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## Genetic Variation in the Midcontinental Population of Sandhill Cranes, *Grus canadensis*

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Three subspecies of sandhill crane (*Grus canadensis*) are recognized in the Midcontinental population, the lesser (*Grus c. canadensis*), Canadian (*G. c. rowani*), and greater (*G. c. tabida*). Blood samples collected on the population's primary spring staging area in Nebraska, U.S.A., were used to resolve the genetic relationship among these subspecies. Phylogenetic analysis of 27 *G. canadensis*, by DNA sequencing of a 675 bp region of the mtDNA, supports the subspecies designations of *G. c. canadensis* and *G. c. tabida*. *G. c. rowani* individuals were intermediate with each of the other two subspecies. Genetic divergence ranged from 6.5 to 14.5% between *G. c. canadensis* and *G. c. tabida*, 0.5 to 6.6% within *G. c. canadensis*, and 0.1 to 6.0% within *G. c. tabida*. Sufficient DNA for analysis was obtained from shed feathers indicating a source of genetic material that does not require the capture or sacrifice of the birds. Other genetic markers and methods, including satellite telemetry, are required for obtaining detailed information on crane distributions as needed to establish effective management units for the MCP.

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**KEY WORDS:** *Grus canadensis*; sandhill crane; genetic variation; Midcontinental population; mtDNA.

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## INTRODUCTION

The Midcontinental population (MCP) of sandhill cranes, *Grus canadensis* L. (1758), in North America consists of approximately 500,000 individuals of three migratory subspecies: lesser (*G. c. canadensis*), Canadian (*G. c. rowani*), and greater (*G. c. tabida*) (Sharp *et al.*, 1999; Tacha *et al.*, 1992). With the exception of the lesser sandhill crane, [*Ardea*] *canadensis* (*G. c. canadensis*), which was formally classified by Linnaeus in 1758, the other subspecies within this population were identified only recently. In 1925, the greater sandhill crane, *Megalormis canadensis tabida* (*G. c. tabida*), was described (Peters, 1925), followed by the Canadian sandhill crane (*G. c. rowani*), in 1965 (Walkinshaw, 1965). The majority of the MCP is believed to be comprised of *G. c. canadensis*, with *G. c. tabida* and *G. c. rowani* making up an estimated 8 and 25% of the population, respectively (Sharp *et al.*, 1999).

Cranes within the MCP breed in northeast Siberia, Alaska, and throughout arctic, subarctic, and temperate regions of Canada west of Hudson Bay. In general, *G. c. canadensis* breeds in the northern subarctic regions of Canada, Alaska, and Siberia, while *G. c. tabida* breeds in northwest Minnesota, southwest Ontario, and southern Manitoba (Meine and Archibald, 1996; Walkinshaw, 1981). *Grus canadensis rowani*, which is thought to be an intermediate subspecies, nests in areas of central Canada, between the breeding areas of *G. c. canadensis* and *G. c. tabida*. The MCP is characterized by its use of the North American Central Flyway during its annual migration between the northern breeding grounds and wintering habitat in the southern and southwestern United States and northern Mexico. Research opportunities at the population level are optimal during the spring, northward migration, when over 80% of the population remains in Nebraska's Platte River Valley for several weeks (Krapu *et al.*, 1984). Unlike the three, nonmigratory and geographically isolated subspecies of sandhill cranes: the Cuban (*G. c. nesiotis*), Florida (*G. c. pratensis*), and Mississippi (*G. c. pulla*), the communal staging area, and overlapping wintering habitat allow for substantial interaction between the subspecies of the MCP.

Subspecific identification of individuals within the MCP is based on morphometry and the geographic separation of breeding habitat. However, subtle differences in morphological characteristics and possible random mating between the subspecies have raised questions concerning the current classification system (Johnson and Stewart, 1973; Tacha *et al.*, 1985). In his description of *G. c. rowani*, Walkinshaw (1965) noted slight overlap in the morphometric measurements of *G. c. rowani* and the other two subspecies, particularly with *G. c. canadensis*. Furthermore, difficulties in distinguishing *G. c. canadensis* from *G. c. rowani* have created problems in census and population monitoring (Drewien *et al.*, 1975; Tacha *et al.*, 1985).

With decreasing habitat in staging and wintering areas, management of the MCP is becoming increasingly important (Currier, 1991; Meine and Archibald,

1996). To further understand the relatedness of these subspecies, and to make optimal management decisions concerning the MCP of sandhill cranes, it is critical to understand their phylogenetic relationships. Molecular genetics may provide insight into resolving these subspecific relationships.

Although the Gruidae have been studied using DNA hybridization, mtDNA cytochrome- $\beta$  analysis, and microcomplement fixation (Ingold *et al.*, 1989; Krajewski, 1989; Krajewski *et al.*, 1999; Krajewski and Fetzner, 1994), phylogenetic analysis of the MCP is lacking. Limited morphometric and geographic investigations, along with DNA hybridization, and biochemical allozyme analyses, have not clarified the relationship between *Grus canadensis* subspecies (Johnson and Stewart, 1973; Krajewski and Fetzner, 1994; Krajewski and Wood, 1995; Tacha *et al.*, 1984). For these reasons, molecular genetic studies at the population level are required.

Phylogenetic analysis of populations frequently is conducted using mtDNA because of its maternal mode of inheritance and relatively rapid rate of evolution (Awise *et al.*, 1984). In addition, mtDNA has been used in other avian taxa (Ellsworth *et al.*, 1995; Krajewski and Wood, 1995; Miranda *et al.*, 1997; Szalanski *et al.*, 2000) including recent studies of genetic variation in whooping cranes (*Grus americana*) (Glenn *et al.*, 1999; Snowbank and Krajewski, 1995).

The purpose of this study was to perform phylogenetic analyses of the MCP of sandhill cranes using a 675 bp region of the mtDNA including 85 bp of the NADH VI region, the complete tRNA<sup>glu</sup> sequence, and 515 bp of the D-loop. Data from the phylogenetic analyses were compared to the classification of each individual determined through morphometry for a comparison of identification techniques. In doing so, we investigated the variation within and among the subspecies of the MCP of *G. canadensis*. In addition, feathers collected in the Platte River Valley in Nebraska were evaluated to assess their usefulness for future DNA studies.

## MATERIALS AND METHODS

Blood samples from 27 sandhill cranes were collected by the U.S. Geological Survey, Northern Prairie Wildlife Research Center (NPWRC) in 1998 and 1999 from the Central Platte River Valley of Nebraska during the population's annual northward migration to their breeding grounds (Table I). The subspecific identity of each crane was determined by morphometric analysis using measurements of the culmen (from posterior edge of nares), tarsus length, and wing chord. Cranes were assigned to the subspecies they most closely resembled based on discriminant functions presented by Johnson and Stewart (1973). The gender of each individual, needed for the appropriate subspecific assignment, was determined either through necropsy or blood analysis, depending upon the sampling method.

Blood samples from 1998 and from tagged and released individuals in 1999 were preserved in 70% ethyl alcohol, while blood from the other 1999 samples

**Table I.** *Grus canadensis* blood samples that were analyzed

Sample	Collection Year	Sample type (blood)	Morphological classification
1	1998	Liquid	<i>canadensis</i>
13	1998	Liquid	<i>canadensis</i>
22 <sup>a</sup>	1999	Liquid	<i>canadensis</i>
33	1998	Liquid	<i>rowani</i>
34 <sup>a</sup>	1999	Liquid	<i>rowani</i>
35 <sup>a</sup>	1999	Liquid	<i>canadensis</i>
46 <sup>a</sup>	1999	Liquid	<i>canadensis</i>
49	1998	Liquid	<i>rowani</i>
110	1998	Liquid	<i>rowani</i>
123	1998	Liquid	<i>tabida</i>
125	1998	Liquid	<i>tabida</i>
126	1998	Liquid	<i>tabida</i>
201	1998	Liquid	<i>canadensis</i>
313	1999	Dried	<i>rowani</i>
343	1999	Dried	<i>canadensis</i>
352	1999	Dried	<i>canadensis</i>
354	1999	Dried	unknown
406	1999	Dried	<i>rowani</i>
407	1999	Dried	<i>rowani</i>
411	1999	Dried	<i>canadensis</i>
415	1999	Dried	<i>rowani</i>
416	1999	Dried	<i>canadensis</i>
618	1999	Dried	<i>rowani</i>
619	1999	Dried	<i>rowani</i>
702	1999	Dried	<i>canadensis</i>
707	1999	Dried	<i>rowani</i>
710	1999	Dried	<i>rowani</i>

<sup>a</sup>Samples tagged and released for telemetry studies by the USGS NPWRC.

(sampled by shooting) were dried on paper towels (Table I). Feather samples also were obtained from roosting sites in the Platte River Valley, and from museum specimens in the University of Nebraska Museum (Lincoln, NE, U.S.A.).

Liquid, preserved blood samples were first incubated (300  $\mu$ L) in 1000  $\mu$ L of RBC lysis solution (Puregene D-5000 DNA Blood Isolation Kit, Gentra Systems, Minneapolis, MN) for 20 min at room temperature and centrifuged. The supernate was removed and the samples were incubated two more times in a similar fashion. The product was then subjected to a chelex extraction (Promega, 1998). DNA was extracted from dried blood samples using a modification of the chelex extraction technique (Promega, 1998). To obtain DNA from the feather samples, 1 cm of the base of the feather shaft was removed and split lengthwise. Extraction then followed the Puregene D-5000 Blood Isolation Kit (Gentra Systems, Minneapolis, MN, U.S.A.) protocol for "fresh or frozen tissue," including proteinase K and RNase treatments.

The primers LND6-1 (5'-CCCCATAATACGGCGAAGGATT-3') (Desmond, 1997) and SCH-R (5'-TGCGCCTCTGGTTCCTATGTCA-3') amplified 675 bp of the 3' end of NADH VI and 5' end of the D-loop region of the mtDNA. The primer SHC-R anneals to a conserved region in the middle of the D-loop region. The 5' ends of the primers are located at 16,621 and 524 on the chicken, *Gallus gallus*, mtDNA genome (Desjardins and Morais, 1990; Genbank x52392). PCR amplification was carried out using 1–20  $\mu$ L of the crude DNA obtained from the extraction per Szalanski *et al.* (2000). The PCR profile was 35 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 60 s. Five microliters of each sample were subjected to electrophoresis on a 1% agarose gel to test for successful amplification. Amplification products and the extracted DNA were stored at –20°C.

DNA was sequenced in both directions by the Iowa State University DNA Sequencing Facility, Ames, IA. Prior to sequencing, amplified DNA was purified using Micron 50 microconcentrator (Amicon Inc, Beverly, MA), and resuspended to a volume of 10.0  $\mu$ L with nanopure water. The GCG (Genetics Computer Group, Madison, WI) GAP program, which utilizes the algorithm of Needleman and Wunsch (1970) to maximize matches and minimize gaps, was used to create a consensus alignment of the forward and reverse sequence of each individual. Genbank accession numbers for the *Grus canadensis* DNA sequences are AF277983–AF277987, AF285769–AF285773, and AF286321–AF286336. The *G. canadensis* sequences were then aligned using *G. americana* (Genbank AF112373, Glenn *et al.*, 1999), *G. japonensis* (Genbank AB017618, Hasegawa *et al.*, 2000), and *G. paradisea* (Genbank AF112372, Glenn *et al.*, 1999) as outgroup taxa.

Unweighted parsimony and maximum likelihood analyses on the alignments were conducted with PAUP\*4.0b2 (Swofford, 1999). Gaps were treated as missing characters for the analyses and the reliability of trees was tested with a bootstrap test (Felsenstein, 1985). Parsimony bootstrap analysis included 100 resamplings using the Branch and Bound algorithm.

For maximum likelihood analysis, the most appropriate model of sequence evolution as described by Sullivan *et al.* (1997) was determined using Hierarchical Likelihood Ratio Tests (hLRTs) in the program Modeltest3.06 (Posada and Crandall, 1998) in conjunction with PAUP\*. The model of sequence evolution chosen for our data by Modeltest, including the transition/transversion ratio, base frequencies, and the gamma distribution shape parameter ( $\alpha$ ), was implemented into PAUP\* for a 100 replicate random addition heuristic search with tree bisection-reconnection (TBR) branch swapping. Starting branch lengths were obtained using Rogers–Swofford approximation method.

## RESULTS

Morphometric analysis classified 11 cranes as *G. c. canadensis*, 12 as *G. c. rowani*, and 3 as *G. c. tabida*. Inability to assign gender to one individual left it of unknown

classification (Table I). DNA sequencing of the mtDNA PCR-amplified product revealed that it ranged in size from 674 to 678 bp among the 27 *G. canadensis* sequenced.

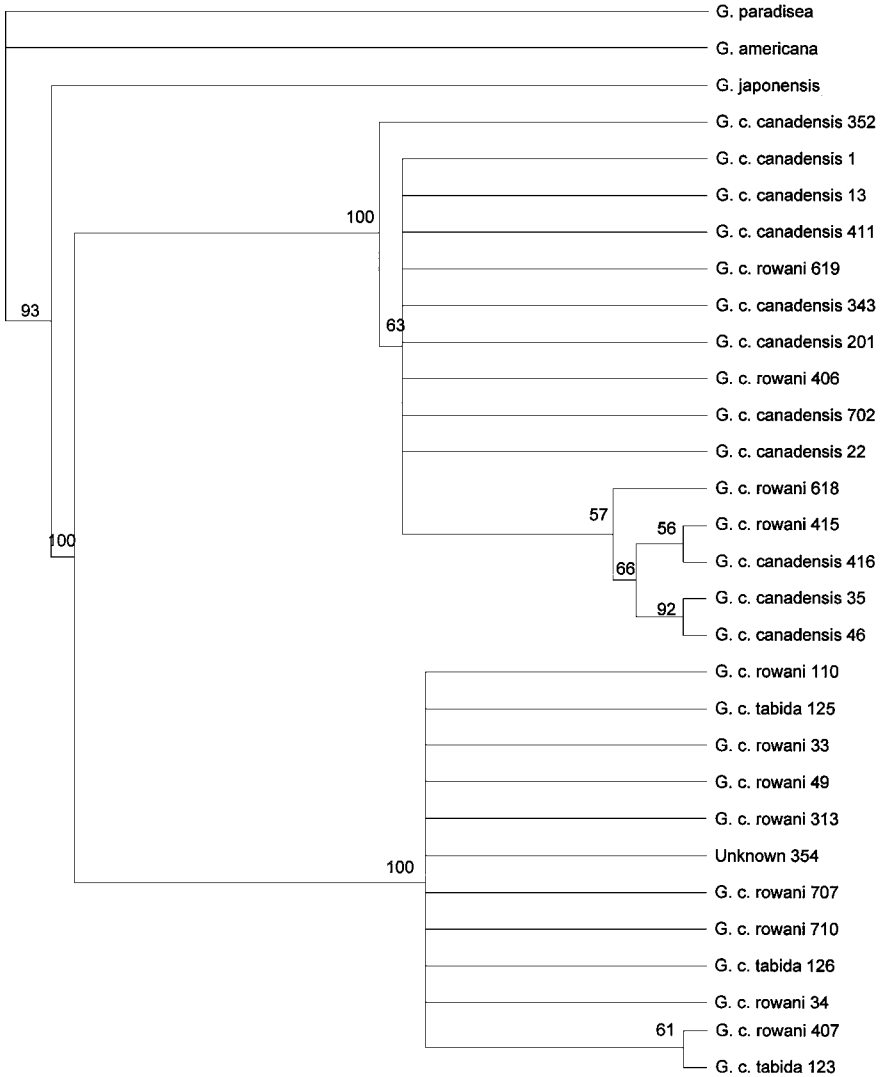
The aligned mtDNA data matrix including the outgroup taxa resulted in a total of 692 characters, including gaps. Of these characters, 242 (35%) were variable and 117 (17%) were parsimony informative. Within the 85 characters of the NADH VI region, 13 were variable with five parsimony informative while within the tRNA<sup>glu</sup> region, 5 of the 73 characters were variable with two parsimony informative. No gaps were present in the protein-coding regions. Excluding the outgroup taxa, 130 (19%) of the 692 characters varied, with 71 (10%) parsimony informative. In the NADH VI region, two sites were variable but not parsimony informative. Finally, one parsimony informative site was located within the tRNA<sup>glu</sup> region of *G. canadensis*.

The mtDNA dataset had only one most parsimonious tree (Fig. 1), (length = 428, CI = 0.67, CI excluding uninformative sites = 0.51), as documented using the Branch and Bound algorithm of PAUP\*. Bootstrap analysis of the aligned *Grus* species resulted in a consensus tree with two well-supported branches (Fig. 1). In all examinations, the outgroup taxa were excluded from *G. canadensis*.

The model found to be optimal for our data by Modeltest was that of Hasegawa–Kishino–Yano (HKY) (Hasegawa *et al.*, 1985) with the gamma shape parameter (HKY + G). The transition/transversion ratio, base frequencies, and the gamma distribution shape parameter as estimated by Modeltest were: Ti/tv = 2.1453; A = 0.3141, C = 0.2816, G = 0.1445, T = 0.2598; and  $\alpha = 0.4101$ . Maximum likelihood analysis found only one tree ( $-\ln$  likelihood = 3072.06933, total number of rearrangements tried = 192874). The maximum likelihood tree was similar to the most parsimonious tree (Fig. 2); in all cases, *G. c. canadensis* was separated from *G. c. tabida*, while *G. c. rowani* was present in both groupings.

The mean genetic distance within *G. canadensis* using the HKY + G method of analysis (Hasegawa *et al.*, 1985) was 5.8%, ranging from 0.1 to 14.5%. Between the two branches of *G. canadensis*, genetic distance averaged 8.8%, ranging from 6.5 to 14.5%. Within the branch representing *G. c. canadensis*, the mean genetic distance was 2.8%, with a range of 0.5–6.6%, and within *G. c. tabida*, the mean distance was 2.3% with a range of 0.1–6.0%.

All *G. c. rowani* collected in 1998 fell into the clade representing *G. c. tabida*. With the exception of a strong pairing between two *G. c. canadensis* samples (35 and 46) little resolution was present in the clades (Fig. 1). No subgroupings distinguished the birds identified via morphology as *G. c. rowani* from either *G. c. canadensis* or *G. c. tabida*. The evaluation of genetic distances within each of the three subspecies as determined by morphology showed a mean genetic distance of 2.9% for *G. c. canadensis*, 5.2% for *G. c. rowani*, and 3.8% for *G. c. tabida*. Genetic distances ranged from 0.5 to 6.6%, 0.1 to 9.7%, and 1.1 to 5.4% for *G. c. canadensis*, *G. c. rowani*, and *G. c. tabida*, respectively.

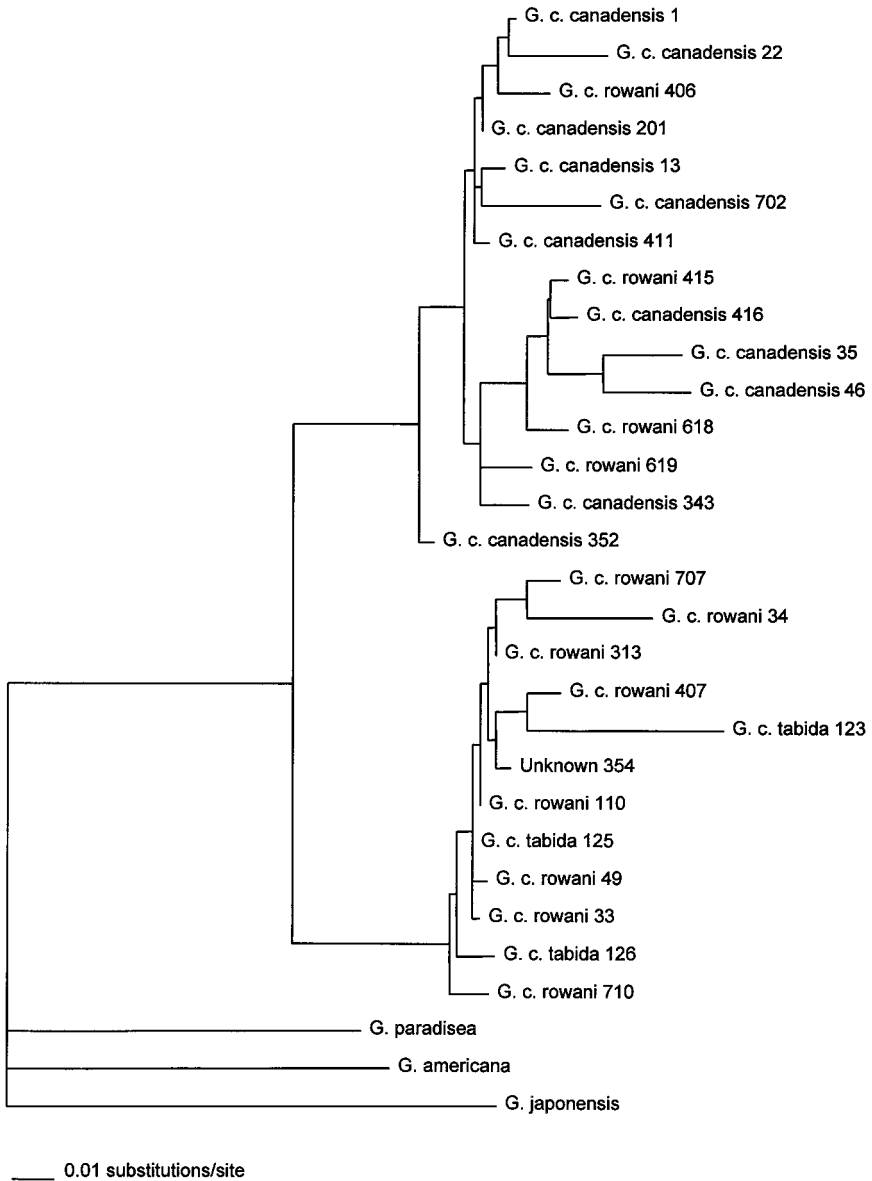


**Fig. 1.** Single most parsimonious tree during an exhaustive search using PAUP\* of mtDNA D-loop sequences with gaps treated as missing characters. Bootstrap values for 100 branch and bound replicates are listed above branches supported at  $\geq 50\%$ .

### DISCUSSION

The results from both the parsimony and maximum likelihood trees indicate a strong separation within the MCP of sandhill cranes (Figs. 1 and 2). Little resolution





**Fig. 2.** Maximum likelihood tree for *Grus canadensis* ( $-\ln$  likelihood = 3072.06933) based on mtDNA sequences. Branch lengths are proportional to the number of substitutions per site.

within the trees lends limited information as to population structure although some subgroupings, present in both trees, indicate associations between individuals that were morphometrically classified as different subspecies. Because of the present classification of this population into three subspecies, it was surprising not to find any indication of a third grouping within the branches.

Though our samples were not collected within the breeding habitat, it is of managerial importance to be capable of identifying these birds to subspecies while they are in their wintering and staging areas. Comparison of the morphologic classification of our sample population upholds the separation of the subspecies *G. c. canadensis* and *G. c. tabida*. Although the inclusion of *G. c. rowani* into the *G. c. canadensis* and *G. c. tabida* clades supports the hypothesis that it is an intermediate form within the MCP, the lack of any division into an independent clade contradicts the current classification system as well as previous morphometric and phylogenetic studies (Johnson and Stewart, 1973; Krajewski and Fetzner, 1994). These findings, however, do support the discrepancies in Krajewski and Fetzner (1994), which paired *G. c. rowani* with *G. c. canadensis* using distance analysis of the cytochrome- $\beta$  region of mtDNA, but separated *G. c. canadensis* from *G. c. rowani* and *G. c. tabida* when analyzed by parsimony analysis. Based upon the comparison of morphometric analysis and genetic data in our study, it seems that the status of *G. c. rowani* is unclear. Its presence in both clades implies that it is either a mixture or hybrid of the other MCP subspecies. This clear division of our sample population, even with a limited sample of *G. c. tabida*, heightens the necessity for further geographic and phylogenetic studies of the MCP of sandhill cranes. Current studies by the USGS NPWRC, using satellite telemetry, will assist in determining the geographic location of the breeding habitats used by the population, give further insight into the phylogenetic and morphological distribution of the population, and determine the extent that the habitats of these subspecies are overlapping.

Although morphometric identification is not definitive (Tacha *et al.*, 1985), it is important to emphasize that the incorporation of *G. c. rowani* into both *G. c. canadensis* and *G. c. tabida* based upon this maternally inherited marker could be due to hybridization events, which result in morphology that differs from the mtDNA make-up of the individual. It is also of note that all *G. c. rowani* collected in 1998 fell into the branch representing *G. c. tabida*, even though those collected in 1999 were distributed throughout both branches. We believe this is most likely due to the method used for sampling the population, but is another reason *G. c. rowani* needs to be examined further using other genetic markers. Similarly, the small sample size of *G. c. tabida* reflects that there are fewer of this subspecies in the MCP. All *G. c. tabida* available at the time of this study were used in the analysis.

The usefulness of this segment of mtDNA that we studied for population analysis of *Grus* spp. is supported by the extent of variation seen through DNA

sequencing of a smaller portion of a similar marker in *G. americana* (Glenn *et al.*, 1999). The mtDNA variation observed in *G. americana* and the MCP of *G. canadensis* supports the use of this marker for future population analysis and management decisions within the Gruidae, but not for use to distinguish among the three subspecies. Future research needs to be performed to identify how and/or if the three subspecies of the MCP can be genetically distinguished from one another.

### Use of Feathers for Genetic Analysis

The collection of blood samples from MCP cranes for analysis is labor intensive, requiring the capture or sacrifice of the birds. For some population studies, shed feathers may provide a suitable alternate source of genetic material. We have been successful in obtaining adequate amounts of DNA from a portion of a single feather shaft using the Puregene D-5000 Blood Isolation Kit (Gentra Systems, Minneapolis, MN) protocol for “fresh or frozen solid tissue” including Proteinase K and RNase treatments. This procedure was successful using both fresh feathers collected from roosting sites as well as museum feathers collected in 1995 (University of Nebraska State Museum, Lincoln, NE, U.S.A.). In our investigation, feathers as small as 7.5 cm in length yielded sufficient DNA for genetic examinations; however, large flight feathers are optimal when available.

Even though Glenn *et al.* (1999) found that the amount of useful DNA obtained from bone and tissue samples of whooping crane museum specimens decreases with age, phylogenetic analysis using feathers potentially can reveal a more complete history of the recent evolution of the sandhill crane genome. More importantly, the collection and analysis of DNA from shed crane feathers on the wintering, staging, and breeding grounds provides a potential means to determine both the geographic distribution of the subspecies and phylogenetic relationships within the MCP. DNA from shed feathers may also offer a means for gathering more accurate estimates of the relative abundance of each subspecies within a particular staging or wintering area assuming the feathers can be sampled in ways that reflect the proportion of each subspecies present. New insight gained from phylogenetic and geographic analyses will provide information critical to management of the MCP of sandhill cranes.

### CONCLUSIONS

Our genetic analysis of the MCP of *G. canadensis* provides evidence for the existence of two valid subspecies, i.e., *G. c. canadensis* and *G. c. tabida*; further investigation is necessary to determine the status of *G. c. rowani* within the population. These results suggest an increased need to find additional tools for partitioning the population into units that can be managed effectively. Ongoing research by the NPWRC using satellite telemetry is establishing where each segment of the

MCP breeds, and is linking breeding areas to migration routes, staging areas, and wintering sites. Results from these studies and further molecular examinations, together with information gained from this study, will provide an improved basis for decisions concerning the management of the MCP of sandhill cranes.

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