

A rangewide population genetic study of trumpeter swans

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Abstract For management purposes, the range of naturally occurring trumpeter swans (*Cygnus buccinator*) has been divided into two populations, the Pacific Coast Population (PP) and the Rocky Mountain Population (RMP). Little is known about the distribution of genetic variation across the species' range despite increasing pressure to make difficult management decisions regarding the two populations and stocks within them. To address this issue, we used rapidly evolving genetic markers (mitochondrial DNA sequence and 17 nuclear microsatellite loci) to elucidate the underlying genetic structure of the species. Data from both markers revealed a significant difference between the PP and RMP with the Yukon Territory as a likely area of overlap. Additionally, we found that the two populations have somewhat similar levels of genetic diversity (PP is slightly higher) suggesting that the PP underwent a population bottleneck similar to a well-documented one in the RMP. Both genetic structure and diversity results reveal that the Tri-State stock, a suspected unique, non-migratory stock, is not genetically different from the Canadian stock of the RMP and need not be treated as a unique population from a genetic standpoint. Finally, trumpeter swans

appear to have much lower mitochondrial DNA variability than other waterfowl studied thus far which may suggest a previous, species-wide bottleneck.

Keywords Trumpeter swan · *Cygnus buccinator* · Microsatellites · mtDNA · Gene flow · Genetic diversity

Introduction

Prior to the 20th Century, trumpeter swans (*Cygnus buccinator*) were abundant throughout most of North America, ranging from Alaska to the Gulf of Mexico and east to Hudson's Bay (Alison 1975). Over-harvest and other factors in the late 1800s and early 1900s led to the significant reduction of the species (Banko 1960). By 1932, the largest known collection of adult trumpeter swans consisted of 57 individuals on a chain of thermal lakes in the vicinity of Yellowstone National Park (Bellrose 1976; Banko 1960). The U. S. government took action to preserve this stock, and in 1935 designated their breeding grounds, Red Rock Lakes, a Migratory Waterfowl Refuge (later, a National Wildlife Refuge). Due to this habitat protection and a moratorium on hunting these birds, trumpeter swan numbers in the Red Rock Lakes Refuge and in the Yellowstone region (now collectively called the "Tri-State" stock) began to rebound. The Tri-State group along with a small stock of trumpeter swans on the Peace River in Alberta, Canada (James 2000, citing MacKay 1981) were thought to be the only surviving birds until 1954 when a group of nesting trumpeter swans was discovered on the Copper River in Alaska (Banko 1960). This Alaskan stock, along with other

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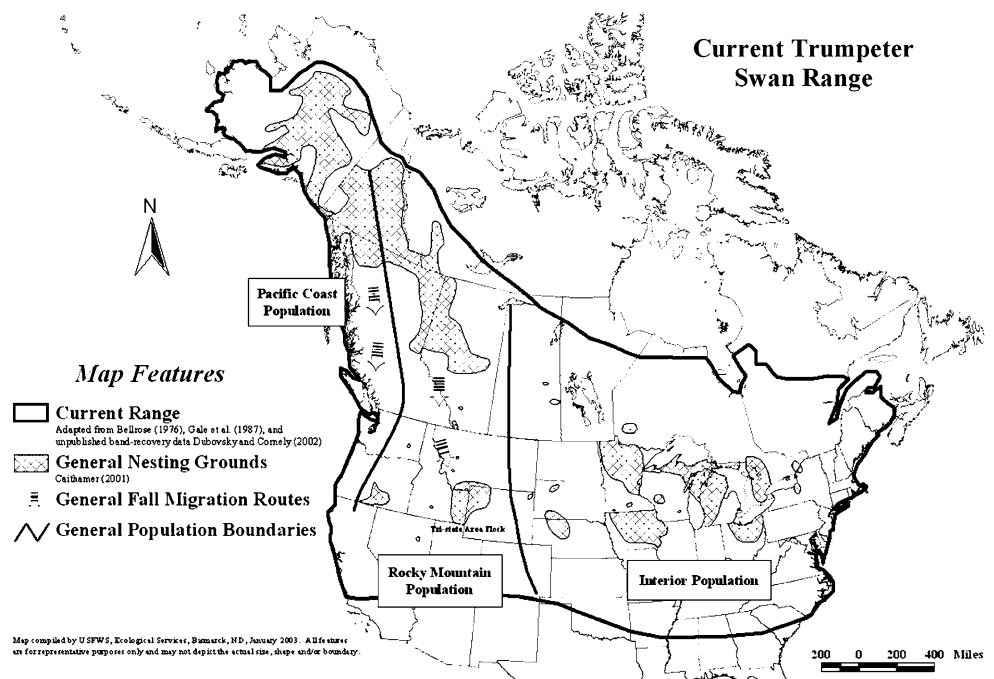
dispersed birds in Alaska and western Canada, had remained undiscovered during the species' decline (Matteson et al. 1995). From these three groups (the Alaskan/Yukon Territory/British Columbian birds, the Alberta birds, and the Tri-State birds) the trumpeter swan expanded its breeding range both by natural dispersal and with the aid of restorative translocation efforts.

Following the discovery of the additional birds and assessment of band-recovery information, trumpeter swans were delineated into three populations for management purposes, largely based on known geographic distributions of swans from various nesting areas and flyway information. These designations may reflect primary migration corridors of the trumpeter swan but do not necessarily define barriers to gene flow. The Pacific Coast Population (PP) is comprised of birds nesting primarily in Alaska and western portions of the Yukon Territory and British Columbia (Fig. 1). These birds are thought to winter on the Pacific coasts of British Columbia and Washington. The Rocky Mountain Population (RMP) is made up of birds from the Tri-State area (i.e., the area near the shared boundaries of Idaho, Montana, and Wyoming), the Grande Prairie–Peace River region of Alberta, and the eastern portions of British Columbia and the Yukon Territory (Fig. 1). Most of these birds winter in the Tri-State region (Shea et al. 2002); the Tri-State birds are therefore considered to be largely non-migratory. A third population, the Interior Population (IP), is

found east of the Rocky Mountains in the central and eastern parts of North America. This population consists of several discrete flocks of swans that are the result of physical translocations of swans into suitable habitat within their historic range (Hansen 1973). Because the IP consists largely of swans derived from translocations, they will not be considered further here.

Although the two flocks comprising the majority of the RMP are spatially disjunct during the nesting season, they are sympatric during winter in the Tri-State region. While the Tri-State flock has a fluctuating but relatively small number of birds (400–500), the Canadian flocks have increased from about 150 birds in the early 1970s to almost 5,000 birds in 2006 (U.S. Fish and Wildlife Service 2006). The RMP as a whole has been increasing in number at an average rate of about 5.4% per year since 1968 (Moser 2006). This increased pressure on the habitat in this region has created a need to alleviate overcrowding of swan habitat and encourage migration via translocations (Hansen 1973; Engelhardt et al. 2000; Shea et al. 2002). Possible explanations for the Tri-State flock's lack of growth include inbreeding depression in the Tri-State flock and environmental factors associated with a compromised habitat due to overuse by wintering swans. The existence or extent of genetic interchange between these two groups remains unknown, and controversy surrounds whether the flocks should be managed in the aggregate or as two distinct entities. Further, the degree of genetic distinctness among trumpeter swans

Fig. 1 Current range, nesting grounds, and migration routes of the trumpeter swan. All birds belong to the Pacific, the Rocky Mountain, or the Interior Populations. The Pacific Population winters on the Pacific Coast in British Columbia, Oregon and Washington. The majority of the Rocky Mountain Population winters in the vicinity of Yellowstone National Park



range-wide is an important parameter to understand given the increasingly complex level and patterns of restoration efforts that often involve translocations of swans and of swan eggs.

Until the latter part of the 20th Century, trumpeter swan management and restoration efforts relied solely on demographic information for guidance. Several studies attempted to determine whether genetic differences exist among trumpeter swan stocks and to compare their genetic variability (Barrett and Vyse 1982, Marsolais and White 1997, Pelizza and Britten 2002). Barrett and Vyse (1982) conducted protein electrophoresis using 19 loci to compare trumpeter swans from the PP, the Tri-State stock of the RMP, and a Canadian stock of the RMP. Of the 19 loci examined, 5 were polymorphic. They found no significant differences in heterozygosity and genetic distance among the three groups sampled. They suggested, however, that the presence of private alleles in low frequency in the PP may indicate unique genetic variability in that group. Pelizza and Britten (2002) used isozyme analysis to compare the PP to the Tri-State stock and to the High Plains stock of the IP in South Dakota (descendants of birds transplanted from the Tri-State stock). They did find a genetic difference between the PP and the other two stocks, yet their sample size was small and only one locus was polymorphic. Marsolais and White (1997) used minisatellites to estimate band-sharing coefficients among four groups (PP, Tri-State stock of RMP, Canadian stock of RMP, and Ontario stock of IP) and determined that the Ontario stock had the least amount of genetic diversity and would benefit from translocations from other groups.

While these studies all provide valuable information, the pattern of genetic variation among populations across the range remains unclear. With increasing pressure on managers to make difficult decisions about whether spatially disjunct groups of nesting birds are genetically unique and which areas to use as sources for translocation and restoration attempts (Engelhardt et al. 2000), resolution of this issue has become vital. To address this issue, we documented genetic variation using rapidly evolving genetic markers (mitochondrial control region DNA sequence and nuclear microsatellites) to screen a number of samples from the most relevant stocks. These markers evolve at rates that are appropriate for resolving structure at the population level (Haig 1998). The objective of our study was to examine genetic variation between the two wild populations (PP and RMP) and also within each population at various sampling locales across the range of the trumpeter swan. Further, we were interested in comparing levels of genetic diversity among sampling

locales. This is important because low levels of genetic variation have been linked to reduced fitness in some species (O'Brien and Evermann 1988, Quattro and Vrijenhoek 1989, Wayne et al. 1991) and may have a negative effect on swans now or in the future.

Materials and methods

Tissue collection and DNA extraction

Trumpeter swan samples were collected on their breeding grounds. Care was taken to avoid sampling individuals that were known relatives (i.e., family groups). In most cases, live birds were caught and sampled (by plucking feathers or drawing blood). In a few cases, tissue was taken from birds found dead in a breeding area. In a few instances where disturbing swans was an issue, a single feather from each nest was taken following the breeding season. Blood quills, feathers, embryos, toe clippings, eggs, and breast muscle were frozen after collection. Blood samples were collected from a brachial vein, placed in EDTA-coated Eppendorf tubes (Brinkmann), and stored at -20°C . Samples from 157 individuals were obtained from several collection sites within the Pacific and Rocky Mountain Populations' primary natural ranges and grouped into seven sampling locales for statistical analyses based on geographic proximity (Table 1, Fig. 2).

DNA was extracted from blood samples with the GenomicPrep Blood DNA Isolation Kit (Amersham Biosciences Corp) using the manufacturer's instructions with modifications following Oyeler-McCance et al. (2005). All other samples (feathers, blood quills, and other tissue) were extracted using the Promega Wizard DNA Purification Kit following the manufacturer's instructions (Promega Corporation).

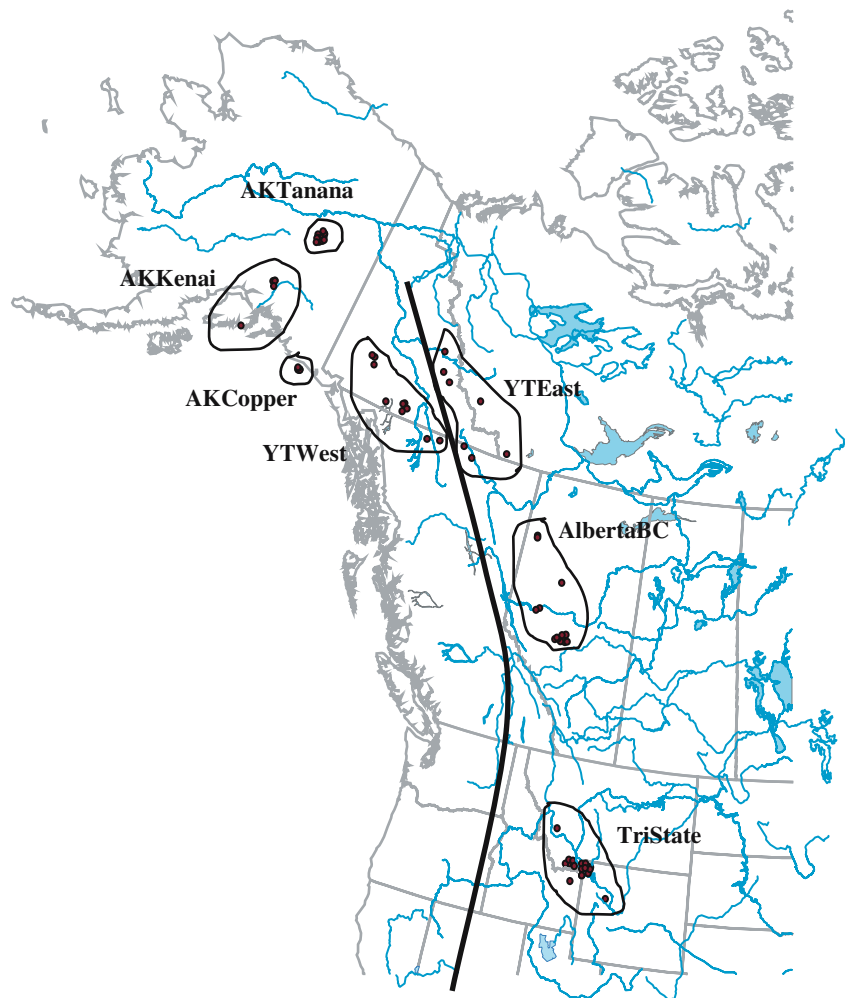
Mitochondrial DNA sequencing

We amplified the entire control region using the primers L16641 (Quinn and Wilson 1993) and H595 (5'-CACTTCAGTGCCATGCTT-3'). In a few cases where the DNA template was degraded, overlapping segments of the control region were amplified separately using swanF1 (5'-GCAGGGGGTATTTGGCTATG-3') with H665 (Quinn and Wilson 1993) and 16522 (5'-GCAAGCTTGGTTCCTCGGTCAGGGCCA-3') with H595. In addition, we amplified a portion of the NADH Dehydrogenase 6 (ND6) gene using primers ThrL (5'-CTCACCCTTCTTAGAGTACCC-3') and H16756 (Quinn and Wilson 1993). PCR components contained

Table 1 Sampling locales, sample sizes, unique locations, and population designations for the β ocks used in the study

State/Province	Unique Location	N	Sample Locale	Population
Alaska	Tanana Valley	20	AKTanana	PaciPc
Alaska	Tokositna Valley	11	AKKenai	PaciPc
Alaska	Kenai National Wildlife Refuge	5	AKKenai	PaciPc
Alaska	Copper River Delta	10	AKCopper	PaciPc
Yukon Territory	Various YT Western Sites	17	YTWest	PaciPc
Yukon Territory	Various YT Eastern Sites	15	YTEast	Rocky Mountain
British Columbia	Fort Nelson/Fort St. John	9	AlbertaBC	Rocky Mountain
Alberta	Various Alberta Sites	26	AlbertaBC	Rocky Mountain
Montana	Red Rock Lakes Wildlife Refuge	21	TriState	Rocky Mountain
Montana	Yellowstone National Park	10	TriState	Rocky Mountain
Montana	Lincoln, Montana	1	TriState	Rocky Mountain
Wyoming	National Elk Refuge	5	TriState	Rocky Mountain
Wyoming	Seedskaelee National Wildlife Refuge	3	TriState	Rocky Mountain
Wyoming	Cora, Wyoming	1	TriState	Rocky Mountain
Idaho	Targhee National Forest	3	TriState	Rocky Mountain

Fig. 2 Trumpeter swan sampling locales. Dots represent discrete wild trumpeter swan β ocks and polygons circumscribe sampling locales. The thick black line represents the administrative boundary between the Rocky Mountain and PaciPc Populations, which was based on migration data and field observations. Major waterways are also shown



0.63 U Promega *Taq* polymerase, 25 μ M of each dNTP and 0.5 μ M of each primer for a total volume of 25 μ l with buffer types varying (Table 2). All PCR thermal profiles began with a preheat step at 94C for 2 min followed by 30

cycles of denaturing at 94C for 40 s, annealing for 1 min 30 s (temperatures varied, Table 2) and extending at 72 C for varying times (Table 2). The final extension step lasted 10 min.

Table 2 PCR conditions and thermoprofiles used for the amplification of parts of the mitochondrial genome. 1 Taq buffer as described in Kahn et al. 1998 (67 mM Tris-HCl pH 8.0, 6.7 mM MgSO₄, 16.6 mM (NH₄)₂SO₄, 10 mM β-mercaptoethanol)

	Entire control region	Control region (Domain I)	Control region (Domain III)	ND6
Primer 1	L16641	swanF1	L16522	ThrL
Primer 2	H595	H665	H595	H16756
Annealing	52 C	60 C	60 C	52 C
Extension Time	2 min 30 s	1 min 20 s	1 min 20 s	2 min
Buffer	1· Taq	Promega	Promega	1 Taq
Size (base pairs)	950	(1.5 mM MgCl ₂)	(1.5 mM MgCl ₂)	Buffer
		534	538	530

PCR products were cleaned with shrimp alkaline phosphatase and exonuclease 1 (USB). Dye terminator cycle sequencing reactions were performed with the Beckman-Coulter Quick Start Sequencing Kit according to the manufacturer's protocol, but halved for a reaction volume of 10 μl. These were precipitated according to the manufacturer's specifications, resuspended in 30 μl of formamide and run on a CEQ 8000 XL Data Analysis System using method LFR-b (Beckman-Coulter). Sequences were aligned with Sequencher 4.2 (GeneCodes).

Because nuclear homologs of mitochondrial sequences (ÖnumtsÖ; Lopez 1994) have been found in other avian species (Sorenson and Quinn 1998) we verified that our sequences were of mitochondrial origin using a long-range PCR similar to the procedure described in Thalmann et al. (2004) on blood samples from two individuals. One segment of the long-range PCR was amplified using primers L16641 (Quinn and Wilson 1993) and H9865 (5'-CGAAGACGTAGGCT TGGATTA-3') and the overlapping second segment was amplified using L9055 (5-CAGTAGCCTTTTAA GCTAG-3') and H595. Each segment was designed to amplify over half of the avian mitochondrial genome, from the ATPase 6/8 region to the control region (Desjardins and Morais 1990). The long-range PCRs utilized LA Taq (Takara Bio Inc., Japan) and included 1.25 U of LA Taq and 0.4 mM of each dNTP in a 25 μl reaction. The thermal profile included a 1 min preheat at 94 C followed by 30 cycles of denaturing at 94 C for 30 s, annealing (59 C for primers L16641 and H9865, 52 C for primers L9055 and H595) for 30 s, and extension at 72 C for 15 min. The long-range PCR products were estimated to be 11 Kb using electrophoresis. PCRs with all primer pairs (see above and Table 2) were performed with both long PCR templates and genomic DNA followed by sequencing. Consistency among these sequences within a single targeted template was viewed as evidence that all primer pairs were indeed amplifying mitochondrial DNA.

Microsatellite analysis

All 157 trumpeter swan samples were screened using 19 nuclear microsatellite loci. Microsatellites were isolated from trumpeter swan samples at the Rocky Mountain Center for Conservation Genetics and Systematics and primer pairs for 16 loci (TSP.1.20.2B, TSP.1.20.5, TSP.1.20.9, TSP.1.20.16, TSP.1.20.43, TSP.1.20.46, TS.1.29.25, TS.1.29.25, TS.1.29.30, TS.1.29.32, TS.1.29.52, TS.1.29.54, TS.1.29.57, TS.4.22.18, TS.8.20.2A, and TS.8.20.13) were designed (St. John et al. 2006). Microsatellite isolation, reaction parameters, primer sequences, thermal profiles, and fragment analysis methods for these are described in St. John et al. (2006). Primers for the remaining three microsatellite loci (CAM3, CAM5, CAM6) were originally designed for the black swan, *C. atratus* (Carew et al. 2003). Screening methods are outlined in St. John et al. (2006). The annealing temperatures of these varied by locus: CAM3 (54 C), CAM5 (56 C), CAM6 (56 C). PCR products were diluted and run on the CEQ8000 XL DNA Analysis System following the manufacturer's protocol (Beckman-Coulter). All loci were run with size standard S400 (Beckman-Coulter) and analyzed using the default Frag 3 method of the CEQ Genetic Analysis Software Package (Version 6.0).

Data analysis

Mitochondrial sequence analysis

An estimate of mitochondrial DNA haplotype diversity (\hat{h}) was calculated following Nei (1987) for each sampling locale using the program ARLEQUIN ver. 2.000 (Schneider et al. 2000). In addition, we used FSTAT 2.9.3.2 (Goudet 1995) to estimate allelic richness, a measure of the number of haplotypes in each sampling locale corrected for unequal sample sizes using a rarefaction method. We examined population structure using an analysis of molecular variance

(AMOVA, Excoffier et al. 1992) in ARLEQUIN with three different groupings. First, each sampling locale was designated as a separate group and analyzed. A second analysis tested how much variation was explained by the separation of TriState birds from all other sampling locales. A third analysis treated each population as a group and tested the PP versus the RMP. We used a Kimura 2-parameter nucleotide substitution model (Kimura 1980) for all calculations. Pairwise F_{ST} values, measures of genetic differentiation between each group, were calculated and their significance was determined by random permutations. To minimize the possibility of committing type I errors due to multiple comparisons, pairwise F_{ST} values were considered to be significant at $P < 0.01$. We used pairwise F_{ST} values to generate an unrooted neighbor-joining network using PHYLIP 3.57 (Felsenstein 1989), which was visualized in TREEVIEW (Page 1996). We examined the relationship between geographic and genetic distance using a Mantel test (Mantel 1967) in ARLEQUIN. Geographic distances were estimated based on linear distances from a North American-centered conical projection.

We conducted phylogenetic analyses in PAUP * version 4.0610 (Swofford 2003) using distance, parsimony, and maximum-likelihood methods using two tundra swan (*C. columbianus*) sequences as outgroups. We used Modeltest 3.7 (Posada and Crandall 1998) to choose the most likely model of evolution given our data for the distance and maximum-likelihood methods using AIC as a criterion for model selection. For the distance analysis, the most appropriate distance model was used to construct a neighbor-joining tree. Support for each node was evaluated using bootstrap analysis with 10,000 bootstrap replicates. A maximum parsimony analysis was conducted using the heuristic search option and 100 replicates of random stepwise additions. One thousand bootstrap replicates were generated to assess support for each node. Finally, we conducted a maximum-likelihood analysis using the best model of evolution chosen by Modeltest. Node support was evaluated using bootstrap analysis with 1,000 replicates.

We also investigated the relationships among haplotypes by generating an unrooted haplotype network using the statistical parsimony software TCS version 1.13 (Clement et al. 2000). The network was constructed following the algorithm of Templeton et al. (1992).

Microsatellite analysis

Microsatellite genotypes were tested for departures from Hardy-Weinberg equilibrium within each popu-

lation using the computer program ARLEQUIN 2.00 (Schneider et al. 2000). ARLEQUIN employs a Markov-chain random walk algorithm (Guo and Thompson 1992) that is analogous to Fisher's exact test but extends it to an arbitrarily sized contingency table. We used 300,000 as the forecasted chain length and 5000 dememorization steps for this analysis. Linkage disequilibrium for each pair of loci was evaluated in each sampling locale across the trumpeter swan's range in GENEPOP (Raymond and Rousset, 1995 Markov chain parameters: 5000 dememorization steps, 500 batches, 5000 iterations per batch).

The amount of genetic diversity per sampling locale was documented several different ways. We calculated mean observed and expected heterozygosity levels per sampling locale using ARLEQUIN. Allelic richness, which adjusts for discrepancies in sample size by incorporating a rarefaction method, was estimated in FSTAT 2.9.3.2. We tested whether the allelic richness differed significantly between all pairs of sampling locales using Wilcoxon matched-pairs signed-ranks tests. In addition, mean number of alleles per locus per sampling locale and percent of polymorphic loci per sampling locale were calculated. We investigated whether our data fit the signature of a population bottleneck by testing for heterozygote deficiencies using the Wilcoxon signed-rank test for the infinite allele model in Program Bottleneck 1.2 (Cornuet and Luikart 1996). This test was conducted within each of the two populations (RMP and PP) as well as with all the data pooled into one population to test for a species-wide bottleneck.

Genetic structure was investigated using AMOVA in ARLEQUIN using the same hierarchical levels as described for the mitochondrial data. Pairwise population F_{ST} significance tests were conducted among all pairs of sampling locales.

We used the software program STRUCTURE 2.00 (Pritchard et al. 2000) as an alternative approach to explore genetic structure. STRUCTURE employs a model-based clustering analysis that groups individuals into genetic clusters without regard to their original sampling locale. We first estimated the number of genetic clusters (K) by conducting 20 independent runs each for $K = 1-10$ with 500,000 Markov Chain Monte Carlo repetitions with a 500,000 burn-in period using the model with admixture, correlated allele frequencies, and no prior information on sampling locales ($\text{popinfo} = 0$). Several different ways of determining the optimal value of K were explored using both the method described by Pritchard et al. (2000) and the delta K method described by Evanno et al. (2005).

Table 3 Number of individuals with a given haplotype in each group. Haplotypic diversity (\hat{h}) values were calculated from Nei (1987) with standard deviations given in parentheses. Allelic richness (A) corrected for unequal sample sizes

Haplotype													
Group	A	B	C	D	E	F	G	H	I	Total	\hat{h}	A	
AKKenai	0	8	0	0	1	5	0	0	1	15	0.638 (0.093)	3.697	
AKCopper	4	3	0	0	1	1	0	0	0	9	0.750 (0.112)	4.000	
AKTanana	0	4	0	0	2	14	0	0	0	20	0.484 (0.112)	2.916	
YTWest	0	0	0	2	6	7	1	1	0	17	0.727 (0.073)	4.533	
Pacific Population	4	15	0	2	10	27	1	1	1	61			
YTEast	0	0	1	7	2	1	0	1	0	12	0.667 (0.141)	4.836	
AlbertaBC	0	0	10	10	0	0	0	15	0	35	0.672 (0.031)	2.998	
TriState	0	0	10	10	0	0	0	1	0	21	0.571 (0.052)	2.679	
Rocky Mountain Population	0	0	21	27	2	1	0	17	0	68			
Total	4	15	21	29	12	28	1	18	1	129			

For all pairs of sampling locales, we calculated the genetic distances of chord distance (Cavalli-Sforza and Edwards 1967) and proportion of shared alleles (Bowcock et al. 1994) using the software MICROSAT (Minch et al. 1995). These genetic distances were used because they have been shown to have a higher probability of obtaining correct tree topologies than other distance measures for microsatellite data (Takezaki and Nei 1996). The genetic distance matrices were used to construct neighbor-joining networks in PHYLIP 3.57 that were viewed using the program TREEVIEW. The strength of support for each node was tested by bootstrapping over loci using the program MICRO-SAT (1,000 permutations). A Mantel (1967) test was used to look for a correlation between genetic distance (F_{ST}) and geographic distance among all pairs of sampling locales using the same geographic distances as described above (ARLEQUIN 2.00, 1,000 permutations). Probabilities for the Mantel test were calculated following Smouse et al. (1986).

Results

Mitochondrial sequence data

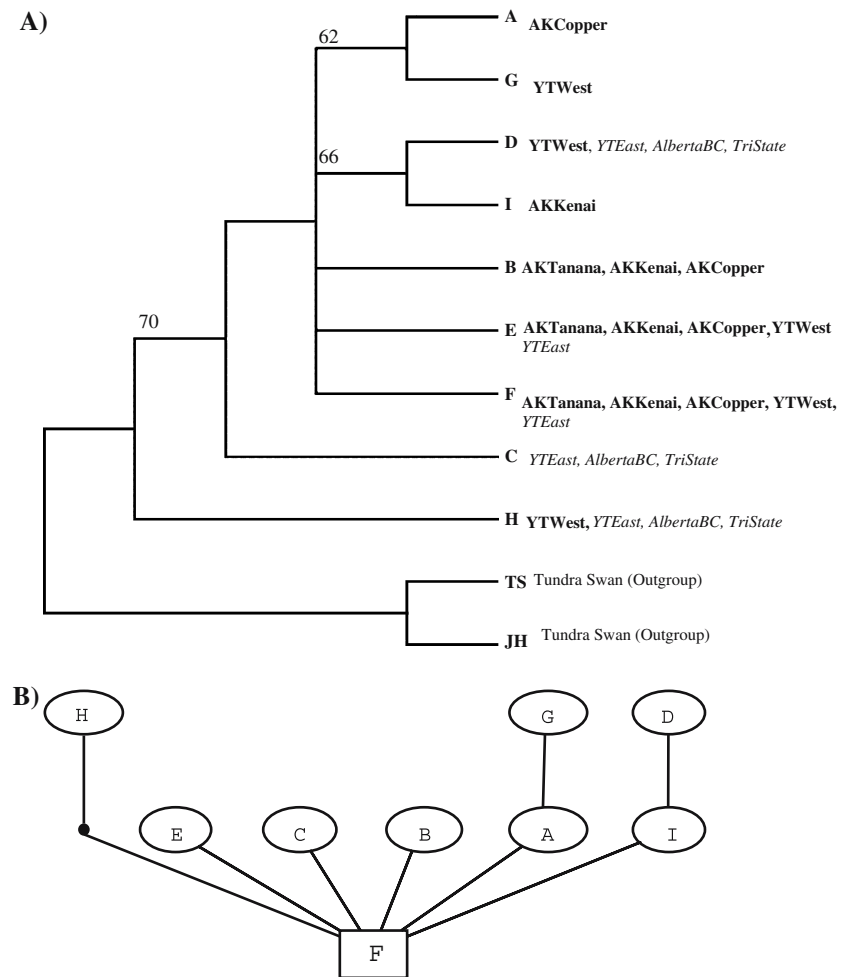
Sequences from PCRs conducted with all primer pairs on both long PCR templates and on genomic DNA were consistent. We viewed this as evidence that our primers were amplifying mitochondrial, rather than nuclear DNA. We compared sequences from 430 base pairs of ND6 and 950 base pairs of the control region. Mitochondrial DNA sequence data were obtained for 129 trumpeter swans (61 from the PP and 68 from the RMP) and two tundra swans. About 28 trumpeter swans were omitted from this portion of the study due to degraded DNA and subsequent problems with amplification. From the trumpeter swans sampled, nine

haplotypes were identified (Genbank accession numbers EF165350-EF165358). Two haplotypes, I and G, were unique to one individual. Each of the remaining haplotypes was shared by at least four individuals (Table 3). Haplotypes were characterized by nine polymorphic sites consisting of eight transitions and one transversion. The two tundra swan sequences differed from one another by six transitions. Both tundra swan sequences differed from the trumpeter swan sequences at 38 fixed sites. These fixed differences included 36 transitions, one transversion and a one base-pair insertion.

The best model of evolution given the data was HKY (unequal frequency of nucleotides and different rates of mutation for transitions and transversions) as chosen by Modeltest. The distance, parsimony, and maximum-likelihood analyses all produced trees with similar topologies. Because of the similar topologies, results from the parsimony analysis alone are presented. Parsimony analysis revealed two most parsimonious trees, one with the topology shown in Fig. 3a. The other tree had identical topology except that the C haplotype was nested within the clade containing A, G, D, I, B, E, and F rather than sister to it. The distance tree also has this topology. The topology of the maximum likelihood tree was identical to the tree in Fig. 3a. The haplotype network (Fig. 3b) revealed that all haplotypes were either one or two steps from the ancestral haplotype F.

Haplotype C was found only in the RMP while haplotypes A, B, G and I were exclusive to the Alaskan groups (Table 3; Figs. 3 and 4). Specifically, A was found only in the AKCopper block and present at high frequency. Haplotypes D and H were prevalent in the RMP but also in a few individuals from YTWest in the PP. Haplotypes E and F were present throughout the PP and only in the YTEast locale of the RMP (Figs. 3a and 4). The TriState group had the same three

Fig. 3 (A) Maximum parsimony tree of trumpeter swan haplotypes. A second most parsimonious tree in which haplotype C was nested within the largest clade was also produced from the analysis. Bootstrap values above 50% are indicated. Locations where each haplotype was found are indicated next to the haplotype. Two tundra swan sequences (*C. columbianus*) were used to root the tree. Sampling locale names from the Pacific Population are in bold and those from the Rocky Mountain Population are italicized. (B) Haplotype network showing the relationship among mitochondrial haplotypes



haplotypes as found in AlbertaBC, yet in different frequencies.

The prst AMOVA designated each sampling locale as a group and showed that 24.11% ($P < 0.001$) of genetic variation was due to differences among sampling locales. Treating the TriState as a group distinct from all other sampling locales, explained the least amount of variation (2.25%, $P = 0.571$). Populations as they were defined earlier, the RMP and the PP, (Table 1) explained the most variation (20.4%, $P < 0.028$) with an additional 9.91% ($P < 0.001$) of the variation attributable to sampling locales. Pairwise analyses of locales (Table 4) revealed that most of the groups within the PP were not significantly different from one another (with the exception of AKKenai and YTWest). Likewise, all of the sampling locales within the RMP were not significantly different from one another (Table 4). All pairwise comparisons of sampling locales between the RMP and the PP were significantly different from each other, with the exception of YTWest and YTEast. A graphical representation of the groups' similarity to one another is represented in a

neighbor-joining network (Fig. 5) derived from F_{ST} values.

Haplotypic diversity (Table 3) differed slightly among locales with the lowest level in AKTanana (0.484) and the highest level in AKCopper (0.750). Allelic richness levels (Table 3) also differed slightly among locales with the lowest occurring in TriState (2.679) and the highest in YTEast (4.836).

A Mantel test comparing the matrix of F_{ST} values and a distance matrix showed a significant correlation coefficient of 0.442 ($P < 0.05$) indicating a weak positive relationship between physical distance and genetic distance.

Microsatellite analysis

The number of microsatellite alleles per locus across all sampling locations ranged from 2 to 15. One locus was removed from further analyses due to significant deviations from Hardy-Weinberg (TS.8.20.13, $P < 0.0001$ in all sampling locales). A second locus was removed due to amplification problems (TS.1.29.52).

Fig. 4 Haplotype frequency for each sampling locale using mitochondrial sequence data. Letters correspond to different haplotypes

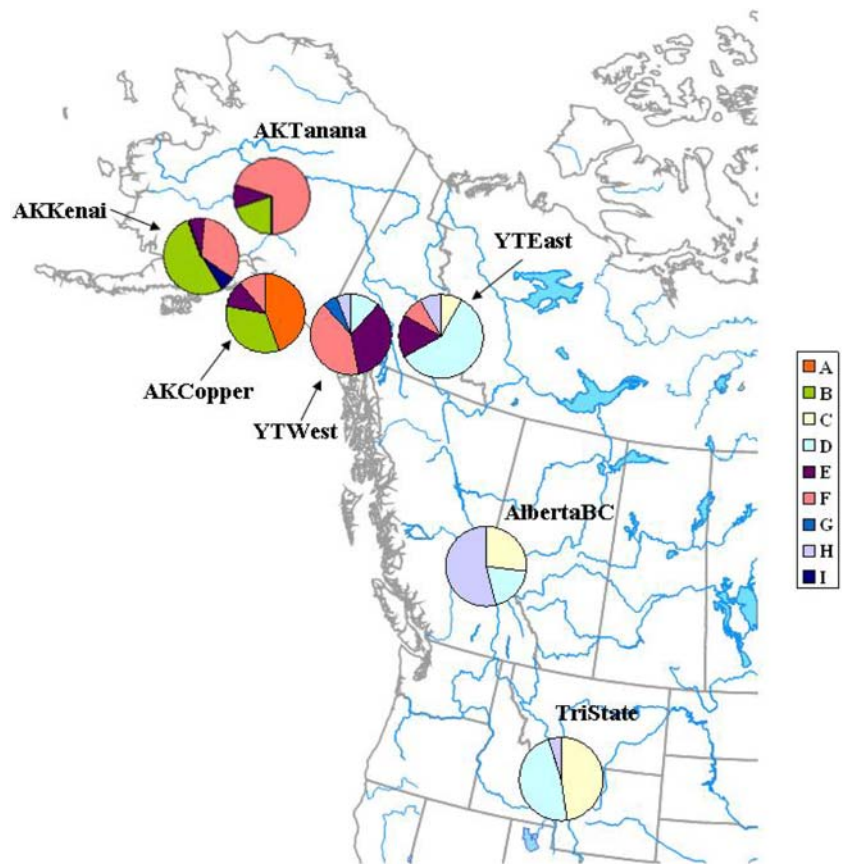


Table 4 Pairwise genetic distance analysis of sampling locales using mitochondrial data. Pairwise F_{ST} values are below the diagonal. P -values calculated from permutation tests are above the diagonal. P -values below $P = 0.01$ (chosen as cutoff for

statistical significance) indicate two sampling locales are significantly different from one another and are shown in bold. Between population comparisons (PP versus RMP) are shaded

	AKTanana	AKKenai	AKCopper	YTWest	YTEast	AlbertaBC	TriState
AKTanana		0.072	0.026	0.015	0.000	0.000	0.000
AKKenai	0.110		0.050	0.002	0.000	0.000	0.000
AKCopper	0.171	0.139		0.016	0.000	0.001	0.000
YTWest	0.093	0.226	0.151		0.017	0.001	0.000
YTEast	0.400	0.395	0.346	0.189		0.021	0.160
AlbertaBC	0.266	0.298	0.275	0.186	0.143		0.012
TriState	0.369	0.380	0.351	0.249	0.048	0.133	

Of the 17 remaining loci, there were no significant departures from Hardy-Weinberg equilibrium (Bonferroni corrected $P = 0.0042$). No significant linkages were found between any pair of loci.

All sampling locales were monomorphic at one or more loci (Table 5). The mean number of alleles per locus ranged from 2.41 alleles (YTWest) to 3.18 alleles (AKKenai; Table 5). Levels of allelic richness, which correct for unequal sample sizes, were similar among sample locales (Table 5) with AKKenai having the highest level of diversity (2.10) and AlbertaBC and TriState with the lowest (1.85). The only significant

differences in allelic richness were that AKKenai was significantly richer than both AlbertaBC and TriState.

Population genetic structure was investigated using AMOVA with the three groups described in the mtDNA analysis. When each sampling locale was treated separately, 4.8% ($P < 0.001$) of the variation was explained by the "among groups" category. The second analysis that tested how much variation was explained by the separation of TriState birds from all other sampling locales, revealed that no variation (0.23%, $P = 0.570$) was explained by this designation. The third analysis testing the PP versus the RMP

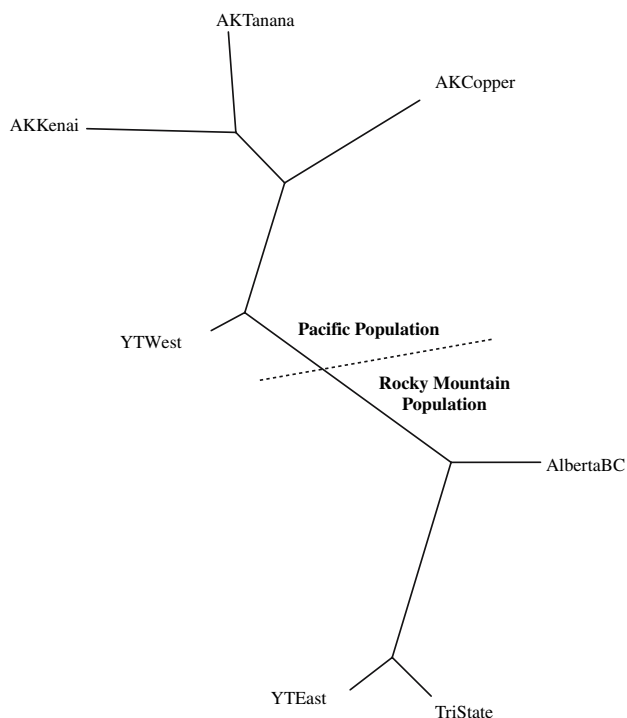


Fig. 5 Neighbor-joining network constructed from pairwise F_{ST} values using mitochondrial sequence data. The dashed line indicates the proposed population boundary

showed that this designation explained the most variation (10.1%, $P < 0.028$) among groups.

Pairwise F_{ST} analysis of sampling locales showed no significant differences among locales within the PP and RMP. All comparisons were significantly different between the RMP and PP with the exception of YTWest versus YTEast and YTWest versus AlbertaBC (Table 6). When considering only within-population comparisons, the highest F_{ST} values occurred in comparisons with AKCopper and the other Alaskan locales.

Using the method suggested by Pritchard et al. (2000), the most appropriate value of K given our data was 5. Using the delta K method of Evanno et al. (2005) our data was best represented by $K = 3$. Further

inspection of the results also suggested that $K = 2$ may be appropriate in that our $\ln P(D)$ values start to plateau at $K = 2$ and because at $K = 3$ most individuals have a roughly equal membership in clusters 2 and 3. Regardless of whether a K of 2, 3, or 5 is chosen as the most appropriate value of K (Fig. 6), a general pattern was observed that individuals in the RMP tended to be very similar and different from those in the PP with the Yukon territory as an area of overlap. Additionally, the AKCopper sampling locale appeared almost uniform in cluster membership at each level of K , whereas the other PP sampling locales had a mixture of cluster membership (Fig. 6).

The test for a species-wide bottleneck (all data pooled into one population) showed a weak signature of a genetic bottleneck that was almost significant ($P = 0.079$). When the two populations were separated and tested we found similar results with the PP population showing a significant sign of a bottleneck ($P = 0.054$) and the RMP showing a trend yet it was not statistically significant ($P = 0.087$).

The neighbor-joining networks constructed using chord distance and proportion of shared alleles showed similar topologies (Fig. 7). The following groupings recur in both trees, AKKenai and AKCopper, YTWest and YTEast, and AlbertaBC and TriState, though bootstrap values were low. The Mantel test showed a positive, though weak, correlation between genetic distance (F_{ST}) and geographic distance ($r = 0.45$, $P = 0.02$).

Discussion

The mitochondrial and nuclear markers revealed congruent patterns of genetic variation across the range of the trumpeter swan. These patterns demonstrate significant differentiation between the PP and the RMP. Specifically, the western (Alaska) locales are well differentiated from the eastern (AlbertaBC and TriState) locales, and the intermediate (Yukon Territory) locales

Table 5 Genetic diversity measures of natural trumpeter swan sampling locales estimated from 17 microsatellite loci. Standard deviations are given in parentheses

Sampling Locale	Mean sample size (SD)	Mean # of alleles per locus (SD)	# of monomorphic loci	Mean observed heterozygosity	Mean expected heterozygosity	Allelic Richness
AKTanana	19.38 (0.99)	2.59 (1.12)	1	0.313	0.390	1.91
AKKenai	15.62 (0.18)	3.18 (2.35)	2	0.411	0.438	2.10
AKCopper	9.88 (0.33)	2.59 (1.23)	3	0.353	0.441	2.05
YTWest	11.54 (4.11)	2.41 (1.23)	3	0.333	0.387	1.94
YTEast	13.32 (1.93)	2.53 (1.18)	1	0.322	0.399	1.94
AlbertaBC	34.26 (3.43)	2.59 (1.58)	2	0.342	0.498	1.85
TriState	36.56 (4.7)	2.82 (1.63)	3	0.327	0.486	1.85

Table 6 Pairwise genetic distance analysis of sampling locales using microsatellite data. Pairwise F_{ST} values are below the diagonal. P -values calculated from permutation tests are above the diagonal. P -values below $P = 0.01$ indicated two sampling

locales were significantly different from one another and are shown in bold. Between population comparisons (PP versus RMP) are shaded

	AKTanana	AKKenai	AKCopper	YTWest	YTEast	AlbertaBC	TriState
AKTanana		0.145	0.017	0.999	0.001	0.000	0.000
AKKenai	0.013		0.098	0.999	0.004	0.000	0.000
AKCopper	0.044	0.021		0.977	0.000	0.000	0.000
YTWest	0.077	0.110	0.046		0.830	0.381	0.007
YTEast	0.081	0.054	0.133	0.022		0.723	0.769
AlbertaBC	0.101	0.099	0.196	0.000	0.008		0.860
TriState	0.087	0.087	0.173	0.033	0.010	0.007	

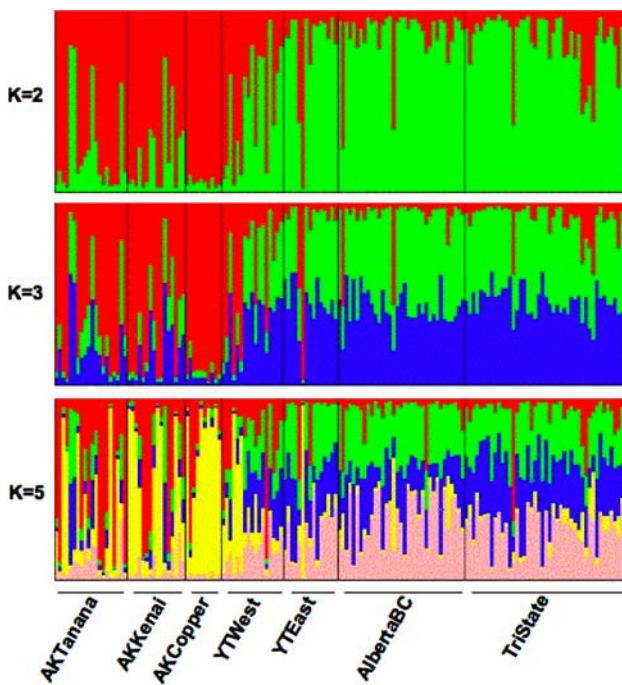


Fig. 6 Results of the STRUCTURE analysis based on 17 nuclear microsatellite loci for three different values of K (2, 3, and 5) which were obtained by eye ($K = 2$), following the delta K method of Evanno et al. ($K = 3$), and following the method of Pritchard et al. 2000. In all analyses, each distinct cluster is represented by a unique color. Each vertical bar represents an individual trumpeter swan. The colors on each vertical bar represent the probability of the individual belonging to each cluster. Individuals are grouped into sampling locales and listed from left to right from Northwest to Southeast along the x axis

likely represent a zone of admixture. Further, there appears to be more structure within the PP than the RMP. These results are consistent with those of Pelizza and Britten (2002) who found a significant difference between the PP and the Tri-State stocks. In addition, our data spans the entire range of naturally occurring trumpeter swans and samples multiple loci, allowing us to better address the boundaries and the histories of the management populations.

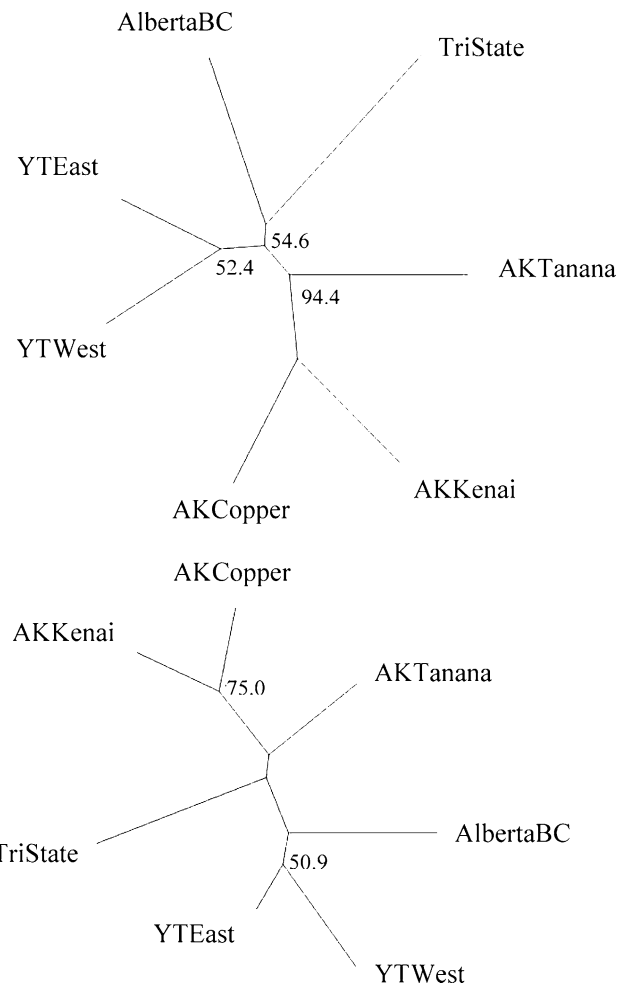


Fig. 7 Neighbor-joining networks constructed using chord distance (top) and proportion of shared alleles (bottom) with data from 17 microsatellite loci. Bootstrap values above 50% are shown

In the mitochondrial data set, haplotypes A, B, G and I were unique to the PP while C was unique to the RMP (Table 3, Figs. 3a and 4). Additionally, the most frequent haplotypes found in the PP were either exclusive to the PP (B) or found only in YTEast within

the RMP range (F and E) (Table 3, Fig. 4). Similarly, the common haplotypes within the RMP were either restricted to the RMP sample locales (C) or shared in very low frequency with YTWest within the PP range (D and H). The AMOVA analysis that grouped the sampling locales at the population level explained the most amount of variability. Pairwise comparisons among all sampling locales revealed that all between-population comparisons (PP versus RMP) were significantly different except between the two Yukon locales (Table 4) where there appears to be gene flow between the two populations. Additionally, there was one significant difference (AKKenai and YTWest) within the PP (Table 4). The neighbor-joining network showed a distinction between the sampling locales in the RMP and PP (Fig. 5).

The nuclear data also demonstrated that there was significant differentiation between the two populations with the Yukon Territory, likely representing an area of admixture between the two populations. As in the mitochondrial data, the greatest amount of variation in the AMOVA was explained when the population designation was included as the level of analysis. In the nuclear dataset, the pairwise locale comparisons (Table 6) were similar to those in the mitochondrial dataset, except that the AlbertaBC and YTWest sampling locales were not significantly different and there were no significant differences within the PP. The STRUCTURE analysis (Fig. 6) showed a large distinction between the Alaska sampling locales and the AlbertaBC and TriState locales regardless of which K value was used. A transition zone between the PP and RMP in the Yukon Territory was evident. While the AlbertaBC and TriState locales had similar patterns among individuals, locales in Alaska were more differentiated (Fig. 6). Both neighbor-joining networks showed similar topologies in that the Alaska locales grouped together, the Yukon locales grouped together, and AlbertaBC and TriState locales grouped together (Fig. 7). Again, the Mantel test found a positive correlation between geographic and genetic distance.

Although the mitochondrial and microsatellite data show remarkably similar patterns of how genetic variation is distributed across the range of the trumpeter swan, there are slight differences between them. The major difference is that the mitochondrial data (Table 4) detect more structure among sampling locales within the RMP and PP than do the nuclear markers (Table 6). This result is not unexpected given the different patterns of inheritance of these two types of genetic markers. In waterfowl it is typically males that move between breeding locations, whereas females tend to be more philopatric to nesting areas

(Cooke et al. 1975). This has been well demonstrated in spectacled eider ducks (*Somateria fisheri*, Scribner et al. 2001) and common eiders (*S. mollissima*, Tiedemann et al. 1999), where mitochondrial data showed much more population structure than corresponding microsatellite data. Further, genetic markers located in the mitochondrial genome also have a lower effective population size due to maternal inheritance, increasing the speed with which genetic drift can cause frequency shifts among varying haplotypes. An additional factor that could lead to minor differences between the two data sets has to do with the number of loci sampled (sampling error). While the mitochondrial genome represents one locus, multiple sites were sampled in the nuclear genome.

Due to these differences inherent to the markers, our nuclear data set is likely to yield a more representative depiction of the structure of trumpeter swan variation, with the Alaska groups being different from the AlbertaBC and TriState groups and with the Yukon Territory as an area of overlap. Further, the higher levels of genetic mixing within each population documented by the microsatellite data likely provide a better depiction of actual gene flow by trumpeter swans. Thus, while there does not appear to be significant gene flow between the locales at the extremes of the range (Alaska locales versus TriState and AlbertaBC) there does appear to be some amount of mixing within each population and in the area of population overlap (Yukon Territory).

In general, we found slightly higher levels of genetic diversity in the sampling locales from the PP (AKCopper and AKKenai for the microsatellite data set and AKCopper and YTWest for the mitochondrial data set) compared to the RMP, although these differences were mostly non-significant. This suggests that even though trumpeter swans underwent a population bottleneck in the early part of the 20th Century (supported by the results of our bottleneck analysis), the species has rebounded in such a way that none of the areas we sampled containing free-ranging PP and RMP birds appeared to consistently have significantly lower genetic variability than any other area. Because trumpeter swans in the PP have similar (only slightly higher) levels of diversity, they, too, likely went through a population bottleneck, which is consistent with demographic information from Alaska and Canada (Matteson et al. 1995). Further, because we found haplotypes unique to each management population, these bottlenecks resulted in at least two remnant groups (Alaska/Northwestern Canada and Tri-State) that have, in turn, expanded to form the two populations found today—the PP and the RMP.

Though diversity levels are similar for the intraspecific comparisons that are the focus of this study, trumpeter swans appear to have much lower mitochondrial DNA variability than other waterfowl studied thus far (Tiedemann et al. 1999; Scribner et al. 2001; Talbot et al. 2003; Ruokonen et al. 2004). This observation of low variability is consistent with the observations of Barrett and Vyse (1982), Meng et al. (1990) and Marsolais and White (1997) who noted low genetic diversity in trumpeter swans across their entire range. This remarkably low diversity coupled with the fact that most mitochondrial haplotypes differed by only one nucleotide change (Fig. 3) suggests that trumpeter swans may have undergone an entire species-wide bottleneck prior to their documented decline of the last century.

Implications for conservation and management

In this study, we identified two distinct populations (PP and RMP) in the western part of North America. This lends support for the current management of the RMP and PP birds as separate populations, with the Yukon Territory being considered a likely region of overlap. Additionally, the populations revealed relatively similar levels of genetic diversity, suggesting that no specific sampling locale mandates special consideration in that regard.

Further, both the mitochondrial and microsatellite data sets suggest that the swans in the TriState locale are not significantly different genetically from swans in the Alberta/BC sampling area. In fact, most analyses (particularly STRUCTURE [Fig. 6], genetic distance [Fig. 7], and pairwise comparisons [Tables 4 and 6]) depict them as being quite similar. Our AMOVA that tested the hypothesis that the TriState locale was different showed that this representation of the data explained the least amount of variation of any test we performed. This suggests that there is likely genetic interchange between these two breeding locations. In addition, the TriState swans were found to have similar levels of genetic variation compared to all other sampling locales indicating that there does not appear to be significantly reduced variation or increased amounts of inbreeding in this area compared to other areas. Therefore, we find no evidence to support differential management treatment between the Tri-State flock and the Canadian flock of RMP based on genetic differentiation.

Additionally, there appears to be more genetic structure within the Alaska locales than between the Alberta/BC and TriState locales. Although the AKCopper flock was not found to be significantly

different from any other Alaskan locale (Tables 4 and 6), it had the highest within Alaska F_{ST} values. Further, the AKCopper flock has a unique haplotype (A) in high frequency and is the only sampling locale in STRUCTURE that is largely comprised of a single cluster. This uniqueness may be indicative of the geography of the region. The Copper River Delta is bordered by mountains, which separate the region from the rest of the Alaskan mainland. This feature may serve as an obstacle to gene flow potentially resulting in isolation, which could, in turn, produce uniqueness by mutation and drift.

Our study documented the genetic variation across the current natural range of the trumpeter swan, determining that there are two largely distinct populations of trumpeter swans whose ranges correspond loosely to the flyways, with the Yukon Territory as a likely area of overlap. Further, although the Tri-State flock exhibits non-migratory behavior, it is not genetically different from the neighboring Alberta/BC flock, nor does it have significantly reduced genetic variation. Interestingly, we documented low levels of genetic variability resulting not only from a well documented population bottleneck in the 20th Century, but also likely a previous species-wide bottleneck. Finally, we found more genetic structure within the Alaskan sampling locales with the Copper River Delta as an area of potential uniqueness. The data from this study can be used in conjunction with large-scale demographic and habitat data to provide an integrated approach to management efforts for the trumpeter swan and aid in restoration attempts.

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