MITOCHONDRIAL DNA VARIATION AMONG WINTERING MIDCONTINENT GULF COAST SANDHILL CRANES

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Abstract: Based on morphological characteristics, 3 subspecies of sandhill cranes (*Grus canadensis*)—the lesser sandhill crane (*G. c. canadensis*), Canadian sandhill crane (*G. c. rowani*), and greater sandhill crane (*G. c. tabida*)—occur within the midcontinent population (MCP) and winter along the Gulf Coast of Texas, USA. Of these subspecies, the greater sandhill crane is the least abundant and the most restricted in distribution in the midcontinent region, making it a subspecies of special concern. Twenty individuals from each subspecies with the highest posterior probabilities of membership in subspecies associated with their morphological discriminant score (i.e., those individuals that were least likely to be misclassified based on the morphological model currently used to assign wintering individuals to subspecies) were chosen from a total sample of 220 birds. We amplified and sequenced a 437-base-pair segment from domain I of the mitochondrial DNA (mtDNA) control region for these 60 birds. Analyses of the resulting 41 different mtDNA haplotypes indicate that birds classified as Canadian and greater sandhill cranes based on their morphology do not differ genetically, but lesser sandhill cranes are genetically distinct from both Canadian and greater sandhill cranes. When Canadian and greater sandhill cranes are grouped together, 55 of the 60 individuals sequenced are consistently classified using morphology and mtDNA. We then used the DNA sequences to develop a simple restriction enzyme assay of this mtDNA segment to survey an additional 160 specimens. These results indicate that (1) mtDNA assays provide reliable discrimination of migratory sandhill cranes, (2) the current subspecific designations of sandhill cranes may not accurately reflect genetic structure in this species, (3) concern for preserving the genetic diversity of sandhill cranes in the midcontinent region may not need to focus on management of greater sandhill cranes wintering in the sampled populations, and (4) studies of individuals from the breeding grounds are clearly needed to determine whether Canadian and greater sandhill cranes are indeed distinct subspecies.

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Sandhill cranes are widely distributed throughout North America, with 6 subspecies generally recognized (Johnsgard 1983, Tacha et al. 1994). Three subspecies—the Mississippi (*G. c. pulla*), Florida (*G. c. pratensis*), and Cuban sandhill cranes (*G. c. nesiotes*)—are relatively rare, nonmigratory, and endemic to the southeastern United States or Cuba. The other 3 subspecies (lesser [*G. c. canadensis*], Canadian [*G. c. rowani*], and greater [*G. c. tabida*]) constitute the largest migratory populations. These populations breed from the northern United States through Canada and Alaska to Siberia and winter from the southern United States through central Mexico (Lewis 1977, Johnsgard 1983). *Grus c. canadensis* is, on average, the smallest migratory subspecies and has the most northerly breeding grounds, whereas *G. c. tabida* is the largest subspecies and has the most southerly breeding grounds (Johnsgard 1983). *Grus c. rowani* is a more recently described subspecies (Walkinshaw 1965) that is intermediate in its breeding range and size compared with *G. c. tabida* and *G. c. canadensis*. Disagreement exists over the validity of *G. c. rowani* (Stephen et al. 1966, Johnson and Stewart 1973, Walkinshaw 1973) because of overlap in body measurements with both *G. c. tabida* and *G. c. canadensis* (Walkinshaw 1949:6–20, 1965; Aldrich 1972; Tacha et al. 1985). Thus, it is difficult to identify the subspecies of some individuals or to know if all 3 subspecies are truly distinct biological entities (Stephen et al. 1966, Miller et al. 1972:11–14, Lewis 1977, Tacha et al. 1994).

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The midcontinent population of sandhill cranes is the largest crane population in the world with more than 500,000 individuals (Tacha et al. 1984, Johnson and Kendall 1997). Because the breeding grounds generally are unknown for wintering individuals, we use the common names for the subspecies when describing information relating to wintering individuals. The MCP includes all 3 migratory subspecies (Braun et al. 1975, Tacha et al. 1994), but the abundance of each subspecies varies widely (Johnson and Kendall 1997). For management purposes, the MCP is divided into 2 wintering subpopulations: (1) western—generally western Texas, composed mostly of lesser sandhill cranes; and (2) Gulf Coast—generally southeastern Texas, composed mostly of Canadian and greater sandhill cranes (Tacha et al. 1986, Ballard et al. 1999). Although the western and Gulf Coast subpopulations are defined by their winter range, individuals from these subpopulations also exhibit east–west fidelity throughout migration (Tacha et al. 1984). The greater sandhill crane is believed to have the fewest individuals with the most restricted distribution in the MCP (Kendall et al. 1997), making it a subspecies of special concern to management agencies.

Effective conservation of the greater sandhill crane subspecies will require accurate delineation of the subspecies. Subspecific recognition is especially important when particular subspecies are threatened, and estimates of subspecific harvest rates within hunted species are needed. Historically, morphological comparison has been the standard procedure used by taxonomists for recognition of bird species and subspecies generally (Ridgway and Friedmann 1941) and crane species specifically (Walkinshaw 1949, 1965; Tacha 1981). Previous research to determine subspecific composition of wintering sandhill crane populations (Guthery and Lewis 1979, Tacha et al. 1986, Ballard et al. 1999) has relied on discriminant function models developed from morphological measurements on a small sample (n = 16) of breeding birds of known sex and subspecies.

Morphological analyses can suffer from several problems such as observer bias, repeatability, and sex-specific differences (Moser and Rolley 1990, Lougheed et al. 1991, Thompson et al. 1999). A variety of genetic techniques have been developed to determine more accurately relationships among groups of organisms (Hillis et al. 1996). Procedures commonly used to distinguish between species and subspecies include protein electrophoresis (e.g., Gaines and Warren 1984, Dessauer et al. 1992); restriction fragment analysis or sequencing of mitochondrial DNA (mtDNA; e.g., Krajewski and Fetzner 1994, Krajewski and Wood 1995); and microsatellite DNA analysis (e.g., Scribner and Bowman 1998). Dessauer et al. (1992) failed to identify any fixed protein differences among the sandhill crane subspecies they sampled. An anonymous protein from the pancreas was identified as fixed between *G. c. canadensis* and *G. c. rowani* (Gaines and Warren 1984). Unfortunately, only 5 cranes were analyzed from 1 (*G. c. canadensis*) population in Alaska; thus, many low-frequency proteins would not have been detected. Mitochondrial DNA and microsatellite DNA loci (Glenn et al. 1997) have not yet been investigated among sandhill crane subspecies.

Additional genetic studies of sandhill crane subspecies clearly are needed (Tacha et al. 1994). We chose to investigate genetic variation of Gulf Coast sandhill cranes using mtDNA sequencing because this method (1) offers a simple and straightforward genetic technique to discriminate among vertebrate subspecies (reviewed in Avise 1994), and (2) it has been used in similar studies (e.g., Zink et al. 2000). Our goals were to determine (1) the level of genetic variation within this subpopulation; (2) how the variation is structured (i.e., how many genetic groups of sandhill cranes winter in this subpopulation); (3) whether the mtDNA groups are congruent with morphologically defined groups; (4) the conditions necessary for a simple, rapid, and objective method to identify the major mtDNA groups; and (5) how the variation discovered may pertain to sandhill crane subspecies.

METHODS

Sample Collection

We collected samples from 220 wintering sandhill cranes from the Gulf Coast (n = 159), South Texas Plains (n = 26), and Rolling Plains (n = 35) regions of Texas from December 1996 through January 1997 (Fig. 1; see Ballard et al. 1999 for additional details). Morphological measures and samples of heart and liver tissues and a remigial feather were taken from the specimens. All tissue samples were acquired using sterile techniques to prevent cross contamination, frozen immediately, and stored at –20 °C until used for genetic analysis. Breeding location and consequent subspecies are not known for these individuals.

Morphology

Sandhill cranes were sexed by gonadal examination. We measured (mm) culmen post-nares (from tip of bill to posterior of nostril), wing chord (unflattened wing from tip of longest primary to anterior edge of joint connecting carpometacarpus and ulna/radius), and tarsus (from posterior edge of tibio-tarsus joint to anterior edge of tarsal-metatarsal joint) for each after-hatch-year (AHY) crane collected. The discriminant models used to classify AHY cranes to subspecies originated from Johnson and Stewart (1973).

DNA Isolation and Amplification

We isolated genomic DNA from muscle samples by digesting 0.1 g in 900 µL of standard proteinase K digestion buffer (Sambrook et al. 1989) followed by a guanidine thiocyanate with diatomaceous earth extraction protocol modified from Carter and Milton (1993; modified protocol available at http://gator.biol.sc.edu/). We obtained polymerase chain reaction (PCR) primers used to amplify mtDNA specifically (Table 1) from Glenn (1997), Glenn et al. (1999), and M. Fain, Southern Illinois University (personal communication).

We amplified the entire control region for 14 individuals using the primers L16598 and HUniv12S. Amplification was carried out in 50 µL volumes with final reaction concentrations of: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 150 µM of each dNTP, 250 µg/mL BSA (Fraction V; Sigma, St. Louis, Missouri, USA), 0.25 µM of each primer, 1 unit AmpliTaq Gold DNA polymerase (PerkinElmer Applied Biosystems, Foster City, California, USA), and 40 ng of DNA. Thermocycling parameters were initial denaturing at 94 °C for 10 min, followed by 30 cycles of 94 °C for 20 sec, 50 °C for 20 sec, and 72 °C for 2 min. Five µL of the resulting PCR reactions were examined for amplification products by electrophoresis for about 1 hr through 1.5% agarose gels containing ethidium bromide and 1× Tris-borate-EDTA (TBE) buffer (Sambrook et al. 1989:6.7–6.13).

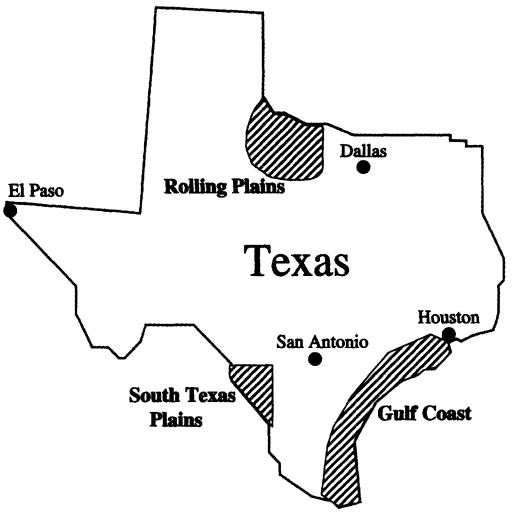

Fig. 1. Collection sites of sandhill cranes wintering in Texas, USA.

Table 1. Oligonucleotides used for PCR amplification and sequencing of sandhill crane mtDNA. Oligonucleotides within the control region are named according to the 3′ base relative to the first base of the whooping crane (*Grus americana*) control region (Glenn 1997, Glenn et al. 1999).

Name	Sequence (5′ to 3′)	Source
L16598[a]	CACCCACACCCTACAACAG	M. Fain
L–17	CCCGAAAAGCCGCTGTTGTA	This study
H393	GAAAGAATGGTCCTGAAGCTAGTAA	Glenn (1997)
L438	CCCCCTACACCCCTAGCACAAC	Glenn (1997)
H454[b]	GCCCTGACCGAGGAACCAGA	Quinn and Wilson (1993)
L594	GCACTTTTGGTTCCCTTTTTTTT	Glenn (1997)
H728	AACTCTTGAGGGCGACGAAC	This study
H833[c]	TGTTAAGAAAGTYAGAGGAAGTGTA	Glenn (1997)
H1026	TTTTGTTTATGTTGGTGTTTTGTTGT	Glenn (1997)
H1298	TAGGGTCCGAGGGCATTTAC	This study
HUniv12S	AGGCATAGTGGGGTATCTAATC	This study

[a] Position not determined relative to whooping crane control region, primer in ND6 (M. Fain, Southern Illinois University, personal communication).
[b] Position 521 relative to the Quinn and Wilson (1993) alignment of chicken and goose mtDNA.
[c] Y = C or T.

We amplified a 437 base pair (bp) section of the control region for all 220 samples using primers L-17 and H393. Amplification was carried out in 50 µL volumes with final reaction concentrations 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, 0.25 µM of each primer, 1 unit *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA), and 40 ng of DNA. Thermocycling parameters were initial denaturing at 94 °C for 3 min, followed by 25–30 cycles of 94 °C for 20 sec, 50 °C for 20 sec, and 72 °C for 30 sec. Initial denaturing was extended to 10 min for amplifications that produced too little product on the first attempt. Five µL of the resulting PCR reactions were examined for amplicons as described above.

DNA Sequencing and Restriction Digests

Sequences from both DNA strands were determined directly from PCR products using BigDye terminator chemistry and an ABI 377XL automated sequencer (Applied Biosystems 1998). All cycle sequencing reactions were carried out in 10 µL volumes with 0.32 µM primer, 50 or 100 ng of PCR product, and BigDye Terminators using ABI specifications except that the terminator mix was diluted 1:1 with halfBD™ (GENPAK, Stony Brook, New York, USA). For the complete control region, the PCR amplicons were purified by polyethylene glycol precipitation (protocol available at http://gator.biol.sc.edu/) and 100 ng was used for sequencing. For the short amplicons, 0.5 µL (approx. 50 ng) of unpurified PCR product was used as template.

Unpurified PCR products were digested with Hae III (Promega, Madison, Wisconsin, USA) restriction enzyme according to the supplier's specifications. Five µL of the digested PCR products were examined after electrophoresis for about 1 hr in 1× TBE buffer in 2% agarose gels containing ethidium bromide. Four µL of 100 bp Ladder (New England Biolabs, Beverly, Massachusetts, USA) was used as a size standard. We examined gels under ultraviolet transillumination and photographed them using an AlphaImager™ (Alpha Innotech, San Leandro, California, USA).

Analyses and Quality Assurance

We imported sequence chromatograms into Sequencher 3.1 (Gene Codes, Ann Arbor, Michigan, USA), where we edited and assembled them into contigs, assuring identical results between complementary strands. Consensus sequences corresponding to the light strand were exported as text files for each individual. We imported the sandhill crane text files plus a Siberian crane (*Bugeranus* [= *Grus*] *leucogeranus*; Genbank accession AF112371) sequence into Sequence Navigator 1.0.1 (PerkinElmer Applied Biosystems, Foster City, California, USA) and aligned them using the Clustal algorithm. We compared sequences with previous results (e.g., Glenn 1997) to assure mitochondrial origin of PCR products.

We used the software application PAUP* 4.0b2a (Swofford 1999) for all phylogenetic analyses. The Siberian crane was defined as the outgroup for all analyses. We investigated phylogenetic relationships among the haplotypes using distance, maximum likelihood, and parsimony analyses. Duplicate haplotypes were removed prior to phylogenetic analysis. We used ModelTest 3.04 (Posada and Crandall 1998) with an alpha of 0.01 to determine the most appropriate model of molecular evolution. Kimura 2-parameter (K2P; Kimura 1980) and Hasegawa-Kishino-Yano (HKY85; Hasegawa et al. 1985) models with among-site rate variation (using a 4-category discrete approximation of the gamma distribution with a shape parameter equal to 0.5) were used in analyses. Neighbor-joining trees were constructed from the resulting genetic distances, and 500 bootstrap replicates were done. For parsimony analysis, we used the heuristic search option with all characters treated as unordered, equally weighted, and gaps treated as a fifth base. We used the 12 sandhill crane haplotypes representing the longest branches and all of the supported groups from the distance and parsimony trees in maximum likelihood analyses.

Quality of sequence and restriction site information was assured by several methods. First, DNA analyses were initially done without prior knowledge of sample subspecies affiliation determined by morphological discrimination (i.e., the DNA tests were done blind). Second, all individuals used for more complete analysis were used again for less complete analysis (i.e., the 14 samples sequenced for the complete control region were included among the 60 sequenced for 437 bp, and all 60 samples sequenced were cut with Hae III). Third, following initial analysis, DNA from 39 samples were reextracted and analyzed independently (replicated), including all individuals with subspecies designations that did not match mtDNA grouping. Finally, all individuals with haplotypes displaying unusually long branch-lengths were resequenced.

RESULTS

Morphology

We used discriminant scores generated from morphological measurements to classify sandhill cranes (n = 215) into the 3 subspecies with the following results: 121 Canadian sandhill cranes, 54 greater sandhill cranes, and 40 lesser sandhill cranes. Subspecific composition of sandhill cranes was highly variable among the sampling locations (Table 2). Canadian sandhill cranes constituted most of the Gulf Coast and Rolling Plains samples, but represented only 23% of the South Texas Plains sample. Greater sandhill cranes constituted 34% of the Gulf Coast sample, but only 1 individual outside of the Gulf Coast region was designated as a greater sandhill crane by the disciminant model. Lesser sandhill cranes constituted most individuals collected in the South Texas Plains, but only 35% of individuals from the Rolling Plains and <6% of the Gulf Coast cranes were lesser sandhill cranes.

Table 2. Subspecific composition of sandhill cranes (*Grus canadensis*) collected from 3 regions of Texas, USA (Nov 1996–Jan 1997), based on morphological discrimination and mitochondrial DNA.

	Technique	
Subspecific designation[a]	Morphology	mtDNA
Gulf Coast (n = 155)[b]		
lesser	5.2%	5.8%
Canadian	60.6%	n/a
greater	34.2%	n/a
Canadian–greater	n/a	94.2%
South Texas Plains (n = 26)		
lesser	76.9%	73.1%
Canadian	23.1%	n/a
greater	0	n/a
Canadian–greater	n/a	26.9%
Rolling Plains (n = 34)		
lesser	35.3%	29.4%
Canadian	61.8%	n/a
greater	2.9%	n/a
Canadian–greater	n/a	70.6%

[a] Discriminant models based on morphological measurements (culmen post-nares, tarsus, and wing chord) of adult sandhill cranes of known sex and breeding origin (D. H. Johnson, U.S. Fish and Wildlife Service, unpublished data) were used to partition cranes into 3 subspecies. Mitochondrial DNA analysis distinguished 2 subspecies of sandhill cranes in this study.

[b] One crane was not distinguished into a unique group (i.e., lesser or Canadian–greater) based on mtDNA restriction enzyme analysis.

Complete Control Region Sequences

Initially, we amplified and sequenced the entire control region and tRNA-Glu, along with portions of ND6 and 12S, for a subset of 14 individuals with posterior probabilities of membership in subspecies from the morphological discriminant scores of >0.99. We deleted sequences from the ND6, tRNA-Glu, and 12S from comparisons because of limited polymorphism and/or difficulty in obtaining high quality sequence data from both strands. The entire control region, except for the first 31 bp that were excluded due to length polymorphisms, was used for comparisons.

Only 2 of the 14 individuals shared identical sequences. We observed a single length polymorphism (deletion) of 1 bp in 4 of the 14 sequences. All other polymorphisms observed were substitutions. As expected, the middle third of the control region (domain II, sensu Baker and Marshall 1997) was the least variable, with an average K2P genetic distance of 0.005 (SD = 0.004). Most of the highly conserved elements of the control region fall within domain II and have been described in detail previously (Glenn 1997, Hasegawa et al. 1999). The 5′ third of the control region (nearest ND6; domain I, sensu Baker and Marshall 1997) was highly polymorphic with an average K2P distance of 0.040 (SD = 0.032), which was more than twice as variable as the 3′ third (average = 0.017; SD = 0.013; domain III, sensu Baker and Marshall 1997). Domain I contains a major insertion–deletion among crane species and has been the focus of studies of other cranes (Glenn 1997, Glenn et al. 1999, Hasegawa et al. 1999). Thus, we concentrated our subsequent efforts on sequence information from domain I.

Sequencing Survey

A larger subset (n = 20 per subspecies) of the original samples with highest posterior probabilities of membership in subspecies (i.e., those that were most confidently assigned to subspecies based on morphology; >0.99 *G. c. canadensis*, >0.99 *G. c. rowani*, >0.93 *G. c. tabida*) from the morphological discriminant scores was subsequently amplified and sequenced (Genbank accessions: AF367871–AF367930). Among these 60 individuals, we discovered 41 different mtDNA haplotypes. HKY85 with unequal rates (i.e., HKY+G) was strongly supported as the best fit model of molecular evolution (JC, F81, and equal rates rejected at $P < 0.001$). We estimated the calculated transition:transversion ratio as 4.79, and the gamma shape parameter as 0.289.

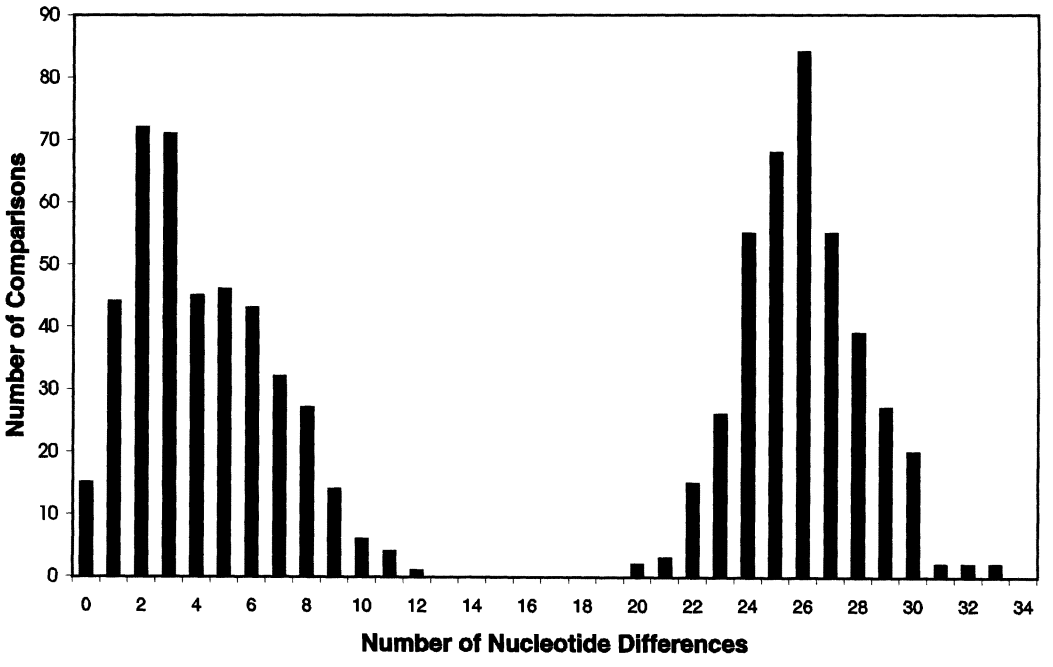

Fig. 2. Distribution of nucleotide differences among mtDNA haplotypes of sandhill cranes.

The 2 haplotypes that occurred in high frequency (#65 and #164) were represented 7 (12%) and 6 (10%) times, respectively. Haplotype #65 was shared by 5 greater and 2 Canadian sandhill cranes. Haplotype #164 was shared among greater sandhill cranes only. One haplotype (#35) was shared among 3 individuals: 2 lesser and 1 Canadian sandhill crane. Six haplotypes were shared among only 2 individuals: 3 haplotypes with both lesser, 1 haplotype with both Canadian, 1 haplotype with both greater, and the final haplotype with 1 Canadian and 1 greater sandhill crane. The remaining 32 haplotypes were sampled in only 1 individual. Thus, 3 haplotypes were shared among individuals that had been identified as different subspecies based on their morphology. Two of those 3 haplotypes were shared by Canadian and greater sandhill cranes.

The Siberian crane sequence differed from the sandhill crane sequences by 69 to 75 nucleotides. Among the 41 sandhill haplotypes, the number of nucleotide differences ranged from 1 to 34 with bimodal distributions of 1 to 13 and 21 to 34 (Fig. 2). Most comparisons between individuals of the same subspecies or individuals of Canadian and greater sandhill cranes had 1–13 nucleotide differences. This mismatch distribution is consistent with an expanding population (compare with results of Zink et al. 2000). Most comparisons between lesser sandhill cranes and individuals of the other 2 subspecies resulted in 21–34 nucleotide differences. The overall distribution strongly suggested the presence of 2 genetically separate entities.

Results of all phylogenetic analyses of these haplotypes were highly supportive of 2 major clades among the Gulf Coast subpopulation of sandhill cranes. The sandhill crane clades were much less differentiated from each other than they were from the Siberian crane (Fig. 3). The 2 clades consisted of group (A) mostly lesser sandhill cranes, and group (B) mostly Canadian and greater sandhill cranes (Fig. 3). Bootstrap support of the basal branches for these 2 mtDNA clades was >90% for distance models assuming equal substitution rates among nucleotide sites (Fig. 3), and ranged from 76% to 93% in models assuming unequal rates (data not shown). There was limited support for additional groups, which is reflected by the short branch lengths associated with them. Phylogenetic relationships of a subset (n = 12) of haplotypes representing the 2 groups were investigated using maximum likelihood analysis, which yielded trees with similar topologies to the distance-based trees (data not shown).

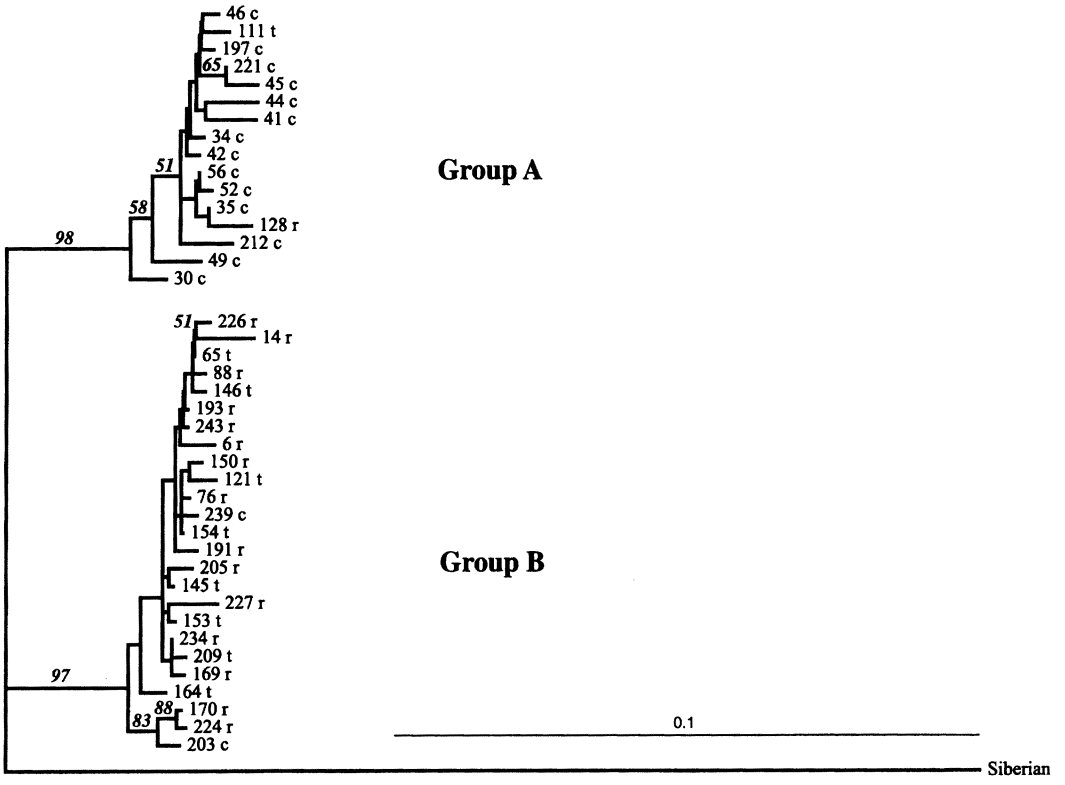

Fig. 3. Rooted phylogram showing that the sandhill crane mtDNA sequences fall into 2 groups. Tree was constructed from Hasegawa-Kishino-Yano (HKY85; 1985) genetic distances using the Neighbor Joining algorithm of PAUP* 4.0b2a (Swofford 1999). Siberian crane was used as an outgroup and demonstrates interspecific differentiation. Bootstrap values ≥50% are given above the branches. Numbers refer to the individual identifications. Letters following the numbers represent the first letter of subspecies, determined by morphological analysis [c = *Grus canadensis canadensis* (lesser), r = *G. c. rowani* (Canadian), t = *G. c. tabida* (greater)].

Restriction Enzyme Survey

Hae III digests of each 437 bp amplicon produced 1 of 3 distinct banding patterns. The 3 restriction patterns (Fig. 4) corresponded to mtDNA groups identified in initial analyses. The restriction digest patterns were of 3 major categories: 39 samples in group A (lesser), 171 in group B (Canadian and greater), and 9 in group C (greater). Inclusion of additional sequences dissolved phylogenetic support for group C, which is now included as part of group B. Only 1 sample had a restriction pattern that was inconsistent with these 3 patterns. Thus, 99.5% of all sandhill cranes could be easily assigned to 1 of the 2 phylogenetically distinct groups by the simple restriction enzyme test developed.

Morphological data were not available for 5 of the 220 samples, and mtDNA was not clear for 1 additional individual (the individual with a unique restriction pattern). By combining Canadian and greater sandhill cranes, classification by morphology and mtDNA were in agreement for 196 (91%) of the 214 individuals in this study. Of these discrepancies, 10 were classified morphologically as lesser and genetically as Canadian–greater, and 8 were classified morphologically as Canadian–greater and genetically as lesser sandhill cranes.

DISCUSSION

The mtDNA control region of sandhill cranes contains a reasonably large amount of genetic variation among individuals. The sequence information obtained from all PCR products is consistent with mitochondrial origin, rather than nuclear origin (compare with results of Quinn 1997). More than 50% of the 60 birds sequenced contained unique haplotypes, but most new haplotypes were not very different from those discovered in the initial sample of 14 individuals. Thus, the amount of variation in this region of mtDNA is appropriate for the questions we wanted to address in this study.

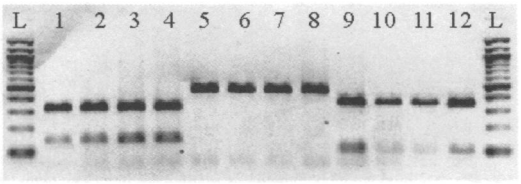

Fig. 4. Hae III restriction enzyme pattern for 12 sandhill cranes, lanes 1–4 = group C (greater); lanes 5–8 = group B (Canadian and greater), lanes 9–12 = group A (lesser). Groups B and C are not phylogenetically distinct, and thus can be combined (see text for details). L = 100 base pair (bp) Ladder (New England Biolabs, Beverly, Massachusetts, USA); bands beginning at the bottom correspond to 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, and 1500 bp. The 500 and 1000 bp bands of the ladder have larger amounts of DNA, obscuring the 900 bp band under the gel conditions used.

Our results clearly demonstrate that sandhill cranes sampled from the eastern wintering range of the MCP represent only 2 distinct genetic groups. The relative length and bootstrap support of the branches separating the 2 groups in the phylogenetic trees and the complete separation of nucleotide differences demonstrate the differentiation between the 2 mtDNA groups. Morphologically defined subspecies identifications were highly similar to the mtDNA groups, except that there was no support for differentiation between Canadian and greater sandhill cranes. The combination of Canadian and greater subspecies has profound management implications because a major objective of managers of the MCP is to reduce harvest of the greater sandhill crane due to its apparent low abundance and restricted distribution.

Eighteen (8.4%) of the 214 samples were inconsistent between the morphological determination and the mtDNA groups. Haplotype #35 was the only haplotype that had morphologically identified individuals shared between the 2 mtDNA groups. Ongoing mtDNA studies of sandhill cranes from their breeding grounds are congruent with our findings (J. Rhymer, University of Maine and K. Jones, University of Illinois-Chicago, personal communications). This level of congruence suggests that the mtDNA groups reflect biologically relevant entities that nearly show reciprocal monophyly (sensu Moritz 1994). The few inconsistent classifications may be due to (1) misclassification at the morphological level, (2) misclassification of the mtDNA, or (3) interbreeding among the subspecies. Obviously, the first explanation seems plausible because it is the concern that prompted this study. The second seems less likely because all inconsistent samples were reanalyzed, and no obvious pattern existed among the inconsistently classified individuals to suggest systematic errors. The third explanation is supported by Tacha et al. (1985), who reported that interbreeding among subspecies in the MCP was common. This will require investigation of biparentally inherited genetic markers and samples from the breeding areas to be addressed appropriately.

If the morphological model works well for some clearly diagnosable individuals, but less well for others, then we would expect more consistency between mtDNA and morphological classification for individuals with the highest morphological posterior probabilities of membership in subspecies. It is clear, however, that the percentage of inconsistent classifications is nearly identical when considering only 20 individuals per subspecies with the highest probabilities based on morphological discriminant scores (8.3% inconsistent classifications) or when considering all individuals in the study (8.4% inconsistent classifications). This suggests that the highest posterior probabilities of membership of the morphological model are not any better than the lower values in classifying individual cranes into appropriate subspecies, which underscores the need for improved assays to determine subspecies of sandhill cranes.

The restriction enzyme assay described here can be used as a fast, simple, inexpensive, objective, and accurate assay to determine in which mtDNA group an individual sandhill crane belongs. Although Hae III works well, we chose it because it reflected mtDNA patterns attributable to the expected 3 subspecies. Unfortunately, additional samples dissolved support for the 3 putative groups. Thus, future investigators may choose to use other restriction enzymes (e.g., Nci I or Mse I), which should simply show 1 pattern for each of the 2 mtDNA groups. It should also be noted that many restriction enzymes produce patterns that are nearly fixed between the 2 groups. Using such enzymes would result in the misclassification of a few individuals (e.g., using Mbo I, the site at position 333 would misclassify individuals 164 and 243 as group A, rather than group B). This illustrates the need for reasonably large sample sizes when choosing restriction enzymes for use as diagnostic markers (compare with results of Walsh 2000).

Although it is not desirable to define subspecies by using ever-more-powerful molecular markers until one uncovers statistically significant variation among the groups being studied, the use of microsatellite loci (Glenn et al. 1997) is appropriate for investigations of sandhill crane subspe-

cies because (1) microsatellites are influenced by both male and female patterns of dispersal, (2) many investigations of similar questions have been and are increasingly addressed with these highly polymorphic markers which would allow useful comparisons, and (3) great amounts of effort (in terms of both time and money) have been expended to conserve sandhill crane subspecies. The decision to decrease conservation efforts for particular subspecies of cranes should only be made with the best possible data. For these reasons, it also may be worthwhile to investigate loci such as MHC (Jarvi et al. 1995).

MANAGEMENT IMPLICATIONS

We demonstrated that the mtDNA of sandhill cranes wintering along the Texas Gulf Coast belong to 2 genetic groups, rather than 3, which were predicted by current subspecies classification methods based on morphology. Consequently, current morphological discrimination models for recognition of migratory subspecies should be reinvestigated and revised as appropriate. Management plans that recognize *G. c. rowani* as a distinct biological entity from *G. c. tabida* in the Gulf Coast subpopulation of the MCP are not supported by our data. Our findings agree with the conclusions of Ballard et al. (1999) who argued that subpopulation management based on concern for the greater sandhill crane may not be warranted. Systematic sampling and testing of mtDNA and microsatellite DNA loci from the other sandhill crane subspecies, along with additional samples from the breeding grounds of all subspecies, would be highly desirable. Such studies would be prudent to ensure proper management and conservation of this species.

Note added in proof: Rhymer et al. (2001) have recently described mtDNA variation among sandhill cranes from the breeding grounds. Their results are consistent with our findings.

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