lian et al. 2001, Funk et al. 2007). Microsatellite markers PPL-4 (F-CTGCAGTGACACAATTCCAG; R-CATCAGCTGTGGATTTGGTC), PPL-10 (F-CTGAAGACTCTGTCATCAGC; R-CATCAGTCTGATGCATCCAG), and PPL-11 (F-GACAAGGATCCGCAATATATCA; R-TTACAACTTGCCAGGTCATG) were isolated using a magnetic-bead-capture enrichment protocol (Glenn and Schable 2005).

Sample screening PCR amplifications were performed using a total reaction volume of 10 μL with the following concentrations: 10 mM Tris-HCl at pH 8.3; 50 mM KCl; 0.001% gelatin; 3.5 mM $MgCl_2$; 100 μM for each of the dNTPs; 0.2 μM of each primer (labeled with 6-FAM or HEX); 100 ng of template; and 1.5 U GoTaq DNA polymerase (Promega, Madison, Wisconsin). The following parameters were used for amplifications: 3 min denaturation at 93°C, followed by 35 cycles of 30 s at 93°C, annealing at 52°C (for C-201, PPL-4, PPL-10, and PPL-11) or 62°C (for CALEX-8, Calex-13, Calex-35, Calex-37) for 30 s, and elongation at 72°C for 1 min. A final 10-min period of elongation at 72°C followed the last cycle. Amplification products were analyzed on an ABI 3100 capillary DNA automated sequencer. ABI GENESCAN ANALYSIS software was used to size fragments based on internal lane standard GeneScan 500 ROX and ABI GENOTYPER software was used to score allele sizes (Applied Biosystems). The final microsatellite data set contained genotypes from 229 individuals sampled from 23 states and provinces (Table 1 and Fig. 1).

Genetic diversity patterns.—We used the program ARLEQUIN, version 3.1 (Excoffier et al. 2005), to quantify genetic diversity measures for each geographic region. Gene and nucleotide diversity values were obtained for mitochondrial sequence data, whereas observed and expected heterozygosity values (H_O and H_E, respectively) were calculated for microsatellite data. Because of sample-size variation at the secondary level, we also used the program HP-RARE (Kalinowski 2005) to obtain rarefaction-based estimates of allelic richness that accounted for the small sample size ($n = 14$) within the Great Lakes region. At the secondary level, tests for deviations from Hardy-Weinberg genotypic proportions and for linkage disequilibrium were performed using GDA, version 1.1 (Lewis and Zaykin 2002).

Phylogenetic analyses.—Two approaches were used to characterize phylogenetic relationships among observed mitochondrial haplotypes. Initially, an appropriate model of DNA sequence evolution was identified using the program MODELGENERATOR (Keane et al. 2006) with the AIC2 model-selection measure and four gamma categories. This analysis indicated that the TrN+I+G nucleotide-substitution model was most appropriate. We performed 100 independent runs of a maximum-likelihood (ML) tree search using the program GARLI, version 0.96 (Zwickl 2006), and obtained nodal support from (1) the consensus of the 100 independent runs and (2) an additional run based on 100 bootstrap replicates. Each run was initialized with a random tree and was terminated on the basis of convergence heuristics set by two GARLI configuration parameters ("genthreshfortopoterm" = 40,000; "scorethreshforterm" = 0.05). We used MRBAYES, version 3.1.2 (Ronquist and Huelsenbeck 2003), to perform Bayesian phylogenetic inference. Analyses included 4 separate runs, each containing 1 cold chain and 3 heated chains using a temperature of 0.15. Runs were performed for 8×10^6

generations and sampled every 1,000 generations (8,000 sampled trees total). The first 4,000 trees were discarded as burn-in. Because MRBAYES does not implement the TrN nucleotide-substitution model, the GTR+I+G model (a superset of the TrN+I+G model) was employed. Resulting NEWICK-formatted trees from both analysis approaches were visualized and annotated using MEGA, version 4.0 (Tamura et al. 2007).

Genetic structure analyses.—Genetic structure patterns were analyzed in several different ways. The Bayesian clustering procedure implemented in STRUCTURE, version 2.2.3 (Pritchard et al. 2000), was used to simultaneously infer the number of genetic clusters suggested by the microsatellite data and likewise probabilistically assign each analyzed individual to one of the inferred clusters. STRUCTURE analyses were performed using values of K (the assumed number of clusters) ranging from 1 to 8. Analyses were performed using an initial burn-in of 2×10^5 steps, followed by 1.5×10^6 Markov-chain Monte Carlo analysis sweeps. Default analysis options including assumption of an admixture model and correlated allele frequencies were used, as suggested by Falush et al. (2003). Ten replicates were performed using each value of K. Values of K that produced the highest average likelihood scores over replicates were summarized and visualized using the programs CLUMPP, version 1.1.1 (Jakobsson and Rosenberg 2007), and DISTRUCT, version 1.1 (Rosenberg 2004), respectively. Independent analyses were performed for (1) the full data set and (2) separately for the subsets of Interior and Atlantic individuals to evaluate the plausibility of hierarchical genetic structure.

Data were analyzed using analysis of molecular variance (AMOVA; Excoffier et al. 1992) as implemented in ARLEQUIN. In these analyses, Φ_{ST} and F_{ST} were calculated (for mitochondrial and microsatellite data, respectively) to quantify differentiation between the Interior and Atlantic groups and among the 5 geographic regions. Pairwise values of Φ_{ST} and F_{ST} were also obtained for each combination of the 5 regions examined. P values for all statistics were obtained using a randomization procedure based on 10,000 randomization replicates. P values from pairwise tests were evaluated using sequential Bonferroni corrections. To facilitate interpretation of the pairwise comparisons, matrices of Φ_{ST} and F_{ST} values were further analyzed using MEGA4 (Tamura et al. 2007) to generate neighbor-joining trees illustrating general patterns of dissimilarity among the 5 regions.

Mantel tests (Mantel 1967) implemented in the program ALLELES IN SPACE (Miller 2005) were used to identify correlations between genetic and geographic distances of individuals. Analyses were performed separately for mitochondrial and microsatellite data, and independent analyses were likewise performed for the Interior and Atlantic regions. For mitochondrial sequence data, inter-individual genetic distances were based on the proportion of mismatched nucleotide sites between pairs of haplotypes. Nei et al.'s (1983) genetic distance measure was used for microsatellite data. We used 10,000 randomizations to obtain P values.

Inference of population history and status.—Microsatellite data were used to evaluate population status with respect to past bottleneck events using the program BOTTLENECK (Cornuet and Luikart 1996). Analyses were performed using Interior and Atlantic birds separately, as well as for each of the 5 geographic regions. Given low observed microsatellite allelic richness (see below), analyses were performed separately using a strict stepwise-mutational model (SMM) and with the two-phase model (TPM) based on a TPM variance of 4 (corresponding to an average of an approximately two-step repeat motif change when nonstepwise changes occur; Di Rienzo et al. 1994) and an assumed proportion of 70% fixed SMM events. We used 10,000 simulation replicates in analyses. Excess heterozygosity compared with theoretical expectations, an indication of past bottlenecks, was evaluated using the Wilcoxon signed-rank test. Likewise, we tabulated the occurrence of distorted allele frequency distributions in different hierarchical units, which is also a useful heuristic for identifying past bottleneck events (Luikart et al. 1998).

RESULTS

Genetic diversity patterns.—In our analyses of mitochondrial sequence variation, 70 unique haplotypes were observed among the 245 individuals examined (Appendix 2; GenBank accession nos. FJ850171–FJ850240). We detected 25 different haplotypes among the 96 Interior birds examined, whereas 49 haplotypes were identified among 149 Atlantic individuals. Only 4 haplotypes were shared between Interior and Atlantic groups. Among Interior birds, gene diversity and nucleotide diversity corresponded to 0.813 and 0.0030, respectively. Corresponding values were higher for Atlantic birds (gene diversity: 0.917, nucleotide diversity: 0.0051). Among the 5 geographic regions, genetic diversity was highest within the Atlantic U.S. group and lowest within the Great Lakes region (Table 2).

Microsatellite markers revealed slightly different trends (Table 2). At the highest hierarchical level, H_O and H_E were higher for the

TABLE 2. Genetic diversity measures for Piping Plover mitochondrial and microsatellite data sets.

	Mitochondrial			Microsatellite[a]			
Subregion	Number of haplotypes	Gene diversity	Nucleotide diversity	Average number of alleles per locus	N_a	H_O	H_E
Prairie Canada	11	0.867	0.0039	2.500	2.410	0.406	0.410
U.S. Great Plains	15	0.829	0.0028	2.250	2.200	0.361	0.386
Great Lakes	7	0.596	0.0020	2.125	2.130	0.384	0.388
Atlantic Canada	16	0.765	0.0032	1.875	1.700	0.231	0.221
Atlantic United States	38	0.961	0.0056	2.250	2.070	0.248	0.262

[a] N_a = average number of alleles per locus based on rarefaction, H_O = observed heterozygosity, and H_E = expected heterozygosity.

Interior group (H_O: 0.3930, H_E: 0.3990) than for the Atlantic group (H_O: 0.2461, H_E: 0.2509). Among the 5 geographic regions, diversity was highest for Prairie Canada samples and lowest for the Atlantic Canada group (Table 2). The microsatellite data were generally characterized by low allelic richness (Table 2 and Appendix 3). Among 40 tests for deviation from Hardy-Weinberg genotypic proportions, 4 significant results at the $\alpha = 0.05$ level were observed: 3 from the Prairie Canada group (loci Calex-8, -37, and -35) and 1 from the U.S. Great Plains group (locus Calex-37). With the exception of locus Calex-8, which displayed slight but significant heterozygote excess in the Prairie Canada group, all other significant tests suggested heterozygote deficiencies. Five of the 140 tests for linkage disequilibrium (5 groups × 28 locus pairs per group) were significant at the $\alpha = 0.05$ level, a result that could be explained by chance alone.

Phylogenetic analyses.—Both phylogeny reconstruction procedures indicated differentiation of Interior and Atlantic birds. Aside from 4 shared haplotypes between regions (haplotypes 1, 2, 18, and 25), the remaining phylogenetic diversity was well partitioned into separable groups defined by geography (Appendix 2 and Fig. 2). Out of 100 independent ML searches, the best tree yielded a likelihood

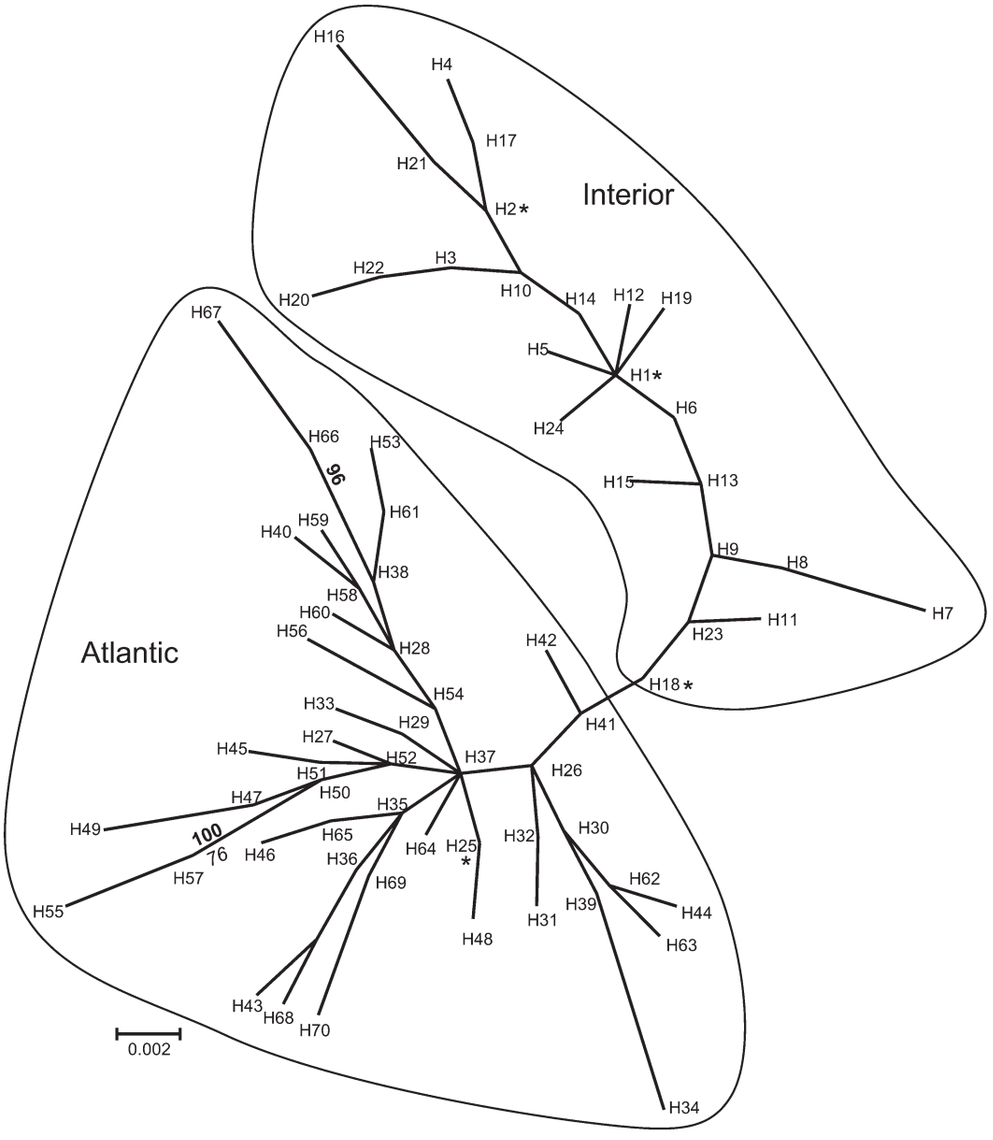


FIG. 2. Unrooted phylogram illustrating the best maximum-likelihood (ML) phylogenetic reconstruction of Piping Plover mitochondrial haplotypes. Locations where haplotypes were observed are provided in Appendix 2. Subtrees containing haplotypes found solely or mostly among Interior rather than Atlantic individuals are highlighted. Asterisks indicate 4 shared haplotypes between Interior and Atlantic birds (haplotypes 1, 2, 18, and 25). Two minor nodes with Bayesian posterior probabilities >95% are indicated in bold, whereas the sole split with bootstrap support >75% (from ML analyses) is indicated in italics.

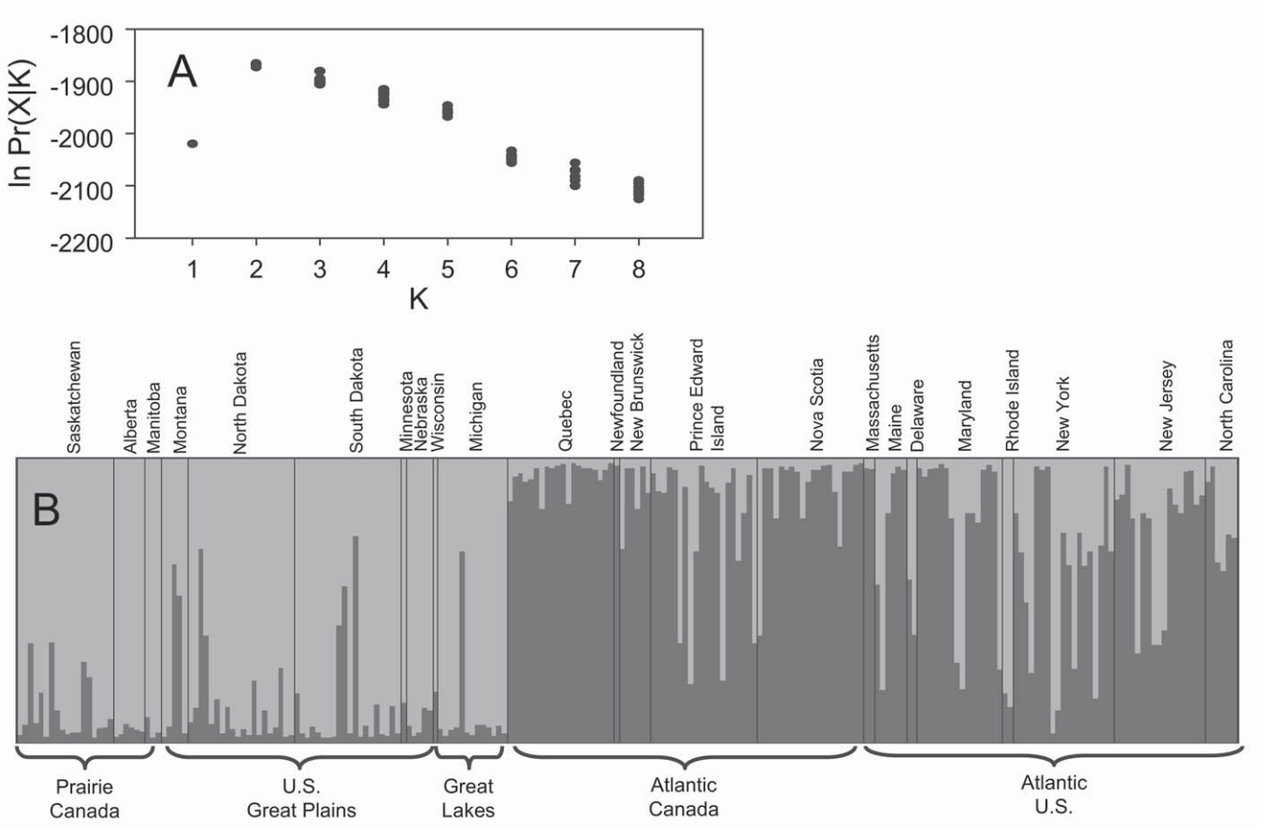


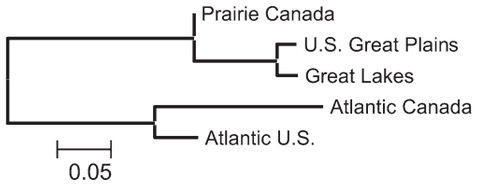
Fig. 3. Analysis in the program STRUCTURE of microsatellite data for Piping Plovers. (A) Evaluation of 10 replicate runs for values of K ranging from 1 to 8 suggested that the $K = 2$ solution (2 genetic clusters) was the most likely solution. (B) Individual cluster-membership coefficients suggested that genetic structure was primarily divided between the Interior and Atlantic groups.

score of –1,293.69. The consensus tree from these runs indicated separation of Interior and Atlantic haplotypes in 100% of searches. The average standard deviation of split frequencies in Bayesian analyses was 0.038, reflecting reasonable convergence of topologies over runs. The posterior probability of the Interior–Atlantic split was 0.78, and only 2 minor nodes had posterior probabilities >0.95. However, ML bootstrap support for the Interior–Atlantic division was <50%, and only 1 split had nodal support >75%. The lack of bootstrap support was likely a function of the low overall divergence between groups. As evidence, haplotypes 18 and 41 (Fig. 2; closely related haplotypes from the Atlantic and Interior groups, respectively) differed by only a single nucleotide. Five of the 6 observed haplotypes observed within Great Lakes birds were allied with other haplotypes found in Interior birds (Appendix 2; haplotypes 1, 13, 15, 23, 24). The sixth Great Lakes haplotype (haplotype 25; found in a single Great Lakes bird) was shared with a few Atlantic individuals and was part of the Atlantic haplotype group (Fig. 2).

Genetic structure analyses.—All analyses suggested strong genetic structure. STRUCTURE indicated that the most likely partitioning of the data exists for the $K = 2$ case (Fig. 3A). When visualized, proportions of individual genomes assigned to each cluster suggested that the two clusters corresponded to separate Interior and Atlantic groups (Fig. 3B). Likewise, and consistent with phylogenetic analyses, individuals from the Great Lakes region (Michigan and Wisconsin) were primarily assigned to the cluster associated with Interior birds. The average cluster-membership probability of individuals was 0.877. Of the 28 misassigned individuals (6 Interior birds and 22 Atlantic birds), none bore a mitochondrial haplotype from the opposite haplotype group. When Atlantic and Interior birds were analyzed separately, runs based on $K = 1$ produced the highest average likelihood scores and suggested no additional structuring.

In comparisons of Atlantic versus Interior birds, Φ_{ST} and F_{ST} (for mitochondrial and microsatellite data, respectively) corresponded to 0.473 and 0.104 ($P < 0.0001$). Likewise, Φ_{ST} and F_{ST} values generated when data were analyzed using 5 regional groups corresponded to 0.426 ($P < 0.0001$) and 0.098 ($P < 0.0001$), respectively. Furthermore, similar patterns were observed between nuclear and mitochondrial marker data sets when comparing all pairwise values of Φ_{ST} and F_{ST} (Fig. 4). In general, pairwise genetic-differentiation values were smaller for contrasts within either the Interior or the Atlantic group than in between-group contrasts. Genetic distances among Interior regions were also smaller than the genetic distance between the Atlantic Canada and Atlantic United States regions. However, within the Interior region, significant F_{ST} values were nonetheless observed for contrasts between

A Mitochondrial	1	2	3	4	5
Prairie Canada (1)	-	0.108	0.074	***<0.0001***	***<0.0001***
U.S. Great Plains (2)	0.018	-	0.057	***<0.0001***	***<0.0001***
Great Lakes (3)	0.040	0.038	-	***<0.0001***	***<0.0001***
Atlantic Canada (4)	0.493	0.534	0.532	-	***<0.0001***
Atlantic U.S. (5)	0.476	0.519	0.480	0.203	-



B Microsatellite	1	2	3	4	5
Prairie Canada (1)	-	***0.009***	0.355	***< 0.0001***	***< 0.0001***
U.S. Great Plains (2)	0.022	-	***0.008***	***< 0.0001***	***< 0.0001***
Great Lakes (3)	0.003	0.038	-	***< 0.0001***	***< 0.0001***
Atlantic Canada (4)	0.151	0.145	0.174	-	***< 0.0001***
Atlantic U.S. (5)	0.121	0.102	0.117	0.062	-

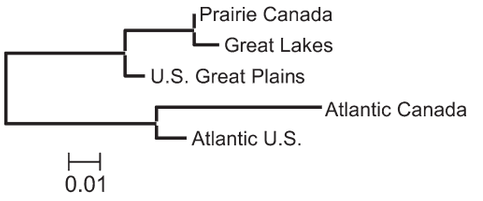


FIG. 4. Pairwise (A) Φ_{ST} and (B) F_{ST} values observed from all pairwise comparisons of the 5 geographic regions examined for Piping Plovers. Actual statistics are contained within the lower off-diagonal elements, whereas congruent *P* values are listed in the upper off-diagonal elements. Significant *P* values after sequential Bonferroni correction are in bold italic type. Neighbor-joining trees to the right of each matrix illustrate relative dissimilarity of the regions based on the pairwise matrices.

Prairie Canada and the U.S. Great Plains and for contrasts between the Great Lakes and U.S. Great Plains (Fig. 4). Consistent with phylogenetic analyses, neighbor-joining trees generated from each matrix indicated that birds from the Great Lakes region were allied with those from the Interior region (Fig. 4).

Mantel tests gave different results for the Atlantic and Interior regions. Among Atlantic birds, significant correlations between genetic and geographic distances were observed for both mitochondrial ($r = 0.237$, $P < 0.001$) and microsatellite ($r = 0.090$, $P < 0.001$) data sets. By contrast, analyses of Interior birds revealed no such patterns (mitochondrial: $r = -0.010$, $P = 0.54$; microsatellite: $r = 0.0663$, $P = 0.059$).

Population history and status.—Tests for the signature of past population bottleneck events provided clear statistical patterns. All analyses performed on Interior birds and the 3 separate partitions of those data detected bottlenecks (Table 3). By contrast, no evidence of bottleneck events was identified among Atlantic individuals.

TABLE 3. Results of analyses designed to infer past bottleneck events in Piping Plovers (microsatellite data). *P* values from tests performed using the stepwise mutational model (SMM) and two-phase model (TPM) are provided, as is an indication of whether or not shifted allele-frequency distributions were observed (consistent with bottlenecks).

Grouping	SMM (*P*)	TPM (*P*)	Shifted allele frequency mode?
All Interior	0.027	0.010	Yes
Prairie Canada	0.027	0.010	Yes
U.S. Great Plains	0.027	0.010	Yes
Great Lakes	0.027	0.027	Yes
All Atlantic	0.594	0.469	No
Atlantic Canada	0.313	0.313	No
Atlantic United States	0.594	0.344	No

DISCUSSION

Subspecies status.—Previous allozyme-based genetic data (Haig and Oring 1988b) were unable to provide support for the presence of separate Interior and Atlantic subspecies that had been proposed on the basis of the geographic distribution of breast-band patterns (Moser 1942, AOU 1945). However, our use of more modern and variable genetic information systems and analyses of a substantially larger data set than Haig and Oring (1988b) revealed differentiation between Interior and Atlantic birds (Figs. 2–4). This pattern is consistent with prior field-based observations that suggested little migration of individuals between regions (Haig and Oring 1988a) and, consequently, provides evidence in support of separate Atlantic and Interior subspecies (*C. m. melodus* and *C. m. circumcinctus*, respectively; AOU 1957). Among 70 unique haplotypes detected (Appendix 2), only 4 (5.7%) were shared between groups. If we assume that "Atlantic" haplotypes are those that were observed solely or mostly among Atlantic individuals (with the complement being true for "Interior haplotypes" and Interior birds), then 93% of Atlantic birds differ from 99% of Interior birds. This pattern exceeds the well-known "75% rule" for defining subspecies (Amadon 1949, Patten and Unitt 2002, Haig et al. 2006). Likewise, given the large Φ_{ST} and F_{ST} values observed between Interior and Atlantic groups (Fig. 4), our data also meet the subspecies definition of Funk et al. (2007), who defined a subspecies as

> a subset of populations with consistent genetic differences from other subsets of populations at multiple independent loci, with genetic differences consisting of significant variation in microsatellite allele and mtDNA haplotype frequencies, the presence of unique alleles or haplotypes, and significant net sequence divergence.

Our data also illustrated that birds from the Great Lakes region are allied with the Interior group and should be taxonomically referred to as *C. m. circumcinctus*. Note that genetic evidence for

two Piping Plover subspecies contrasts with weak genetic differentiation among U.S. Snowy Plovers (Funk et al. 2007). Given the observed differentiation between *C. m. melodus* and *C. m. circumcinctus*, we suggest that future research evaluating adaptive divergence or reproductive isolation of subspecies may be informative.

Regional genetic structure.—Results of genetic structure analyses obtained from STRUCTURE were inconsistent with those produced by conventional analyses based on allele frequencies. The former indicated that $K = 1$ was most likely within each region; however, the latter suggested that different patterns of genetic structure exist within each subspecies. This difference is likely attributable to the presence of weak (but significant) genetic structure or underlying isolation-by-distance patterns, neither of which is easily detected by STRUCTURE (see sections 4.4 and 4.5 of STRUCTURE documentation; Latch et al. 2006, Schwartz and McKelvey 2009).

Within the Interior group, pairwise Φ_{ST} and F_{ST} values were markedly lower than comparable values generated for contrasts between Atlantic Canada and the Atlantic United States (Fig. 4). This pattern may reflect higher individual gene flow (reduced breeding-site fidelity) in Interior birds than in Atlantic birds. However, despite producing lower pairwise values, significant F_{ST} values were nonetheless observed in the Interior region for 2 of the 3 pairwise contrasts based on microsatellite data (Fig. 4). This result suggests that the Interior subspecies does not represent a single panmictic entity. Field observations appear to corroborate this idea, in that >20 years of bird-banding studies have never identified Great Lakes birds breeding west of Lake Superior (or vice versa) (J. Dingledine, USFWS, and F. Cuthbert, University of Minnesota, pers. comm.). Consequently, long-distance gene flow, when it occurs, may be episodic and insufficient to maintain demographic connectivity of regions.

Spatial genetic-structure patterns were also in agreement with pairwise Φ_{ST} and F_{ST} values, in that Atlantic birds showed evidence of isolation-by-distance patterns. In this case, our data suggest that dispersal, when it occurs, is generally associated with movement to relatively proximal breeding territories. By contrast, Interior birds showed no overt spatial genetic-structure signals. This pattern is consistent with the reduced genetic differentiation among Interior subregions (Fig. 4) and may reflect reduced breeding-site or natal-site fidelity when previously occupied sites are unavailable because they are flooded, dry, overgrown with vegetation, or otherwise disturbed by human activities. Overall, differences between Interior and Atlantic birds may reflect regional variation in habitat stability. Atlantic regions may provide more reliable, long-term habitat for Piping Plovers that is less likely to demonstrate extreme spatiotemporal variability. By contrast, the Interior region of North America experiences substantial temporal climatic variation that may cause flooding or complete desiccation of alkali lakes and other wetlands (Espie et al. 1998, Haig et al. 2005). Furthermore, human-controlled variable flooding regimes on the Missouri River may periodically force dispersal of many birds nesting in the Great Plains if habitat becomes unavailable because of inundation, vegetation encroachment, or other habitat disturbances (North 1986; Schwalbach et al. 1993, as cited in Espie et al. 1998).

Genetic diversity, population status, and history.—Piping Plover genetic diversity appeared to be comparable to the range of values observed in two Snowy Plover subspecies from the continental U.S. and Caribbean (Funk et al. 2007). Snowy Plover is a species of conservation concern, although only one Distinct Population Segment is ESA-listed (USFWS 1993). In our analyses, mitochondrial control-region nucleotide diversity (Table 2) ranged from 0.0020 to 0.0056 (mean = 0.0044) compared with a range of 0.0006 to 0.0083 at the same locus in Snowy Plover (mean = 0.0042). Average expected microsatellite heterozygosity within each region (Table 2; range: 0.2211–0.4098, mean = 0.3334) likewise fell within the range of values in Snowy Plover (range: 0.249–0.539, mean = 0.453). The slightly higher average value in Snowy Plover likely reflects differences in allelic richness of the microsatellite loci investigated in the present study and by Funk et al. (2007). Among 8 microsatellite loci examined here, numbers of alleles ranged from 2 to 5 (mean = 2.875) across our full data set. By contrast, an average of 4.8 alleles per locus (range: 2–10) were present among the 10 loci examined by Funk et al. (2007).

Our evaluations of population status and history suggested differences between Interior and Atlantic Piping Plovers (Table 3), which is consistent with our phylogenetic and genetic-structure analyses. The microsatellite data indicated that each of the 3 Interior subregions (and all Interior birds combined) showed evidence of past genetic bottlenecks, whereas no such patterns were observed among Atlantic birds. Note that tests based on the SMM tend to be highly conservative compared with those based on the TPM (Cornuet and Luikart 1996). Consequently, the significance of both SMM- and TPM-based analyses, coupled with the heuristics provided by observed allele-frequency distribution shifts, provide compelling evidence for past bottlenecks within the Interior region.

The timing and intensity of influential historical events can be difficult to infer solely on the basis of genetic data. For example, detection of bottlenecks depends on the interplay of a multivariate combination of statistical and population parameters that primarily include (1) size of the pre-bottleneck population, (2) size of the post-bottleneck population, (3) duration of the bottleneck event, (4) number of sampled individuals for analyses, and (5) number of loci examined (Cornuet and Luikart 1996, Luikart et al. 1998). Historical records and prior census data (Haig and Oring 1985, Haig and Plissner 1993, Plissner and Haig 2000, Haig et al. 2005, Elliott-Smith et al. 2009), however, tend to corroborate the general inferences provided by our analyses. For example, in the mid-1980s, it was estimated that <2,000 Piping Plover breeding pairs existed in North America (Haig and Oring 1985), only ~17 of which inhabited the Great Lakes region (USFWS 1985, Haig and Oring 1988a). These estimates are thought to reflect severe population declines that began in the early 20th century (Haig and Oring 1985, 1988a) and likely resulted in the significant bottleneck events we detected among Interior birds in general. However, available data do not allow us to provide more explicit temporal estimates, nor do they allow us to determine whether the same (or different) bottleneck events influenced all Interior regions. That said, data from 4 international censuses over 15 years suggest that population increases have occurred among Interior birds in recent decades. For example, between 1991 and 2006, the number of birds counted in the Great Lakes increased by 175% (from 40 to 110 birds; Haig and Plissner 1993, Haig and Elliott-Smith et al. 2009). Comparable trends have been observed in Prairie Canada and the U.S. Great Plains, where census data also indicated recent increases in population size (3,467 birds in 1991 vs. 4,662 in 2006; Haig and Plissner

1993, Elliott-Smith et al. 2009). Combined, this information may indicate population increases that followed prior bottleneck events within the Interior region.

In contrast to Interior birds, Atlantic populations exhibited no evidence of genetic bottlenecks. If our hypothesis regarding the relative stability of Atlantic versus Interior habitats is correct, Atlantic birds may not have experienced historical population reductions or bottlenecks of the magnitude experienced by Interior birds. As evidence, census data (Haig and Plissner 1993, Elliott-Smith et al. 2009) indicate that the Atlantic Canada population has changed little from 1991 to 2006 (513 birds in 1991 and 457 in 2006), which suggests relatively stable current population sizes. However, the Atlantic U.S. population appears to have almost doubled in size in recent years (1,462 birds were observed in 1991 and 2,828 in 2006; Haig and Plissner 1993, Elliott-Smith et al. 2009). Absence of a consistent bottleneck signal in this case may indicate a normal population-growth trajectory that was not preceeded by a prior reduction in population size. Alternatively, if a bottleneck actually has occurred within the region, it was apparently not of sufficient magnitude and duration or not in the right time frame to be detected, given the sampling effort (numbers of individuals and loci) encompassed by our study.

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APPENDIX 1. Collection years and U.S. state or Canadian province of each individual used in either mitochondrial or microsatellite analyses (y = yes, n = no). Collection years are unknown for 15 Piping Plover specimens included here.

Genetic sample ID	Mitochondrial data	Microsatellite data	Year collected	State or province
SK1	y	y	1997	Saskatchewan
SK2	y	y	1997	Saskatchewan
SK3	y	y	1997	Saskatchewan
SK4	y	y	1997	Saskatchewan
SK5	y	n	2002	Saskatchewan
SK6	y	y	2002	Saskatchewan
SK7	y	y	2002	Saskatchewan
SK8	y	y	2002	Saskatchewan
SK9	y	y	2002	Saskatchewan
SK10	y	y	2002	Saskatchewan
SK11	y	y	2002	Saskatchewan
SK12	y	y	2002	Saskatchewan
SK13	y	y	2002	Saskatchewan
SK14	y	y	2002	Saskatchewan
SK15	y	y	2002	Saskatchewan
SK16	y	y	2001	Saskatchewan
SK17	y	y	2002	Saskatchewan
SK18	y	y	2002	Saskatchewan
SK19	y	y	2002	Saskatchewan
AB1	y	y	2001	Alberta
AB2	y	y	2001	Alberta
AB3	y	y	2001	Alberta
AB4	y	y	2001	Alberta
AB5	y	n	2001	Alberta
AB6	y	y	2001	Alberta
AB7	y	y	2001	Alberta
MB1	y	y	1999	Manitoba
MB2	y	y	2000	Manitoba
MB3	y	y	2000	Manitoba
MT1	y	y	1995	Montana
MT2	y	y	1995	Montana
MT3	y	y	1995	Montana
MT4	y	y	2008	Montana
MT5	y	y	2008	Montana
NE1	y	y	—	Nebraska
NE2	y	y	—	Nebraska
NE3	y	y	—	Nebraska
NE4	y	y	1996	Nebraska
NE5	y	y	—	Nebraska
NE19	y	n	1996	Nebraska
ND1	y	y	1995	North Dakota
ND2	y	y	1994	North Dakota
ND3	y	y	1992	North Dakota
ND4	y	y	1993	North Dakota
ND5	y	y	1995	North Dakota
ND6	y	y	1995	North Dakota
ND7	y	y	1995	North Dakota
ND8	y	y	1995	North Dakota
ND9	y	y	1995	North Dakota
ND10	y	y	1995	North Dakota
ND11	y	y	1995	North Dakota
ND12	y	y	1995	North Dakota
ND13	y	y	1996	North Dakota
ND14	y	y	1995	North Dakota
ND15	y	y	1995	North Dakota
ND16	y	y	1995	North Dakota
ND17	y	y	1995	North Dakota
ND18	y	y	1995	North Dakota
ND19	y	y	1995	North Dakota
ND20	y	y	1995	North Dakota
SD1	y	n	1993	South Dakota
SD2	y	y	1988	South Dakota
SD3	y	n	1988	South Dakota
SD4	y	y	1988	South Dakota
SD5	y	y	1988	South Dakota
SD6	y	y	1988	South Dakota
SD7	y	y	—	South Dakota
SD8	y	y	1995	South Dakota
SD9	y	y	1995	South Dakota
SD10	y	n	1995	South Dakota
SD11	y	n	1995	South Dakota
SD12	y	y	1995	South Dakota
SD13	y	n	1995	South Dakota
SD14	y	n	1995	South Dakota
SD15	y	n	1995	South Dakota
SD16	y	n	1995	South Dakota
SD17	y	y	1996	South Dakota
SD18	y	y	—	South Dakota
SD19	n	y	—	South Dakota
SD20	y	y	1996	South Dakota
SD21	n	y	1994	South Dakota
SD22	n	y	1994	South Dakota
SD23	n	y	1994	South Dakota
SD24	n	y	1994	South Dakota
SD25	n	y	1994	South Dakota
SD26	n	y	1994	South Dakota
SD27	n	y	1994	South Dakota
SD28	n	y	1994	South Dakota
MN1	y	y	1994	Minnesota
WI1	y	y	2001	Wisconsin
MI1	y	n	1991	Michigan
MI2	y	y	1993	Michigan
MI3	y	y	1993	Michigan
MI4	y	y	1994	Michigan
MI5	y	y	—	Michigan
MI6	y	y	1999	Michigan
MI7	y	y	1999	Michigan
MI8	y	n	1999	Michigan
MI9	y	y	1992	Michigan
MI10	y	y	—	Michigan
MI11	y	y	—	Michigan
MI12	y	y	—	Michigan
MI13	y	y	1994	Michigan
MI14	y	n	1992	Michigan
MI15	y	y	1992	Michigan
MI16	y	y	2001	Michigan
NS1	y	y	2001	Nova Scotia
NS2	y	y	2002	Nova Scotia
NS3	y	y	2002	Nova Scotia
NS4	y	n	2003	Nova Scotia
NS5	y	y	2003	Nova Scotia
NS6	y	y	2000	Nova Scotia
NS7	y	n	2001	Nova Scotia
NS8	y	n	2004	Nova Scotia

(*continued*)

APPENDIX 1. Continued.

Genetic sample ID	Mitochondrial data	Microsatellite data	Year collected	State or province
NS9	y	y	2000	Nova Scotia
NS10	y	y	2002	Nova Scotia
NS11	y	y	2002	Nova Scotia
NS12	y	y	2002	Nova Scotia
NS13	y	n	2003	Nova Scotia
NS14	y	y	2000	Nova Scotia
NS15	y	y	2003	Nova Scotia
NS16	y	y	2003	Nova Scotia
NS17	y	y	2004	Nova Scotia
NS18	y	y	2004	Nova Scotia
NS19	y	y	2003	Nova Scotia
NS20	y	y	2003	Nova Scotia
NS21	y	y	2004	Nova Scotia
NS22	n	y	2004	Nova Scotia
NS23	n	y	2004	Nova Scotia
NS26	n	y	2004	Nova Scotia
NF1	y	n	2004	Newfoundland
NF2	y	y	2000	Newfoundland
NB1	y	y	2001	New Brunswick
NB2	y	y	2003	New Brunswick
NB3	y	y	2004	New Brunswick
NB4	y	y	2004	New Brunswick
NB5	y	y	2004	New Brunswick
NB6	y	y	2004	New Brunswick
PEI1	y	y	1994	Prince Edward Island
PEI2	y	y	1994	Prince Edward Island
PEI3	y	y	1994	Prince Edward Island
PEI4	y	y	1994	Prince Edward Island
PEI5	y	y	1994	Prince Edward Island
PEI6	y	y	1995	Prince Edward Island
PEI7	y	y	1994	Prince Edward Island
PEI8	y	y	1994	Prince Edward Island
PEI9	y	y	1994	Prince Edward Island
PEI10	y	y	1995	Prince Edward Island
PEI11	y	y	1994	Prince Edward Island
PEI12	y	y	1995	Prince Edward Island
PEI13	y	y	1994	Prince Edward Island
PEI14	y	y	1995	Prince Edward Island
PEI15	y	y	1994	Prince Edward Island
PEI16	y	y	1995	Prince Edward Island
PEI17	y	y	1994	Prince Edward Island
PEI18	y	y	1994	Prince Edward Island
PEI19	y	y	1994	Prince Edward Island
PEI20	y	y	2005	Prince Edward Island
QB1	y	y	1995	Quebec
QB2	y	y	1995	Quebec
QB3	y	y	1995	Quebec
QB4	y	y	1995	Quebec
QB5	y	y	1995	Quebec
QB6	y	y	1995	Quebec
QB7	y	y	1995	Quebec
QB8	y	y	1995	Quebec
QB9	y	y	1995	Quebec
QB10	y	y	2006	Quebec
QB11	y	y	2005	Quebec
QB12	y	y	2005	Quebec
QB13	y	n	2004	Quebec
QB14	y	y	2004	Quebec
QB15	y	y	2004	Quebec
QB16	y	y	2003	Quebec
QB17	y	y	2001	Quebec
QB18	y	y	2001	Quebec
QB19	y	y	2000	Quebec
QB20	y	y	1999	Quebec
QC21	n	y	2001	Quebec
ME1	y	n	1994	Maine
ME2	y	y	1999	Maine
ME3	y	y	1999	Maine
ME4	y	y	1996	Maine
ME5	y	y	—	Maine
ME6	y	y	—	Maine
ME7	n	y	—	Maine
MA1	y	y	1999	Massachusetts
MA2	n	y	—	Massachusetts
DE1	y	y	1996	Delaware
DE2	y	y	1996	Delaware
MD1	y	y	1995	Maryland
MD2	y	y	1994	Maryland
MD3	y	y	1994	Maryland
MD4	y	y	1994	Maryland
MD5	y	y	1994	Maryland
MD6	y	y	1994	Maryland
MD7	y	y	1997	Maryland
MD8	y	y	1997	Maryland
MD9	y	y	1996	Maryland
MD10	y	y	1998	Maryland
MD11	y	y	1998	Maryland
MD12	y	y	1998	Maryland
MD13	y	y	1998	Maryland
MD14	y	y	1996	Maryland
MD15	y	y	1996	Maryland
MD16	y	y	1996	Maryland
MD17	y	n	1996	Maryland
RI1	y	y	1995	Rhode Island
RI2	y	y	1995	Rhode Island
RI3	y	n	1995	Rhode Island

(*continued*)

APPENDIX 1. Continued.

Genetic sample ID	Mitochondrial data	Microsatellite data	Year collected	State or province
NY1	y	y	1996	New York
NY2	y	y	1996	New York
NY3	y	y	1996	New York
NY4	y	y	1996	New York
NY5	y	y	1996	New York
NY6	y	y	1996	New York
NY7	y	y	1996	New York
NY8	y	y	1997	New York
NY9	y	y	1997	New York
NY10	y	y	1997	New York
NY11	y	y	1997	New York
NY12	y	y	1997	New York
NY13	y	y	1997	New York
NY14	y	n	1997	New York
NY15	y	y	1997	New York
NY16	y	y	1997	New York
NY17	y	y	1997	New York
NY18	y	y	1997	New York
NY19	y	n	1997	New York
NY20	y	y	1997	New York
NY21	n	y	1997	New York
NJ1	y	y	1995	New Jersey
NJ2	y	y	1995	New Jersey
NJ3	y	y	1995	New Jersey
NJ4	y	y	1995	New Jersey
NJ5	y	y	1995	New Jersey
NJ6	y	y	1995	New Jersey
NJ7	y	y	1995	New Jersey
NJ8	y	y	1996	New Jersey
NJ9	y	y	1992	New Jersey
NJ10	y	y	1992	New Jersey
NJ11	y	y	1992	New Jersey
NJ12	y	y	1992	New Jersey
NJ13	y	n	1994	New Jersey
NJ14	y	y	1995	New Jersey
NJ15	y	y	1995	New Jersey
NJ16	y	y	1995	New Jersey
NJ17	y	y	1991	New Jersey
NJ18	y	y	1992	New Jersey
NJ19	y	n	1995	New Jersey
NJ20	y	n	1996	New Jersey
NJ21	y	n	1996	New Jersey
NJ22	y	n	1997	New Jersey
NC1	y	y	1995	North Carolina
NC2	y	y	1995	North Carolina
NC3	y	n	1994	North Carolina
NC4	y	y	1995	North Carolina
NC5	y	y	1995	North Carolina
NC6	y	y	1995	North Carolina
NC7	y	n	1995	North Carolina
NC8	y	n	1995	North Carolina
NC9	y	y	1996	North Carolina

APPENDIX 2. Geographic locations where 70 unique Piping Plover haplotypes were detected. Only haplotypes 1, 2, 18, and 25 were shared between Interior and Atlantic birds. Abbreviations: AB = Alberta, SK = Saskatchewan, MB = Manitoba, MT = Montana, ND = North Dakota, SD = South Dakota, NE = Nebraska, MN = Minnesota, WI = Wisconsin, MI = Michigan, QB = Quebec, NF = Newfoundland, NB = New Brunswick, PI = Prince Edward Island, NS = Nova Scotia, ME = Maine, MA = Massachusetts, DE = Delaware, MD = Maryland, RI = Rhode Island, NY = New York, NJ = New Jersey, and NC = North Carolina.

	Prairie Canada			Northern Great Plains					Great Lakes		Atlantic Canada					Atlantic United States								
Haplotype	AB	SK	MB	MT	ND	SD	NE	MN	WI	MI	QB	NF	NB	PI	NS	ME	MA	DE	MD	RI	NY	NJ	NC	Total
1	1	7	1	2	6	9		1	1	10				2										40
2	2	2	1		6	4				1		1	2		4									23
3	2																							2
4	1		1																					2
5	1																							1
6		3			2																			5
7		1																						1
8		1																						1
9		3																						3
10		1																						1
11		1																						1
12				1			1																	2
13				1						1														2
14					3																			3
15					2	1				1														4
16					1																			1
17						2																		2
18						1	1																1	3
19						1																		1
20						1																		1

(*continued*)

Appendix 2. Continued.

	Prairie Canada			Northern Great Plains					Great Lakes		Atlantic Canada					Atlantic United States								
Haplotype	AB	SK	MB	MT	ND	SD	NE	MN	WI	MI	QB	NF	NB	PI	NS	ME	MA	DE	MD	RI	NY	NJ	NC	Total
21							1																	1
22							2																	2
23							1			1														2
24										1														1
25										1							1		3		1			6
26											11	1	3	7	9	2					2	2	1	38
27											7			4										11
28											2													2
29													1											1
30														2										2
31														1										1
32														2										2
33														1										1
34														1										1
35															1						1	4		6
36															1					1	2	3		7
37															4						1	2		7
38															1						2	1	2	6
39															1									1
40																1								1
41																1								1
42																1								1
43																1								1
44																		1						1
45																		1	1					2
46																			5			5		10
47																			1					1
48																			1					1
49																			1					1
50																			2					2
51																			1					1
52																			2					2
53																				2	1			3
54																					3			3
55																					1			1
56																					1			1
57																					1			1
58																					1			1
59																					1			1
60																					1			1
61																					1			1
62																						1		1
63																						1		1
64																						1		1
65																						2		2
66																							1	1
67																							1	1
68																							1	1
69																							1	1
70																							1	1
Total	7	19	3	4	20	19	6	1	1	16	20	2	6	20	21	6	1	2	17	3	20	22	9	245

APPENDIX 3. Observed allele frequencies at 8 microsatellite loci in each of 5 geographic regions described in the text.

		Geographic region					
	Allele size	Prairie Canada	U.S. Great Plains	Great Lakes	Atlantic Canada	Atlantic United States	Total
Locus C-201	168	0.222	0.353	0.250	0.136	0.371	0.274
	170	0.667	0.588	0.500	0.864	0.579	0.669
	172	0.111	0.059	0.250	0.000	0.050	0.057
n		27	51	14	66	70	228
Locus PLL-11	223	0.370	0.451	0.357	0.597	0.739	0.566
	227	0.630	0.549	0.643	0.403	0.261	0.434
n		27	51	14	67	69	228
Locus PLL-4	180	0.327	0.370	0.143	0.067	0.044	0.162
	186	0.673	0.630	0.857	0.530	0.732	0.650
	188	0.000	0.000	0.000	0.403	0.225	0.188
n		26	50	14	67	69	226
Locus PLL-10	216	0.212	0.128	0.286	0.000	0.000	0.070
	218	0.789	0.873	0.714	1.000	1.000	0.930
n		26	51	14	67	70	228
Locus Calex-13	148	0.259	0.190	0.321	0.311	0.228	0.253
	149	0.741	0.810	0.679	0.689	0.772	0.747
n		27	50	14	66	68	225
Locus Calex-8	210	0.037	0.000	0.000	0.000	0.000	0.004
	212	0.630	0.847	0.786	1.000	0.900	0.879
	214	0.019	0.000	0.000	0.000	0.000	0.002
	216	0.315	0.153	0.214	0.000	0.100	0.115
n		27	49	14	67	70	227
Locus Calex-37	162	0.000	0.000	0.000	0.015	0.064	0.024
	163	0.111	0.157	0.179	0.000	0.000	0.059
	164	0.778	0.804	0.821	0.970	0.914	0.884
	165	0.111	0.039	0.000	0.015	0.000	0.026
	166	0.000	0.000	0.000	0.000	0.021	0.007
n		27	51	14	67	70	229
Locus Calex-35	130	0.130	0.343	0.107	0.000	0.036	0.109
	132	0.870	0.657	0.893	1.000	0.964	0.891
n		27	51	14	67	70	229