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Effects of a Population Bottleneck on Whooping Crane Mitochondrial DNA Variation

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Abstract: *The Whooping Crane (*Grus americana*) is an endangered bird that suffered a severe population bottleneck; only 14 adults survived in 1938. We assessed the genetic effect of this human-caused bottleneck by sequencing 314 base pairs (bp) of the mitochondrial DNA control region from cranes that lived before, during, and after this bottleneck. The maximum length of DNA amplifiable from museum specimens was negatively correlated with age, and only 10 of 153 specimens yielded the entire 314 bp sequence. Six haplotypes were present among the prebottleneck individuals sequenced, and only one of these persists in the modern population. The most common modern haplotype was in low frequency in the prebottleneck population, which demonstrates the powerful effect of genetic drift in changing allele frequencies in very small populations. By combining all available data, we show that no more than one-third of the prebottleneck haplotypes survived the human-caused population bottleneck. High levels of variation of substitution rates among nucleotide sites prevented us from estimating the prebottleneck population size. Our data will be incorporated into the captive breeding program to allow better management decisions regarding the preservation of current genetic diversity. These data offer the first glimpse into the genetic toll this species has paid for human activities.*

Efectos de un Cuello de Botella Poblacional en la variación ADN mitocondrial de la Grulla Americana

Resumen: *La Grulla Americana (*Grus americana*) es un ave amenazada que sufrió un cuello de botella poblacional severo con tan solo 14 adultos sobreviviendo en 1938. Evaluamos el efecto genético de este cuello de botella causado por humanos secuenciando 314 pb de la región control del ADN mitocondrial de grullas que vivieron antes, durante y después de este cuello de botella. La longitud máxima de ADN amplificable de especímenes de museo estuvo negativamente correlacionada con la edad, y solo 10 (de 153) especímenes produjeron la secuencia completa con los 314 pb. Cinco haplotipos estuvieron presentes entre los individuos pre-cuello de botella secuenciados y solo uno de estos persiste en las poblaciones modernas. El haplotipo moderno más común era de baja frecuencia en la población pre-cuello de botella, lo cual demuestra el poderoso efecto de la deriva génica en frecuencias cambiantes de alelos en poblaciones muy pequeñas. Al combinar todos los datos a la mano, mostramos que no más de una tercera parte de los haplotipos pre-cuello de botella sobrevivieron a un cuello de botella ocasionado por humanos. Los altos niveles de la tasa de variación entre sitios, impidió la estimación del tamaño poblacional previo al cuello de botella. Nuestros datos pueden ser incorporados al programa de reproducción en cautiverio para permitir decisiones de manejo mejor enfocadas para la preservación de la diversidad genética actual. Estos datos ofrecen un primer vistazo a el precio genético que esta especie ha pagado debido a actividades humanas.*

Introduction

Population bottlenecks, natural or human-induced, can have significant genetic effects, such as reduced effective population size (Wright 1938), loss of heterozygosity (Nei et al. 1975), and loss of alleles (Maruyama & Fuerst 1985),

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which may lead to decreases in competitiveness, disease resistance, and survival (Soulé 1986; Avise 1994; Keller et al. 1994). Populations that have been reduced in size due to recent human activities have become a focus of conservation genetic studies (Bonnell and Selander 1974; Ralls et al. 1979; Briscoe et al. 1992; Taylor et al. 1997). Although laboratory and manipulative experiments have demonstrated some effects of bottlenecks on controlled populations (Hedrick et al. 1996), studies are needed that directly examine the genetic consequences of bottlenecks on natural populations (Arden et al. 1997).

The Whooping Crane (*Grus americana*) is North America's tallest bird and the rarest of the world's 15 crane species (Johnsgard 1983). Whooping Cranes inhabited much of North America prior to its settlement by Europeans (Fig. 1; Allen 1952). The extent of gene flow among these early populations is unknown. Habitat alterations on the primary breeding grounds by European settlers, combined with hunting for food, sport, and market, led to the inexorable depletion of Whooping Crane populations (Allen 1952). All living Whooping Cranes descend from a single population that breeds in Wood Buffalo National Park (WBNP), Canada, and winters in the Aransas National Wildlife Refuge (ANWR), Texas (Fig. 1; Allen 1952, 1956). The ANWR Whooping Crane population consisted of only 14 adults in the fall of 1938 and has been carefully monitored each year since (Allen 1952; Doughty 1990). Additional Whooping Cranes persisted as a year-round resident population along the Louisiana Gulf coast until 1950 (Doughty 1990), but all of the Louisiana Whooping Cranes and their descendants have subsequently perished. As of February 1997, 160 Whooping Cranes were wintering in the ANWR area, 100 were in captive breeding populations, and 73 were in reintroduced wild populations (Jones & Mirande 1997; Stehn 1997).

Whooping Cranes offer a unique opportunity to study the loss of genetic variation in a natural population that experienced a severe bottleneck because the census size of the population during its crash and subsequent rebound have been carefully documented (Allen 1952; Doughty 1990). Whooping Cranes have also been the focus of several studies of genetic variation (e.g., Dessauer et al. 1992; Longmire et al. 1992; Snowbank & Krajewski 1995; Glenn et al. 1997). Because offspring from all sur-

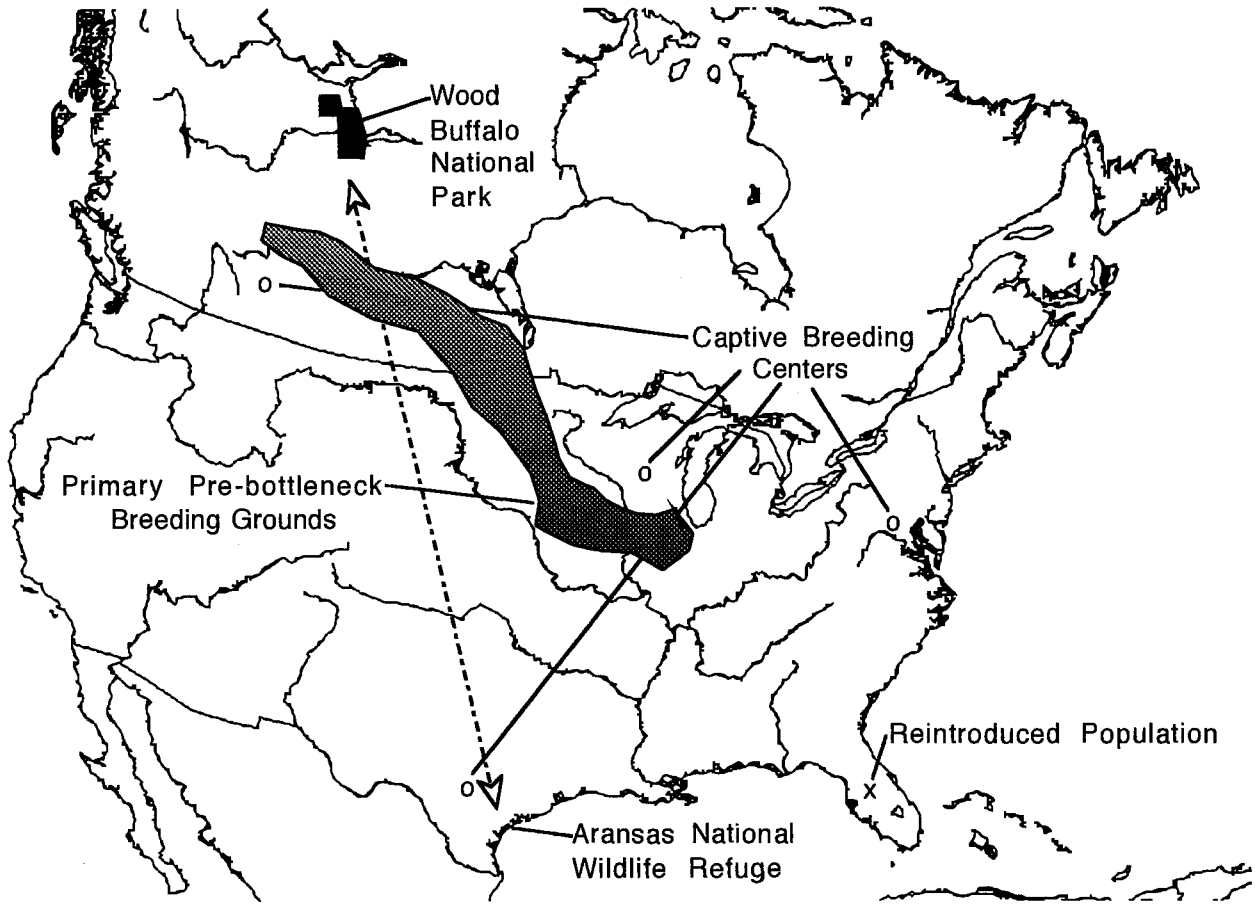

Figure 1. Former primary breeding grounds (shaded area; Allen 1952) and current distribution (named locations) of Whooping Cranes. Whooping Cranes were formerly found throughout much of North America (Allen 1952).

viving maternal lineages were captured for the captive population (Mirande et al. 1991; Jones & Mirande 1997), a complete census of the species' remaining mitochondrial DNA (mtDNA) diversity can be obtained from a small number of individuals (Snowbank & Krajewski 1995). In addition, hundreds of Whooping Cranes have been deposited in museum collections before, during, and after the bottleneck (Hahn 1954; Glenn 1997). The large size and number of specimens also allow a unique opportunity to explore the techniques used to recover DNA from museum specimens. We amplified and characterized the most variable portion of mtDNA from museum skins to make direct comparisons of pre- and postbottleneck genetic variation in Whooping Cranes (Taylor et al. 1994).

Methods

Tissue Samples

Tissue samples from 153 museum specimens were collected (Table 1; Glenn 1997). Most samples, 131, consisted of skin snips (usually <5 cm^2) with attached feathers. One muscle and 11 bone samples were collected from skeletal specimens. From 10 specimens, skin, bone, and foot pad were taken for a comparison of DNA recovery from different tissues. Fresh tissue or blood samples were available from 158 individuals (Table 1; Glenn 1997). All samples were collected in accordance with the laws regarding endangered species.

Composite nesting areas (CNAs)—territories where nesting through fledging occurs—were established for the entire WBNP population through field studies begun in the 1950s, and captive breeding has emphasized genetic representation of all the original CNAs (Mirande et al. 1991). Thanks to this effort, the maternal history of most birds collected after 1966 is known or can be inferred (Snowbank & Krajewski 1995; Glenn 1997). A sample of 17 individuals was selected that should contain all possible mitochondrial lineages still persisting within Whooping Cranes. Thus, two groups of Whooping Cranes were examined to assess the effect of the bottleneck: (1) the prebottleneck sample, consisting of 113 museum specimens collected before 1938, and (2) the postbottleneck sample, consisting of 10 fresh blood samples and 7 museum specimens collected after 1966.

DNA Extraction

We isolated genomic DNA from fresh tissue using one of three protocols: phenol/chloroform/isoamyl alcohol (PCI) extraction and ethanol precipitation (Sambrook et al. 1989), guanidine thiocyanate and silica (Boom et al. 1990), or diatomaceous earth (Carter & Milton 1993). Explicit protocols are available from the Internet (ftp://onyx.si.edu/protocols/; file names TSilicaDNA.rtf and MUD- DNA.rtf).

We used a digestion protocol modified from that of Pääbo et al. (1988) to isolate genomic DNA from a portion of skin approximately 10 × 5 mm (about 10 mg) from all museum skins (including attached feather quills). Isolation was followed by PCI extraction. We used a digestion buffer containing 1 mM Ca^{2+} with no EDTA to improve the efficiency of proteinase K digestion (Sambrook et al. 1989). All bone samples were digested in the buffer described by Pääbo et al. (1988). Extracts were washed and concentrated with TLE (10 mM Tris pH 8; 0.2 mM EDTA) in Microcon-30 filters (Amicon, Danvers, Massachusetts, U.S.A.), and the final volume was adjusted to 50–200 μL. Additional extraction protocols were tested on skin and foot pad tissues by digestion in buffer from Pääbo et al. (1988), our modified buffer, and our modified buffer without collagenase. Each extraction was followed by PCI extraction and concentration as above. We also used an SDS-Urea extraction (Densmore & White 1991) and QiaAmp Tissue Preps (Qiagen Inc., Chatsworth, California, U.S.A.) according to the manufacturer's mouse-tail protocol. (Explicit protocols may be obtained from the same server referenced for the fresh tissue procedure under the file name OldDNA.rtf.)

Contamination Control

Contamination control strategies generally followed Thomas (1994; but see Glenn 1997). All supplies used for museum specimens were purchased new and kept in laboratories that had not contained bird DNA or polymerase chain reaction (PCR) amplicons. All pre-PCR manipulations were conducted in a dedicated pre-PCR laboratory once species-specific primers had been obtained. The DNA was isolated from museum samples in a second dedicated pre-PCR laboratory that never contained fresh bird DNA or tissue.

To control carry-over contamination, all PCR reactions contained dUTP rather than TTP (Longo et al. 1990). The exceptions to this strategy were (1) the initial amplification, cloning, and sequencing of the complete control region, which was completed by late 1994 (before the pre-PCR lab was completed), and (2) final amplification efficiency tests using TTP and dUTP, which were performed after all sequences had been obtained. Five additional precautions were taken to prevent carry-over contamination. First, the amplification, cloning, and sequencing of the complete control region for primer design used a single individual (USNM 542868) with haplotype 1. This individual was used as a positive control for all PCR experiments. Second, all museum skin samples were extracted once before PCR of individuals other than the positive control was done. Third, the primers designed to amplify the 314 base pair (bp) fragment were specific to Whooping Cranes under the conditions

employed in this study (DNA from all other crane species and a variety of other birds and mammals were tested). Fourth, all initial extractions were amplified for four of the fragments before any were sequenced, so no amplicons containing TTP (from the sequencing reactions) existed before most PCR amplifications were completed. Finally, all samples were examined for congruence of sequences from all amplified fragments. Thus, all haplotypes other than the positive control (haplotype 1), could be attributed absolutely to DNA derived from the extractions, not re-amplification of previous PCR amplicons, clones, or sequencing reactions.

Table 1. Whooping Crane samples that yielded at least 250 bp of mtDNA sequence.

Studbook ID	*Museum ID*[a]	*Tissue*	*Locality*[b]	*Year*[c]	*Haplotype*[d]
Prebottleneck	AMNH 354211	Skin	—	—	2 or 6[e,f]
	HMCZ 042510	skin	South Dakota	1893	3
	USNM 019171	bone	Texas	1893	3
	USNM 212985	skin	Kansas	1910	3
	FMNH 401920	skin	Nebraska	1922	3
	USNM 371331	skin	Nebraska	—	3
	USNM 273926	skin	Nebraska	1923	5
	USNM 035445	foot	—	—	6
	FMNH 405302	skin	Illinois	1891	7
	USNM 288519	bone	Kansas	1923	7
	USNM 007333	bone	NWT	1860s	8
	JFBM 017948	skin	—	—	9[e,f]
Postbottleneck					
1020		blood	WBNP	1985	1
1027		blood	WBNP	1992	1
1041		blood	WBNP	1992	1
1042		blood	WBNP	1985	1
1048	USNM 567748	skin	WBNP	1971	1
1054	USNM 567747	skin	WBNP	1971	1
1055	USNM 567744	skin	WBNP	1974	1
1057	USNM 567745	skin	WBNP	1974	1
1059	USNM 567743	skin	WBNP	1974	1
1060	USNM 567746	skin	WBNP	1974	1
1062		blood	WBNP	1985	1
1100		blood	WBNP	1992	1
1032		blood	WBNP	1985	2
1047	USNM 567749	skin	WBNP	1971	2
1031		blood	WBNP	1992	3
1128		blood	WBNP	1992	3
1195		blood	WBNP	1992	3
Additional samples					
—	USNM 395058	skin	ANWR	1948	1[f]
1002	USNM 599623	skin	ANWR	1949[b]	1[f]
—	USNM 428576	skin	ANWR	1951	1[f]
1019	USNM 601126	wing/skin	ANWR	1970	1[f]
—	USNM 481801	skin	Kansas	1965	1[f]
1038	USNM 532714	skin	WBNP	1969	1[f]
—	USNM 542868	frozen muscle	WBNP	—	1[f]
1011	USNM 428124	bone	Louisiana	1950	3[e,i]
—	USNM 419881	skin	ANWR	1951	3[e]
1018	USNM 480442	skin	Louisiana	1964	3[e,j]
—	USNM 491260	bone	Louisiana	1970	3[e,j]
—	USNM 420012	skin	ANWR	1951	4[e]

[a] *AMNH, American Museum of Natural History; HMCZ, Harvard Museum of Comparative Zoology; USNM, United States National Museum (Smithsonian Institution); FMNH, Field Museum of Natural History; JFBM, James Ford Bell Museum of Natural History (University of Minnesota).*
[b] *NWT, Northwest Territories, Canada; WBNP, Wood Buffalo National Park (locality for all birds descended from the WBNP/ANWR population unless specifically collected on the ANWR); ANWR, Aransas National Wildlife Refuge.*
[c] *Year the bird, blood, or wing was collected.*
[d] *See Table 2 for haplotype information (keyed to number provided here).*
[e] *Incomplete sequence (amplicons C, K, L).*
[f] *Excluded from analysis.*
[g] *Incomplete sequence (amplicons C, J, L, K).*
[h] *Year brought into captivity.*
[i] *Mac, the last wild Whooping Crane from Louisiana; died at ANWR.*
[j] *Captive offspring of Josephine.*

DNA Amplification and Sequencing

An alignment of the complete control-region sequence of one Whooping Crane and four other crane species (Glenn 1997) was used to design seven primer pairs for use in amplification and sequencing attempts (Fig. 2). Hot Start PCR using Hot Beads (Lumitek, Salt Lake City, Utah, U.S.A.) and highly stringent PCR conditions was performed in Perkin-Elmer/Cetus 480 thermocyclers equipped with Cycler Mate heated lids (BioLogic Engineering, Inc., Shelton, Connecticut, U.S.A.). The PCR was carried out in 50-μL volumes of 50 mM KCl, 10 mM Tris-HCl pH 9, 1% Triton X-100, 1.5 mM $MgCl_2$, 150 μM of each dNTP, 250 μg/mL BSA (Fraction V, Sigma, St. Louis, Missouri, U.S.A.), and 0.5 μM of each primer with 1 unit *Taq* DNA polymerase (Promega, Madison, Wisconsin, U.S.A.) and 50 ng of modern DNA or 2.5 μL of museum DNA extract. The highest annealing temperature (55° for amplicons A, B, C, I, and K; 60° for amplicons J and L), lowest Mg^{2+} concentration, and lowest number of cycles (25–30 for modern DNA; 30–35 for museum DNA) yielding consistent amplification were employed. Controls with uracil DNA glycosylase (UDG) included liquid $MgCl_2$ rather than Hot Beads, 1 unit of UDG, 30 minutes at 37° and 10 minutes at 94° before cycling. The PCR products were examined by electrophoresis through 2% agarose gels containing 1× TBE buffer and ethidium bromide (Sambrook et al. 1989).

Inhibition of PCR by museum extracts was tested by adding 50 pg of modern Whooping Crane DNA to a PCR of each DNA extraction and attempting to amplify amplicon B (Fig. 2; Glenn 1997). Only DNA extractions that did not previously amplify this fragment were assayed. Amplification products were electrophoresed and scored visually relative to controls on 2% agarose minigels stained with ethidium bromide.

The PCR products >150 bp were purified by PEG precipitation (Applied Biosystems 1994), whereas those <150 bp were purified by agarose gel electrophoresis followed by spin filtration (Glenn & Glenn 1994). Sequences were determined directly from PCR products on an ABI 373 stretch automated sequencer (Applied Biosystems 1994) using Taq FS dye terminator chemistry with 10% dimethyl sulfoxide added.

Data Analyses

Analyses were performed to determine if the pattern of genetic variation in the extant postbottleneck population is different from that of the prebottleneck sample. We used Tajima's test (Tajima 1989*a*) to analyze patterns of nucleotide diversity, and the routine Proc Freq of SAS (SAS Institute, Cary, North Carolina, U.S.A.) to assess the significance of observed haplotype frequency differences. We investigated relationships among the haplotypes using parsimony analysis and median networks. For parsimony analysis we used the branch and bound search of PAUP* (Swofford 1997), with all characters treated as unordered and equally weighted. We constructed median networks for the haplotypes according to Bandelt et al. (1995).

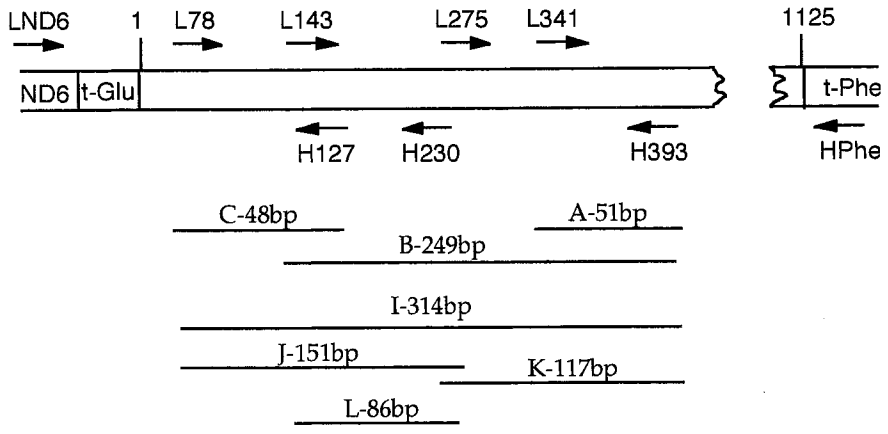

Figure 2. Primers and amplicons used to survey variation in the mtDNA control region of Whooping Cranes. Numbering begins with the first base 3′ of tRNA-Glu. Primer numbers correspond to the 3′ base of primers. Length of amplicons excludes primers. Primer sequences are (5′ to 3′): L-ND6, CCC ATA ATA CGG CGA AGG ATT AGA; L-78, TAY ATG CCA CAT AAT ACA TTA CAC TA; H-127, CAT GCA CTG GTA TGT GTC TCT TG; L-143, ATC AAT GCA AGA GAC ACA TAC CAG; H-230, CCG TAT ATT TTG AGG GAG TWG T; L-275, GCA GTG CCT TAG AAC AAA CTA TGA; L-341, GTC TCT CGG ACC AGG TTA TTT ATT; H-393, GAA AGA ATG GTC CTG AAG CTA GTA A; H-Phe, TGC TTT GTG GGT TAA GCT A. LND-6 and H-Phe provided by T. Parsons.

(W = A or T, Y = C or T)

Results

The complete control region of Whooping Cranes is 1125 bp. Comparing sequences among the crane species sequenced revealed substantial variation. As expected, the 5′ third and 3′ third contained substantially more variation than the middle third. The most interspecific variation occurred in the first third of the control region, which included an indel of about 50 bp. Thus, we focused our efforts on the 314 bp section between positions 78 and 393 of the Whooping Crane mtDNA control region (Fig. 2; Glenn 1997; Genbank accessions AF114338-AF114377; Genbank numbers for the complete mtDNA sequences were assigned as follows: Siberian Crane [*Grus* (formerly *Bugeranus*) *Bugeranus leucogeranus*], AF112371; Blue or Stanley Crane [*Anthropoides paradisea*], AF112372; and Whooping Crane, AF112373).

Degradation of Museum DNA

Among museum specimens with precisely known ages, 32 of 35 specimens <80 years old yielded amplicons B or I (249 or 314 bp; Fig. 2), whereas only one of 58 specimens >80 years old yielded amplicons of these lengths (Fig. 3). Thus, there was a substantial decrease in PCR amplifiability of long DNA fragments from specimens of increasing age.

We tested several possible explanations for the decrease in long amplicons. First, tests for inhibitory effects of the extracts on PCR demonstrated that only 14 of 142 museum skin or muscle extractions showed substantial PCR inhibition. Only 5 of the 14 completely inhibited PCR under the tested conditions; of these, only 1 was >80 years old. Thus, increasing inhibition among older samples was not responsible for the observed correlation.

We then chose for further study a representative subsample of 10 museum specimens spanning the age of the specimens investigated (31–140 years old). Four DNA extraction protocols were tested on skin samples from these birds. No significant correlation of PCR amplification and DNA extraction method was found (Glenn 1997). Our standard method (digestion with collagenase and $CaCl_2$ in the buffer) did, however, give the highest proportion of extracts supporting synthesis of small fragments (amplicons A). We also tested two additional types of tissue, foot pad and bone, which have been reported to be good sources of ancient and museum DNA (Cooper et al. 1992; Mundy et al. 1996). Again, we found no significant difference in the recovery of amplifiable DNA (Glenn 1997). Replacement of dUTP with TTP in our PCR protocol also had no effect (Glenn 1997).

Finally, we tested the efficiency of primer pairs. Although the primer pairs differed in PCR efficiency, no consistent trend with amplicon size was found. Dilution series tests with Whooping Crane genomic DNA derived from frozen tissue indicated that the amount of template needed for consistent amplification varied from 5 pg (amplicons A, B, and C) to 50 pg (amplicons I and K) to 500 pg (amplicons J and L).

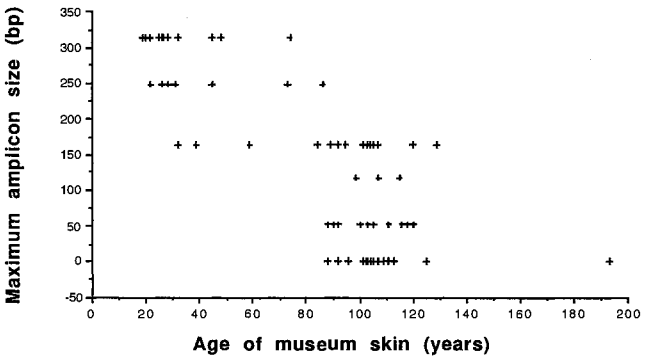

Figure 3. Maximum amplicon size versus age for Whooping Crane museum specimens. Only DNA extractions derived from skins with no PCR inhibition are shown. The 164 bp amplicon derives from primers L438 and H603 (Glenn 1997). A best-fit simple linear decay curve of $y = 3.625 - 2.716x$ *yields* $r^2 = 0.715$.

Effects on Haplotype and Nucleotide Diversity

We identified three haplotypes, 1, 2, and 3, with four polymorphic sites in the 314 bp region sequenced in the sample of 17 postbottleneck individuals, which represent all surviving lineages (Table 2). One additional haplotype that is now extinct, 4, was discovered among seven museum specimens salvaged during 1948–1965. Only 10 of the 113 prebottleneck museum specimens yielded data for the entire 314 bp of sequence (Table 2). There were five haplotypes, 3 and 5–8, with four polymorphic sites among these 10 prebottleneck samples (Table 2). One of two additional prebottleneck samples that yielded 270 bp of sequence represented another unique haplotype, 9. If we assume that all postbottleneck haplotypes were present in the prebottleneck population (although some were undetected in the prebottleneck sample), then there were at least nine prebottleneck haplotypes with eight polymorphic sites (Table 2). Thus, it is clear that the number of Whooping Crane haplotypes has decreased dramatically, dropping from at least nine before the bottleneck to three in the surviving population.

In contrast, the bottleneck has had little effect on the nucleotide diversity of Whooping Crane mtDNA. We examined two standard measures of average nucleotide diversity: $\hat{\pi}$, the average number of pairwise nucleotide differences among haplotypes per bp (Nei 1987), and $\hat{\theta}$, which is based on the observed number of segregating sites (Watterson 1975; Table 2). Within the prebottleneck sample, $\hat{\pi}$ is nearly equal to $\hat{\theta}$. Thus, Tajima's test statistic (*D*), the standardized difference between $\hat{\pi}$ and $\hat{\theta}$ (Tajima 1989*a*), is close to zero.

Table 2. Mitochondrial DNA haplotypes detected among Whooping Crane samples.

Haplotype	Variable sites 1122223 90701374 40703608	Prebottleneck frequency[a]	Postbottleneck frequency[a]
1	TCTGATCC[b]	0	12 (61)
2	CCCAATCC[b]	0[c] [0.5][c]	2 (14)
3	TCCAATCT	5	3 (21)
4	CTCAGTTC	0	0[d] [1][d]
5	TCCAATTT	1	0
6	CCCAACCC[b]	1[c] [1, 5][c]	0
7	CCCAATTT	2	0
8	CCCAATCT	1	0
9	CTCAG--T	0[e] [1][e]	0
Total		10 [12][f]	17 (96)
Variable sites		4 [6][f]	4 (4)
$\hat{\theta}$		0.0045	0.0038
$\hat{\pi}$		0.0045	0.0045
Tajima's *D*		−0.0379	0.5913

[a] *Numbers in parentheses are living captives that descend from composite nesting areas with offspring of known haplotypes.*
[b] *Position 348 was unambiguously T for both strands of haplotypes 3, 5, 7, 8, and 9. For haplotypes 1, 2, 4, and 6, this site usually gave a stronger C peak on the light strand and a stronger T peak on the heavy strand. This pattern was consistent; only three individuals yielded a stronger C peak for both strands.*
[c] *An additional partial sequence from AMNH 354211 that could be haplotype 2 or 6. Excluded from calculations of* $\hat{\pi}$, $\hat{\theta}$, *and* D.
[d] *One individual from museum sample USNM 420012 (see Table 1). Excluded from calculations of* $\hat{\pi}$, $\hat{\theta}$, *and* D *because it was only detected immediately following the bottleneck, in 1951, and does not survive today.*
[e] *One individual from JFBM 17948. Excluded from calculations of* $\hat{\pi}$, $\hat{\theta}$, *and* D *because only 270 bp were amplified and sequenced.*
[f] *Total of 12 individuals with 6 variable sites if partial sequences (c and e above) are included.*

Discussion

Our discovery of mtDNA variation is in contrast to the report of Snowbank and Krajewski (1995), who found no variation among Whooping Crane mtDNA they examined. Although we sequenced only a portion of the region they surveyed with restriction enzymes, we discovered variation because sequencing is much more powerful than restriction-fragment analysis of most PCR products. In retrospect, haplotype 1 can be distinguished from haplotypes 2 and 3 with a restriction enzyme (e.g., Sma I) that was not used by Snowbank and Krajewski. No restriction enzymes, however, exist to distinguish haplotypes 2 and 3.

Degradation of Museum DNA

The decrease of maximum PCR amplicon size with age and the lack of inhibition by the older extracts suggests that DNA is decaying with time in museum specimens or changing in a way that limits its utility as a template for PCR. We recovered amplifiable DNA genuinely deriving from Whooping Crane skins much less frequently than might have been expected from previous studies of museum specimens (Thomas et al. 1990; Ellegren 1991; Hagelberg et al. 1991; Cooper et al. 1992; Taylor et al. 1994). Previous studies, however, have examined few specimens older than 80 years (n = 0–5 in Thomas et al. 1990; Ellegren 1991; Hagelberg et al. 1991; Cooper et al. 1992; Taylor et al. 1994) and have often utilized shorter amplicons, which increases the chance of successful amplification. Because of the specific sequence of the region studied, primers used for amplicons of about 100 bp were either inefficient for PCR or amplified regions with no variation. Thus, the variable positions in the region under study here could be amplified only rarely in amplicons of smaller size. Although it is possible to obtain more template by simply using more tissue for DNA extraction and by refining the PCR conditions to amplify from less template, these strategies increase the potential for contamination to affect results and increase the damage to valuable, often irreplaceable, museum specimens. Our results, therefore, suggest that traditional museum preservation and storage techniques yield specimens that serve as viable genetic warehouses (Pääbo et al. 1989; Diamond 1990; Graves & Braun 1992) for only a limited time.

Apparent Lack of Population Structure

Full interpretation of our results requires knowledge about the structure of the prebottleneck Whooping Crane population(s). For instance, the loss of haplotypes could derive entirely from the loss of other populations (i.e., the WBNP population has the same diversity as it did before the bottleneck). Unfortunately, the small size of our prebottleneck sample precludes statistical inferences about population structure of the prebottleneck Whooping Crane population. The USNM 007333 is the only prebottleneck Whooping Crane clearly from the WBNP area. Seven other birds, those from South Dakota, Nebraska, Kansas, and Texas, are likely to derive from subpopulations that at least came into contact with birds from WBNP. Because USNM 007333 and USNM 420012 have haplotypes that are now extinct, it is clear that the WBNP/ANWR population lost haplotypes.

Evidence against strong population subdivision comes from two lineages from the nonmigratory Louisiana population that were also sequenced for the entire 314 bp of amplicon I. Both of these birds shared the most common prebottleneck haplotype with migratory Whooping Cranes, haplotype 3 (Table 1). In addition, all three haplotypes in the modern sample occurred in both current breeding areas of WBNP, Sass and Klewi, indicating that there is no microgeographic structuring of the extant natural population. We therefore assume, for the purpose of analysis, that all Whooping Cranes derive from one large, panmictic population, recognizing the influ-

ence of this assumption on the validity of our conclusions.

Effects on Haplotype and Nucleotide Diversity

Tajima's D is often used to test for deviations from selective neutrality, and the low D value obtained is consistent with the hypothesis that the prebottleneck Whooping Crane population was in an approximate neutral mutation-random drift equilibrium. There are, however, two caveats. First, the estimation of $\hat{\pi}$ and $\hat{\theta}$ is based on a mutation model that assumes that multiple substitutions do not occur at the same nucleotide site, an assumption that appears to be invalidated by our data. Second, the estimation of $\hat{\pi}$ and $\hat{\theta}$ from the postbottleneck sample is based on a constructed sample rather than a random sample. Within the postbottleneck sample, $\hat{\pi}$ exceeds $\hat{\theta}$; thus, D is positive due to the loss of rare alleles, as expected. The D value, however, is not significantly different from zero (Tajima 1989*a*). The sampling scheme should not significantly bias the estimation of these parameters because the effective sizes of the CNAs do not vary.

The contrasting patterns of haplotype and nucleotide diversity are consistent with a recent reduction in population size and subsequent evolution of the Whooping Crane population by random genetic drift. The difference between these two measures occurs because haplotype diversity (i.e., the number of haplotypes in the population) is strongly affected by the elimination of many rare haplotypes, which happens almost instantaneously after the reduction of population size, whereas nucleotide diversity reflects the less dramatic change in the *average* differences among the haplotypes (Nei et al. 1975; Maruyama & Fuerst 1985). As an additional consequence of losing rare haplotypes, $\hat{\theta}$, which is estimated from the number of segregating sites in the sample, is expected to decline more rapidly than $\hat{\pi}$, which is estimated as the *average* number of pairwise differences per nucleotide site between all pairs of haplotypes (Tajima 1989*b*). Our data are consistent with these theoretical expectations.

Significant Shifts in Haplotype Frequencies

Although our results did not indicate significant differences in nucleotide diversity between pre- and postbottleneck populations, a direct comparison of the frequencies of individual haplotypes showed a significant effect. Haplotype frequencies in the prebottleneck population were estimated from the prebottleneck sample (Table 2). The frequency of Whooping Crane haplotypes among surviving lineages was estimated both by sampling one chick from each founding CNA and by counting the total number of captive individuals believed to descend from each founding CNA. These estimates are in good agreement (Table 2). An exact test was performed on the 4×2 contingency table of the number of individuals observed for the pre- and postbottleneck samples in four haplotype categories (haplotypes 1, 2, 3, and 5-8; Table 2). The pre- and postbottleneck frequencies were significantly different ($p < 0.0001$).

The most dramatic frequency shift occurred for haplotype 1. This haplotype was not observed in the prebottleneck population but was found in 12 of 17 (71%) founding maternal lineages of the extant population. To test the hypothesis that this frequency shift was due to random drift associated with bottlenecks, we simulated a Wright-Fisher model (Ewens 1979) for a simple bottleneck scenario with parameters similar to the observed Whooping Crane bottleneck. Bottlenecks of six generations were simulated. Female population sizes of 35, 18, 7, 10, 13, and 23 were assumed in generations 1-6 respectively. These values are half the observed census size of all migratory Whooping Cranes, beginning in 1916 and resampling every 11 years (approximately the Whooping Crane generation time; Allen 1952; Doughty 1990). All haplotypes were lumped together, except the rare ones. The initial frequency of the rare haplotype was assumed to be 0.1 because, although haplotype 1 was undetected in the prebottleneck sample, a conservative estimate of its frequency is the inverse of the sample size (i.e., the same frequency as the singleton class of haplotypes in the prebottleneck sample). Haplotypes in each generation were selected at random, with replacement, from the previous generation.

The proportion of bottlenecks with frequency shifts from 0.1 to 0.71 or greater was estimated from 100,000 simulated bottlenecks. Two percent of the simulations from this model produced a frequency shift for the rare haplotype at least as great as the observed frequency shift for haplotype 1. All reasonable changes to the parameters of the simulations gave similar results. Because our survey indicates there were several rare haplotypes with frequencies around 0.1 in the prebottleneck population (Table 2) and because the probability that any rare haplotype might experience such a frequency shift is the sum of probabilities for all such haplotypes, the simulations suggest that the frequency shift of haplotype 1 is consistent with a simple bottleneck effect (i.e., a reduction in population size and genetic drift). For a more complicated model with overlapping generations, which is more appropriate for Whooping Cranes, the probability value resulting from simulation is expected to be higher because the effective population size is smaller when generations overlap (Hill 1972).

Heterogeneity of Nucleotide Substitution Rate

It would be desirable to estimate parameters such as the prebottleneck effective population size from our data, but they do not conform to the assumptions of the models used for such estimations, especially the assumption

of single substitutions per site. Our data are consistent with the high level of among-site rate variation that seems to characterize the evolution of mtDNA control-region sequences (Wakeley 1993). For example, the minimum spanning network of the haplotypes (Fig. 4) illustrates that multiple shortest paths exist among haplotypes, indicating multiple substitutions at three sites. Also although only 8 of the 314 sites varied, a parsimony reconstruction of the relationships among the nine haplotypes revealed 25 most parsimonious trees of length 11 (i.e., a minimum of 11 substitutions), again suggesting that several sites were multiply substituted.

The small number of variable sites prohibited the use of maximum likelihood to evaluate models of rate heterogeneity, but the parsimony-inferred distribution of substitutions per site allowed a gamma-distributed rates model to be tested (Sullivan et al. 1995). If evolutionary rates are gamma-distributed, the distribution of substitutions per site will follow a negative binomial, whereas uniform rates will produce a Poisson distribution. The Whooping Crane data fit a gamma-distributed rates model (α = 0.044) significantly better than a uniform rates model as judged by a likelihood ratio test (df = 1, $\chi^2 = 11.93$, $p < 0.001$, Sullivan et al. 1995). This method is expected to underestimate the amount of rate heterogeneity among sites (Wakeley 1993), yet the estimated gamma shape parameter (α = 0.044) indicates a level of rate heterogeneity among the most extreme ever reported.

Management Implications

Our data allow a direct empirical comparison of genetic variation before and after critical endangerment of a long-lived vertebrate. The recent bottleneck has had a

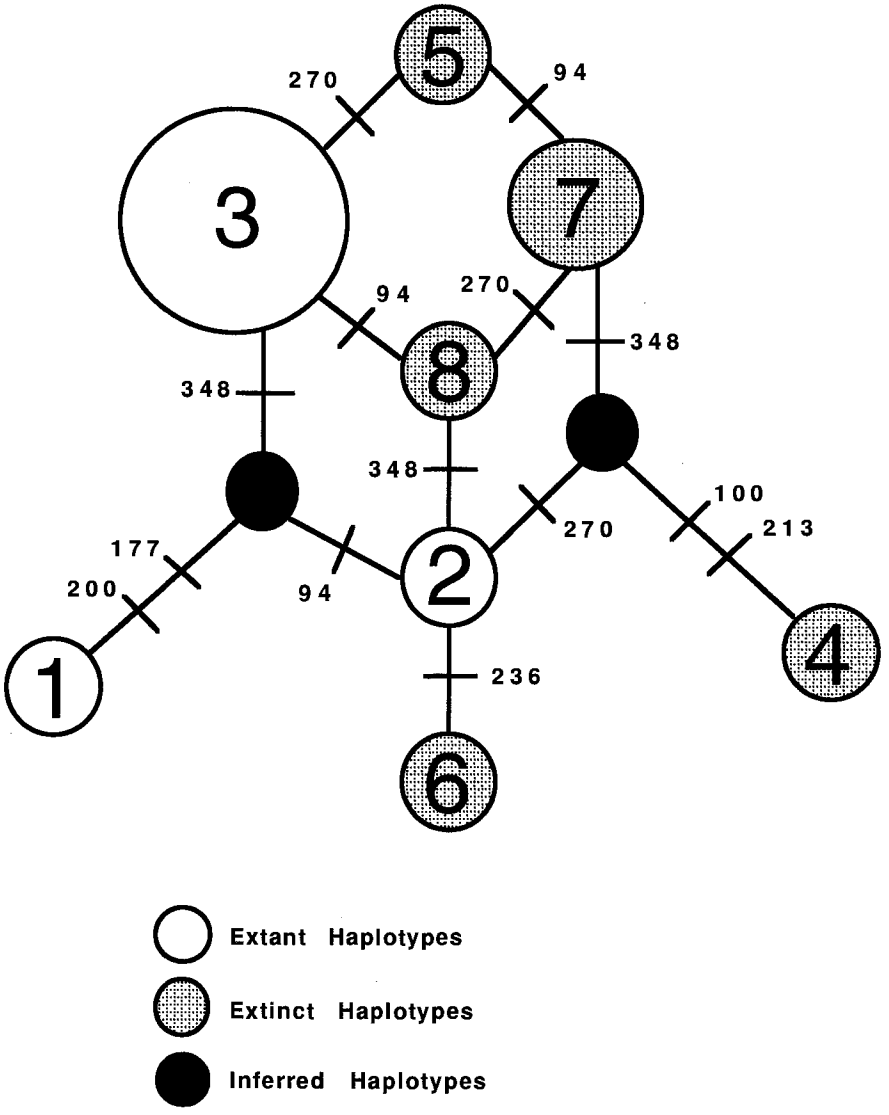

Figure 4. Median network (Bandelt et al. 1995) of Whooping Crane haplotypes. Haplotypes are connected by line segments proportional to the number of substitutions between haplotypes. Positions of inferred substitutions are indicated as slashes through the line segments. Haplotypes are drawn proportionately to their frequency in the prebottleneck sample. Haplotypes not detected in the prebottleneck sample but observed in post-bottleneck birds (1, 2, and 4) are drawn to the same scale as singly detected haplotypes. Haplotype 9 should be near haplotype 4 but cannot be unambiguously connected to the network.

dramatic effect on the number and frequency of mtDNA haplotypes in the modern Whooping Crane population. Significant efforts, however, are now being made to preserve as much genetic diversity in this species as possible, and a multi-stage decision matrix is in place for making captive breeding decisions (Mirande et al. 1991). The discovery of variation in the mtDNA of modern Whooping Cranes allows the maintenance of mtDNA diversity to be added to this matrix. More important, this locus is being used to add significant information to the studbook data of Whooping Cranes so that all individuals can now be assigned to known maternal lineages. The discovery of shared haplotypes among the prebottleneck resident Louisiana population and the prebottleneck migratory population supports the idea that these subpopulations exchanged genes, and it supports current attempts to recreate a nonmigratory population. The Whooping Crane represents a classic case of species endangerment due to human activities. Its slow but steady recovery stands as a tribute to the long-term commitment of individuals, government agencies, and private organizations to bring this bird back from the brink of extinction (Cannon 1996).

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Literature Cited

Allen, R. P. 1952. The Whooping Crane. Research report 3. National Audubon Society, New York.

Allen, R. P. 1956. A report on the Whooping Crane's northern breeding grounds. A supplement to research report 3. National Audubon Society, New York.

Applied Biosystems. 1994. 373 DNA sequencing system user's manual. Part number 903204, revision. A. Perkin Elmer, Foster City, California.

Arden, S. L., D. M. Lambert, A. G. Rodrigo, and I. G. McLean. 1997. The effects of population bottlenecks on multilocus DNA variation in robins. Journal of Heredity **88:**179–186.

Avise, J. C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Bandelt, H-J., P. Forster, B. C. Sykes, and M. B. Richards. 1995. Mitochondrial portraits of human populations using median networks. Genetics **141:**743–753.

Bonnell, M. L., and R. K. Selander. 1974. Elephant seals: genetic variation and near extinction. Science **184:**908–910.

Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology **28:**495–503.

Briscoe, D. A., J. M. Malpica, A. Robertson, G. J. Smith, R. Frankham, R. G. Banks, and J. S. F. Barker. 1992. Rapid loss of genetic variation in large captive populations of *Drosophila* flies: implications for the genetic management of captive populations. Conservation Biology **6:**416–425.

Cannon, J. R. 1996. Whooping Crane recovery: a case study in public and private cooperation in the conservation of endangered species. Conservation Biology **10:**813–821.

Carter, M. J., and I. D. Milton. 1993. An inexpensive and simple method for DNA purifications on silica particles. Nucleic Acids Research **21:**1044.

Cooper, A., C. Mourer-Chauvire, G. K. Chambers, A. von Haeseler, A. C. Wilson, and S. Pääbo. 1992. Independent origins of New Zealand moas and kiwis. Proceedings of the National Academy of Sciences, USA **89:**8741–8744.

Densmore, L. D., III, and P. S. White. 1991. The systematics and evolution of the Crocodylia as suggested by restriction endonuclease analysis of mitochondrial and nuclear ribosomal DNA. Copeia **1991:**602–615.

Dessauer, H. C., G. F. Gee, and J. S. Rogers. 1992. Allozyme evidence for crane systematics and polymorphisms within populations of Sandhill, Sarus, Siberian, and Whooping Cranes. Molecular Phylogenetics and Evolution **1:**279–288.

Diamond, J. M. 1990. Old dead rats are valuable. Nature **347:**334–335.

Doughty, R. W. 1990. Return of the Whooping Crane. University of Texas Press, Austin.

Ellegren, H. 1991. DNA typing of museum birds. Nature **354:**113.

Ewens, W. J. 1979. Mathematical population genetics. Springer, Berlin.

Glenn, T. C. 1997. Genetic bottlenecks in long-lived vertebrates: mitochondrial and microsatellite DNA variation in American alligators and Whooping Cranes. Ph.D. dissertation. University of Maryland, College Park.

Glenn, T. C., and S. J. Glenn. 1994. Rapid elution of DNA from agarose gels using polyester plug spin inserts (PEPSIs). Trends in Genetics **10:**344.

Glenn, T. C., R. S. Ojerio, W. Stephan, and M. J. Braun. 1997. Microsatellite DNA loci for genetic studies of cranes. Pages 36–45 in R. P. Urbanek and D. W. Stahlecker, editors. Proceedings of the 7th North American Crane workshop. North American Crane Working Group, Grand Island, Nebraska.

Graves, G. R., and M. J. Braun. 1992. Museums: storehouses of DNA? Science **255:**1335–1336.

Hagelberg, E., I. C. Gray, and A. J. Jeffreys. 1991. Identification of the skeletal remains of a murder victim by DNA analysis. Nature **352:** 427–429.

Hahn, P. 1954. Where is that vanished bird? Royal Ontario Museum, University of Toronto, Ontario.

Hedrick, P. W., R. C. Lacy, F. W. Allendorf, and M. E. Soulé. 1996. Directions in conservation biology: comments on Caughley. Conservation Biology **10:**1312–1320.

Hill, W. G. 1972. Effective size of populations with overlapping generations. Theoretical Population Biology **3:**278–289.

Johnsgard, P. A. 1983. Cranes of the world. Indiana University Press, Bloomington.

Jones, K. L., and C. Mirande. 1997. The genealogy of the Whooping Crane (*Grus americana*). International Crane Foundation, Baraboo, Wisconsin.

Keller, L. F., P. Arcese, J. N. M. Smith, W. M. Hochachka, and C. C. Stearns. 1994. Selection against inbred song sparrows during a natural population bottleneck. Nature **372:**356–357.

Longmire, J. L., G. F. Gee, C. L. Hardekopf, and G. A. Mark. 1992. Establishing paternity in Whooping Cranes (*Grus americana*) by DNA analysis. Auk **109:**522–529.

Longo, M. C., M. S. Berninger, and J. L. Hartley. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene **93:**125–128.

Maruyama, T., and P. A. Fuerst. 1985. Population bottlenecks and nonequilibrium models in population genetics. II. Number of alleles in a small population that was formed by a recent bottleneck. Genetics **111:**675–689.

Mirande, C. R., R. Lacy, and U. Seal. 1991. Whooping Crane (*Grus americana*) conservation viability assessment. Captive Breeding Specialist Group, Apple Valley, Minnesota.

Mundy, N. I., C. S. Winchell, and D. S. Woodruff. 1996. Tandem repeats and heteroplasy in the mitochondrial DNA control region of the Loggerhead Shrike (*Lanius ludovicianus*). Journal of Heredity **87:**21–26.

Nei, M. 1987. Molecular evolutionary genetics. Equation 10.5. Columbia University Press, New York.

Nei, M., T. Maruyama, and R. Chakraborty. 1975. The bottleneck effect and genetic variability in populations. Evolution **29:**1–10.

Pääbo, S., J. A. Gifford, and A. C. Wilson. 1988. Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Research **16:** 9775–9787.

Pääbo, S., R. G. Higuchi, and A. C. Wilson. 1989. Ancient DNA and polymerase chain reaction: the emerging field of molecular archaeology. Journal of Biological Chemistry **264:**9709–9712.

Ralls, K., K. Brugger, and J. Ballou. 1979. Inbreeding and juvenile mortality in small populations of ungulates. Science **206:**1101–1103.

Sambrook J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Snowbank, S. A., and C. Krajewski. 1995. Lack of restriction-site variation in mitochondrial-DNA control region of Whooping Cranes (*Grus americana*). Auk **112:**1045–1049.

Soulé, M. E. editor. 1986. Conservation biology: the science of scarcity and diversity. Sinauer Associates, Sunderland, Massachusetts.

Stehn, T. 1997. 1996/97 winter Whooping Crane survey. Aransas National Wildlife Refuge, Austwell, Texas.

Sullivan, J., K. E. Holsinger, and C. Simon. 1995. Among-site rate variation and phylogenetic analysis of 12S rRNA in sigmodontine rodents. Molecular Biology and Evolution **12:**988–1001.

Swofford, D. 1997. PAUP* 4.0.0d55. Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, Massachusetts.

Tajima, F. 1989*a*. The effects of change in populations size on DNA polymorphism. Genetics **123:**597–601.

Tajima, F. 1989*b*. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics **123:**585–595.

Taylor, A. C., W. B. Sherwin, and R. K. Wayne. 1994. Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. Molecular Ecology **3:**277–290.

Taylor, A. C., A. Horsup, C. N. Johnson, P. Sunnucks, and B. Sherwin. 1997. Relatedness structure detected by microsatellite analysis and attempted pedigree reconstruction in an endangered marsupial, the northern hairy-nosed wombat, *Lasiorhinus krefftii*. Molecular Ecology **6:**9–19.

Thomas, R. H. 1994. Analysis of DNA from natural history museum collections. Pages 311–321 in B. Schierwater, B. Streit, G. P. Wagner, and R. DeSalle editors. Molecular ecology and evolution: approaches and applications.. Birkhauser Verlag, Basel, Switzerland.

Thomas, W. K., S. Pääbo, F. X. Villablanca, and A. C. Wilson. 1990. Spatial and temporal continuity of kangaroo rat populations shown by sequencing mitochondrial DNA from museum specimens. Journal of Molecular Evolution **31:**101–112.

Wakeley, J. 1993. Substitution rate variation among sites in hypervariable region 1 of human mitochondrial DNA. Journal of Molecular Evolution **37:**613–623.

Watterson, G. A. 1975. On the number of segregating sites in genetical models without recombination. Theoretical Population Biology **7:** 256–276.

Wright, S. 1938. Size of population and breeding structure in relation to evolution. Science **87:**430–431.