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Mitochondrial phylogeography, subspecific taxonomy, and conservation genetics of sandhill cranes (*Grus canadensis*; Aves: Gruidae)

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Abstract

Six subspecies of sandhill cranes (*Grus canadensis*) have been denoted based on perceived morphological and/or breeding locality differences among them. Three subspecies are migratory, breeding from the high arctic in North America and Siberia (lesser sandhill, *G. c. canadensis*), south through central Canada (Canadian sandhill, *G. c. rowani*) and into the northern United States (greater sandhill, *G. c. tabida*). A review of sandhill crane taxonomy indicates that the size variation, on the basis of which these subspecies were named, may be clinal and not diagnostic. The other three subspecies, all listed as endangered or threatened, are non-migratory, resident in Florida (*G. c. pratensis*), Mississippi (*G. c. pulla*), and Cuba (*G. c. nesiotes*). We used analysis of mitochondrial DNA control region (CR) sequences to determine whether haplotypes representing current subspecies show any genetic cohesion or are more consistent with a pattern of clinal variation in morphology. CR sequences indicate that only two highly divergent (5.3%) lineages of sandhill cranes occur in North America: one lineage composed only of arctic-nesting *G. c. canadensis*, the other of the remaining North American subspecies (we lack data on the Cuban population). The deep split between lineages is consistent with an estimated isolation of approximately 1.5 Mya (mid-Pleistocene), while the distribution of mutational changes within lineages is consistent with an hypothesis of rapid, post-Pleistocene population expansions. No other phylogeographic structuring is concordant with subspecific boundaries, however, analysis of molecular variance indicates that there is significant population genetic differentiation among all subspecies except *G. c. tabida* and *G. c. rowani*, which are indistinguishable. We suggest that recognition of the recently named *G. c. rowani* be abandoned.

Introduction

Named subspecies carry at least the connotation of phenotypic uniformity in a specific geographic region, i.e. they are (or should be) predictive (Barrowclough 1983). However, they may not accurately reflect the patterns of intraspecific geographic variation in some avian species. For instance, formal subspecific recognition has occasionally been given to minor geographic differences in color or size, in part, due to inadequate sampling of localities across the species range and/or inadequate sampling of individuals within localities, e.g. uneven sampling of sex/age classes (Barrowclough and Flesness 1996). As a result, variation that is clinal rather than representing well-differentiated populations has been recognized taxonomically and some subspecies delineations may not reflect actual patterns of differentiation.

The sandhill crane (*Grus canadensis*) presents an interesting case in which the naming of subspecies has had direct consequences for both game management and endangered species conservation. There are

six putative subspecies, three of which are migratory and three non-migratory. The migratory subspecies (lesser sandhill crane, *G. c. canadensis*; Canadian sandhill crane, *G. c. rowani*; greater sandhill crane, *G. c. tabida*) currently have relatively robust populations and are hunted. Eastern and western populations of *G. c. tabida* were close to extirpation by the 1930s due to hunting, agricultural expansion, and drainage of wetlands, but have recovered in recent decades (Meine and Archibold 1996). The three non-migratory subspecies are all listed as threatened or endangered. The Florida sandhill crane (*G. c. pratensis*) is listed as Threatened under the United States Endangered Species Act (ESA) and is listed in Appendix II of the Convention on International Trade in Endangered Species (CITES) agreement. The Mississippi (*G. c. pulla*) and Cuban (*G. c. nesiotes*) sandhill cranes are listed as Critically Endangered (ESA) and in CITES Appendix I.

History of sandhill crane taxonomy

Linnaeus (1758) named the sandhill crane *Ardea canadensis*, but Brisson (1760) transferred this species, along with three other cranes, into his genus *Grus*. The type locality of *G. canadensis* is Southampton Island in northern Hudson Bay (Figure 1a), and members of this population and others northwest of it and into Siberia have since been denominated *G. c. canadensis* ("lesser sandhills", because of their relatively small size). Meyer (1794) described the nonmigratory sandhill crane population of Florida as *G. c. pratensis* ("Florida sandhills"), and Bangs and Zappey (1905) named the population resident on Cuba and the Isle of Pines *G. c. nesiotes* ("Cuban sandhills"). Peters (1925) recognized the migratory sandhill cranes that bred in the southwestern United States in Nevada as *G. c. tabida* ("greater sandhills", because of their relatively large body size). Peters (1934) included these four subspecies in his Check-List of Birds of the World, and Walkinshaw (1949) constructed a key to their identification based on morphometric and plumage characters. Walkinshaw (1949) provided the first detailed survey of sandhill crane breeding and wintering localities, and noted that body sizes in populations of *G. c. canadensis* and *G. c. tabida* appeared to form a cline in which the smallest birds breed farthest to the north (Figure 1a).

Sandhill crane taxonomy was relatively stable until Walkinshaw (1965) described a new subspecies, *G. c. rowani* ("Canadian sandhills") from the prairie provinces of Canada (delineated by dashed lines in Figure 1a). He distinguished Canadian sandhills from other subspecies by the light gray shafts of their primary feathers and by a variety of external morphometric traits. This taxonomic revision proved controversial with some researchers (Stephen 1967; Tacha 1981; Tacha et al. 1985, 1992) concluding that a substantial number of individuals could not be correctly categorized to subspecies on the basis of external measurements (but see Johnson and Stewart 1973). It was generally agreed, however, that *G. c. canadensis* individuals were more distinct than were those of *G. c. tabida* and *G. c. rowani*. Aldrich (1979) reviewed the morphometric basis for distinguishing *G. c. rowani* from *G. c. canadensis* and *G. c. tabida*, and although he concurred that *G. c. rowani* is intermediate between the other two subspecies, he concluded that differentiation among these forms was sufficient to recognize *G. c. rowani* as "a practical unit in crane management" (p. 140).

The last taxonomic addition to the group was the resident sandhill crane population in Jackson County, Mississippi, described as a distinct subspecies, *G. c. pulla* ("Mississippi sandhills"), primarily on the basis of their dark plumage (Aldrich 1972). In his seminal monograph on cranes, Walkinshaw (1973) recognized all six subspecies, maintaining that size, plumage coloration, and breeding distribution were sufficient for diagnosis, and Johnsgard (1983) listed the three migratory forms as subspecies in his monograph on cranes.

Baldwin (1977) compared anatomical and physiological features of chick development among *G. c. canadensis*, *G. c. rowani*, *G. c. tabida*, and *G. c. pratensis*, and concluded that each is "a unique genetic unit" (p. v), however, Gaines and Warren (1984) were the first to actually assay genetic variation within sandhill crane subspecies. They compared cranes from a *G. c. rowani* breeding area with those from a *G. c. canadensis* breeding area, and found an apparently fixed allelic difference at an anonymous pancreatic protein locus (P1). The same locus showed strong frequency differences between wintering crane populations in the Texas Panhandle (primarily *G. c. canadensis*) and southern coastal Texas (primarily *G. c. rowani* and *G. c. tabida*). Unfortunately, no *G. c. tabida* individuals were sampled from their breeding range. Dessauer et al. (1992) found seven polymorphic allozyme loci in a survey of *G. c. tabida*, *G. c. pratensis*, and *G. c. pulla* individuals – they did not sample *G. c. canadensis* or *G. c. rowani*. Alleles at

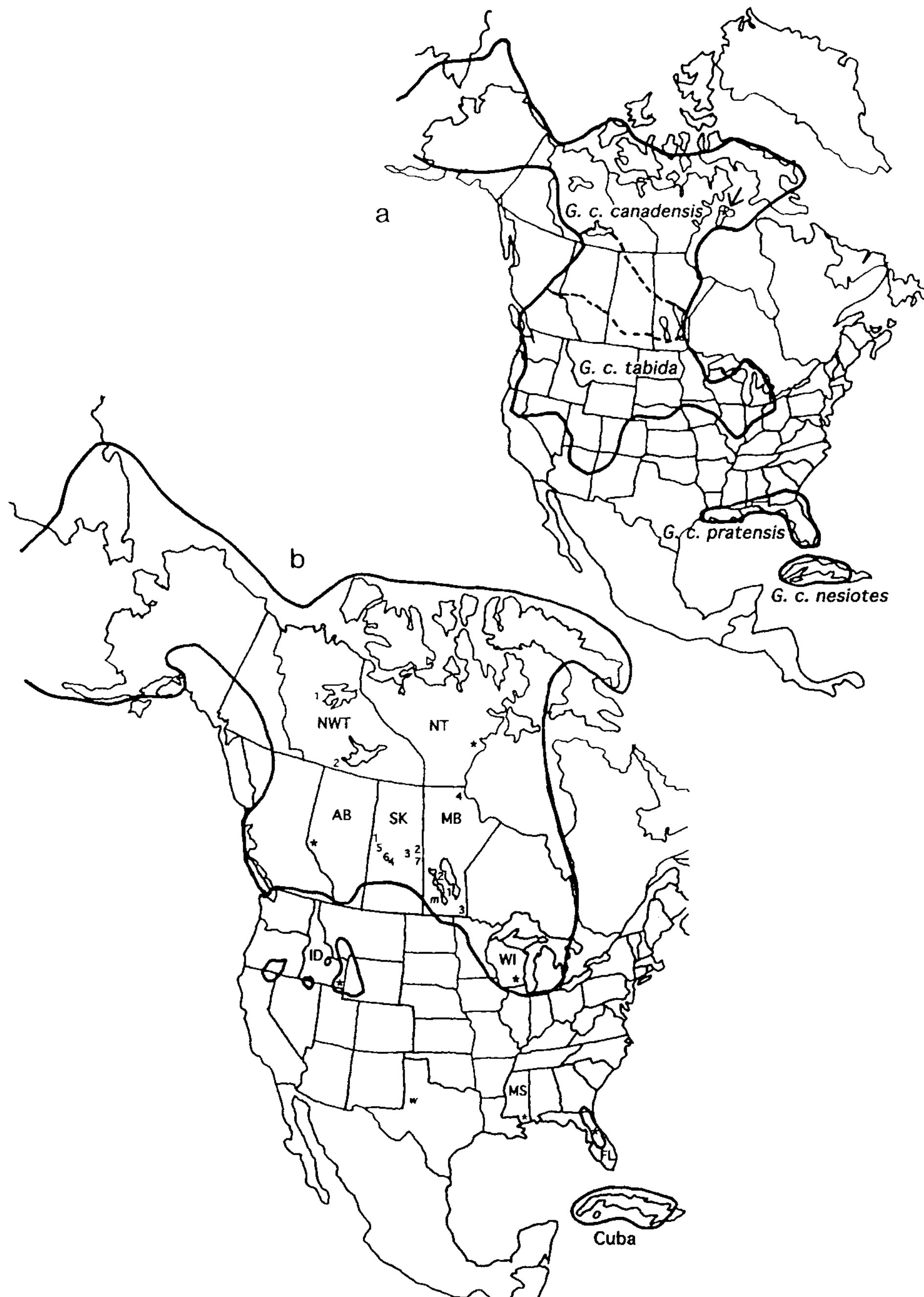

Figure 1. Geographical distribution of sandhill crane subspecies: a) based on pre-1920 breeding records of four subspecies recognized at that time (after Walkinshaw, 1949). Arrow indicates type locality (*) on Southampton Island in northern Hudson Bay. Area between dotted lines represents Walkinshaw's distribution of intermediate sized cranes (subsequently named, *G. c. rowani*, Walkinshaw 1965); b) current distribution of six recognized subspecies (after Meine and Archibald 1996), including sampling sites: Northwest Territories (NWT), Nunavut (NT), Alberta (AB), Saskatchewan (SK), Manitoba (MB), Idaho (ID), Wisconsin (WI), Mississippi (MS, *G. c. pulla*), Florida (FL, *G. c. pratensis*), fall migration (*m*), and winter (*w*) (asterisk and numbers represent sample populations listed in Table 1).

three of these loci showed some frequency differences among subspecies, but there were no diagnostic alleles for any of them, and phylogenetic analyses of these data showed no significant differences among subspecies.

The most recent summaries of sandhill crane taxonomy (Ellis et al. 1996; Meine and Archibald 1996) continue to recognize six subspecies, although Meine and Archibald (1996: 105) acknowledge that the "*G. c. canadensis-rowani-tabida* group is probably clinal, with gradual changes in morphological characters and no positive means of distinguishing among them". Although less well studied, the non-migratory *G. c. pratensis*, *G. c. pulla*, and *G. c. nesiotes* appear to overlap in size with *G. c. rowani* and *G. c. tabida* (Baldwin 1977).

No quantitative analysis, morphological or genetic, has been undertaken to assess patterns of variation in all six putative subspecies. We use analysis of mitochondrial DNA (mtDNA) control region sequences to determine whether currently recognized subspecies delineations are supported by molecular data or whether genetic variation is more consistent with a pattern of gradually changing (clinal) variation in morphology across the species range.

Materials and methods

Samples

Sandhill cranes were sampled across their breeding range in North America (Figure 1b). Muscle or heart tissue samples were obtained from *G. c. canadensis*, *G. c. rowani* and *G. c. tabida* at 15 breeding localities across Canada (Table 1). In addition, blood samples were obtained from sandhill cranes in western and eastern breeding populations of *G. c. tabida* in the northern USA (Grays Lake, Idaho, n = 5, and Wisconsin, n = 6). Blood samples from two of the nonmigratory subspecies were obtained from unrelated birds in captive breeding flocks; *G. c. pulla* (Patuxent Wildlife Research Center, n = 5) and *G. c. pratensis* (International Crane Foundation, n = 9). No *G. c. nesiotes* samples are currently available, but efforts are underway to obtain samples of this subspecies from the population of sandhill cranes in Cuba. Four birds in zoos, considered *G. c. canadensis* based on morphology, were also included (Fort Worth Zoo: wild caught birds, wintering in west Texas near Muleshoe Wildlife Refuge, n = 2; Akron Zoo: no information on origin, however birds were registered in the 1994 ISIS Bird Abstract as bona fide *G. c. canadensis*, n = 2). Heart tissues were also obtained from putative *G. c. rowani* (based on morphology), shot by hunters during early fall migration in western Manitoba (Big Grass Marsh, n = 4). As there is no clear sister taxon to sandhill cranes (Krajewski and King 1996), the Stanley crane (*Anthropoides paradisea*) from Africa and White-naped crane (*Grus vipio*) from southeast Asia were used as outgroups. These species are representatives from two of the other four species groups of gruine cranes.

Molecular analysis

DNA was isolated from each sample using standard procedures (Rhymer et al. 1994) and the majority of domains I and II of the mitochondrial control region was amplified using primers developed for sandhill cranes by M. Fain for another study (L32:5′-GTACTGGATTACATTCAG-3′, located downstream from the hairpin loop of C's at the 5′ end of the control region; H778: 5′-ACGAATACCATGTATGC-3′, located toward the 3′ end of the central conserved domain of the control region, upstream of CSB-1). To ensure that the 650 bp region sequenced was mitochondrial in origin, sequences were initially obtained from overlapping amplicons with multiple pairs of primers across ND6, the control region, 5′ end of 12S rRNA, and the intervening tRNAs. Sequences were identical for all amplicons, and all sequences appeared functional (proper structure for RNAs, and no indels or stop codons in the ND6 sequence).

DNA (∼5–10 ng) was amplified in a total volume of 25 μl with AmpliTaq (Perkin-Elmer) DNA polymerase, using a 5 min denaturing step at 94 °C, followed by 35 cycles of 94 °C for 45s, 50 °C for 1 min, 72 °C for 1 min, and finishing with a 5 min extension at 72 °C. PCR products were purified with Nanosep™ microcentrators (30K) and direct sequencing was done on an ABI automated sequencer (model 373 Stretch). Sequences have been deposited in Genbank/EMBL (Accession nos. AY049054, AY049055).

Relationship of mtDNA control region sequences

In all, 73 sandhill crane sequences were analyzed. Sequences were edited by eye and aligned using the Clustal algorithm in SequenceNavigator™ (Applied Biosystems Inc.) and by eye. Phylogenetic relationships were estimated using maximum likelihood

Table 1. Sandhill Crane population estimates and sampling localities from across North America

Subspecies	Census population size	Locality	Abbreviation	(n)
G. c. canadensis	450,000	**Northwest Territories**		
		Fort Norman	NWT1	(2)
		Nunavut		
		Rankin Inlet	NT	(2)
		Manitoba		
		North Knife River	MB4	(1)
		Texas (winter)		
		Muleshoe Refuge	*canadensis*	(2)
		Unknown (Akron Zoo)	*canadensis*	(2)
G. c. rowani	[unknown]*	**Manitoba**		
		Riverton	MB1	(1)
		Gypsumville	MB2	(9)
		Big Grass Marsh (fall migration)	*rowani*	(4)
		Saskatchewan		
		Green Lake	SK1	(4)
		Lobstick Lake	SK2	(3)
		Tobin Lake	SK3	(2)
		Foxford	SK4	(2)
		Big River	SK5	(1)
		Weirdale	SK6	(2)
		Leaf Lake	SK7	(1)
		Alberta		
		Rocky Mountain House	AB	(4)
		Northwest Territories		
		Fort Providence**		
		NWT2	(2)	
G. c. tabida (eastern)	45,000	**Manitoba**		
		Prada	MB3	(4)
		Wisconsin	WI	(6)
G. c. tabida (western)	30,000	**Idaho**		
		Gray's Lake	ID	(5)
G. c. pratensis	5,000	**Florida** (captive)[†]		
		International Crane Foundation	FL	(9)
G. c. pulla	150	**Mississippi** (captive)[†]		
		Patuxent Wildlife Research Center	MS	(5)

*Cannot be estimated for *G. c. rowani* as range is uncertain (Meine and Archibald 1996).
**Considered intermediate (*G. c. rowani*) according to Walkinshaw (1949) (see Figure 1a).
[†]Unrelated founders.

analysis with the Hasegawa et al. (1985) model of substitution plus gamma (HKY + Γ) to account for among-site rate variation, empirical base frequencies, ti/tv ratio (5.2:1) and $\alpha(0.22)$ estimated from the data, and stepwise addition, as implemented in PAUP* 4.0b4 (Swofford 1999). In addition, the neighbor-joining method with Tamura and Nei's (1993) substitution model with a gamma distribution, and random addition of haplotypes was used. Levels of resolution on nodes were estimated by 1000 random bootstrap replications of the data. Maximum parsimony analysis was not included because the number of unique haplotypes exceeds the number of parsimony informative sites. Rates of evolution in resulting lineages were compared using Tajima's (1993) test.

Patterns of subspecies structure, gene flow and effective population size

Population genetic structure was inferred by analysis of molecular variance (AMOVA; Excoffier et al. 1992) provided within the program package ARLEQUIN v.1.1 (Schneider et al. 1997), assuming that subspecies of sandhill cranes would correspond to genetic structure among populations. The null distributions to test significance of the variance components and the pairwise *F*-statistic equivalents (ϕ_{ST}) were constructed from 10,000 permutations of the data. Gene flow among subspecies, expressed as estimated number of female migrants per generation ($N_{ef}m_f$, where N_{ef} is the genetic effective population size of females and M_f is the female migration rate) was estimated from $\phi_{ST} = 1/(1 + 2N_{ef}m_f)$ (Slatkin 1991). Maximum likelihood estimates of $N_{ef}m_f$ were also calculated using the program MIGRATE v. 0.7 (Beerli and Felsenstein 1999). This method uses a coalescent theory approach to estimate past asymmetric migration rates between populations, taking into account history of mutations, uncertainty of the genealogy and different subpopulation sizes. Although absolute numbers of migrants between populations estimated with these methods may not be accurate, they are likely to be useful for general comparisons within a few orders of magnitude (Whitlock and McCauley 1999).

Measures of genetic diversity for each subspecies were estimated by calculating haplotype diversity (*h*), nucleotide diversity (π), number of polymorphic sites (*s*), number of pairwise differences (*k*) and ti/tv using ARLEQUIN v.1.1 (Schneider et al. 1997). Funnel plots (log(n) versus the response variable) were done to determine if these parameters were actually correlated with differences in sample sizes, masking potential differences among subspecies (Light and Pillemer 1984). The parameter $\theta = 2N_{ef}\mu$, where μ is the neutral mutation rate per site per generation, should correspond to the observed nucleotide diversity (π) if populations are in equilibrium and sequences are not under selection (Watterson 1975; Tajima 1983). Theta was also estimated from the coalescent Metropolis-Hastings Markov chain Monte Carlo method (θ_{MHMC}) of Kuhner et al. (1995) using the computer program FLUCTUATE v.1.3 (Kuhner et al. 1998). This program uses geneological relationships among haplotypes allowing for historically fluctuating population sizes, and N_{ef} can be estimated once values for θ_{MHMC} and μ are available.

Mismatch distributions (distribution of pairwise substitution differences between pairs of haplotypes in a population) were analyzed using the demographic expansion model of Rogers and Harpending (1992) as implemented in ARLEQUIN v.1.1 (Schneider et al. 1997). Recent population expansions or bottlenecks will generate a unimodal distribution, while long-term stable populations or slowly declining populations will have a multimodal mismatch distribution (Rogers 1995; Rogers et al. 1996). The mismatch distribution is described by $\theta_0 = 2N_0u$; $\theta_1 = 2N_1u$; and $\tau = 2ut$ where the initial effective population size, N_0, suddenly changes in size to N_1 at τ units of mutational time, calculated in terms of *u*, the mutation rate per generation of the entire nucleotide sequence studied and *t*, the number of generations since expansion. Tau (τ) is estimated by ($m - \theta_0$), where *m* is the mean of the observed mismatch distribution (Rogers 1995).

Results

Phylogeography and sequence divergence among sandhill crane subspecies

Of the 650 bp of sandhill crane mtDNA control region sequenced, 67 (10.3%) of the sites were variable, but only 44 (6.8%) were phylogenetically informative. Neighbor-joining and maximum likelihood analyses produced largely concordant trees, so only the neighbor-joining tree is presented here (Figure 2). The only significant phylogenetic divergence among subspecies is the deep split between arctic-nesting *G. c. canadensis* (lineage I) with 100% bootstrap support and all other subspecies (lineage II), which cluster 93% of the time. An exception to

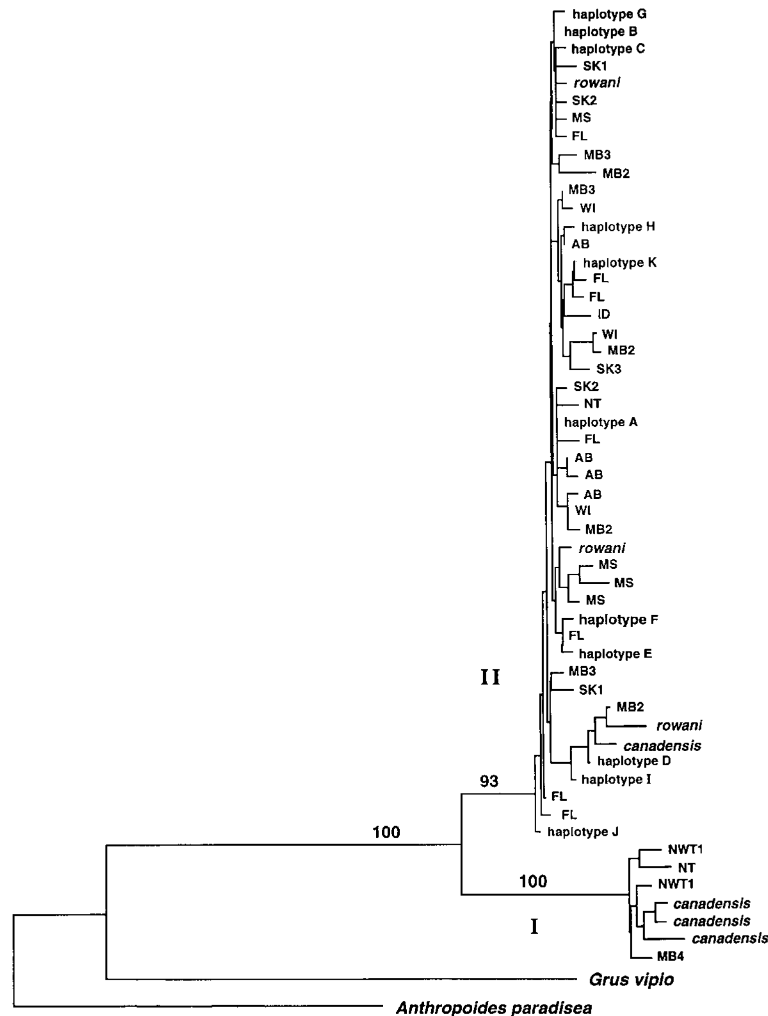

Figure 2. Neighbor-joining tree of unique sandhill crane haplotypes from mtDNA control region sequences. The tree was rooted with Stanley (*Anthropoides paradisea*) and white-naped (*Grus vipio*) cranes. Samples are denoted by the localities where they were found and haplotypes with ≥2 individuals are designated A–J (see Tables 1 and 2 for details). Bootstrap values over 65 are noted above nodes.

this pattern of phylogeographic structuring was the clustering of two *G. c. canadensis* with cranes in lineage II – one bird was from the Akron Zoo (denoted *canadensis* based on morphology) and one from Fort Rankin, Nunavut (NT) (Figure 2). The split between lineages is substantial; differences among haplotypes ranged from 4.3% to 6.4% (average 5.3%). Within lineages, percent divergence among haplotypes is relatively shallow, averaging 0.87% (range: 0.6–1.1%) in lineage I and 0.64% (range 0.1–1.9%) in lineage II.

No other phylogeographic structure is evident. Fifty-four different haplotypes were found among 73 individuals; 43 (59%) sandhill cranes had unique haplotypes, with another 11 haplotypes being shared among birds within and among populations, and even among putative subspecies (Table 2). In fact, phylogenetic relationships among haplotypes do not coincide with subspecies boundaries or with geographic localities within subspecies in lineage II (Figure 2). The migratory subspecies (*G. c. tabida* and *G. c. rowani*) do not show any significant phylogenetic divergence from one another nor from those that are non-migratory residents in Florida (*G. c. pratensis*) and Mississippi (*G. c. pulla*).

Table 2. Geographic origin of sandhill cranes with shared haplotypes

Haplotype	Locality	Population	(n)	Subspecies	Designation
A	Saskatchewan	SK2, SK3	(2)	*G. c. rowani*	Canadian
	Manitoba	MB2	(2)	*G. c. rowani*	Canadian
	Northwest Territories	NWT2	(1)	*G. c. rowani*	Canadian
	Wisconsin	WI	(1)	*G. c. tabida*	Eastern Greater
B	Saskatchewan	SK4	(1)	*G. c. rowani*	Canadian
	Manitoba	MB3	(1)	*G. c. rowani*	Canadian
	Northwest Territories	NWT2	(1)	*G. c. rowani*	Canadian
	Idaho	ID	(1)	*G. c. tabida*	Western Greater
	Mississippi	MS	(1)	*G. c. pulla*	Mississippi
C	Saskatchewan	SK1, SK6	(3)	*G. c. rowani*	Canadian
D	Manitoba	MB2	(2)	*G. c. rowani*	Canadian
E	Saskatchewan	SK6	(1)	*G. c. rowani*	Canadian
	Manitoba	MB2	(1)	*G. c. rowani*	Canadian
F	Saskatchewan	SK7	(1)	*G. c. rowani*	Canadian
	Manitoba	Fall migration	(1)	*G. c. rowani*	Canadian
G	Manitoba	MB1	(1)	*G. c. rowani*	Canadian
	Wisconsin	WI	(1)	*G. c. tabida*	Eastern Greater
H	Saskatchewan	SK5	(1)	*G. c. rowani*	Canadian
	Idaho	ID	(1)	*G. c. tabida*	Western Greater
I	Saskatchewan	SK4	(1)	*G. c. rowani*	Canadian
	Idaho	ID	(1)	*G. c. tabida*	Western Greater
J	Idaho	ID	(1)	*G. c. tabida*	Western Greater
	Wisconsin	WI	(1)	*G. c. tabida*	Eastern Greater
K	Florida	FL	(2)	*G. c. pratensis*	Florida

Subspecies structure and gene flow

While phylogenetic analyses did not reveal divergence among subspecies, except for *G. c. canadensis*, an analysis of molecular variance did reveal highly significant genetic structure among subspecies (fixation index, $\phi_{ST} = 0.48$, $P < 0.0001$; Table 3). This high among-subspecies variance is largely due to the divergence between haplotype lineages I and II, but even when *G. c. canadensis* (essentially lineage I) is removed from the analysis, the among-subspecies variance for the remaining four subspecies is still statistically significant, although substantially reduced ($\phi_{ST} = 0.06$, $P < 0.01$) (Table 3). Thus, the majority of molecular variance (94%) in sandhill cranes is distributed among haplotypes within subspecies.

Pairwise comparisons of the fixation indices among subspecies revealed that populations of *G. c. tabida* and *G. c. rowani* are indistinguishable genetically ($\phi_{ST} = -0.007$, $P = 0.5$) (Table 4). On the other hand, there is significant structure for the non-migratory *G. c. pulla* and *G. c. pratensis* in all pairwise comparisons. Suprisingly, these two subspecies, considered the same until recently (Aldrich 1972), were more distinct from one another than either was from *G. c. tabida* or *G. c. rowani*. The number of female migrants/generation estimated from methods based on ϕ_{ST} and maximum likelihood differ somewhat, but the same patterns of gene flow emerge from these analyses: virtually no exchange between *G. c. canadensis* and the other four subspecies, but substantial gene flow between between *G. c. tabida* and *G. c. rowani* (Table 5). The maximum likelihood method estimates gene flow in both directions between each pair of subspecies. This asymmetric analysis, which takes genealogy into account, indicates that most gene flow is from *G. c. tabida* to the controversially defined *G. c. rowani*. The only other relatively high estimate of

Table 3. Analysis of molecular variance (AMOVA) of mitochondrial DNA haplotype variation of sandhill crane subspecies

Source of variation	d.f.	Variance components	% of variation	*P*	Fixation index (ϕ_{ST})
Lineages I and II					
Among subspecies	4	2.48	48.5	<0.0001	0.48
Within subspecies	68	2.63	51.5	<0.0001	
Lineage II only					
Among subspecies	3	0.15	6.5	<0.01	0.06
Within subspecies	60	2.10	93.5	<0.0001	

exchange between subspecies is from *G. c. pratensis*, the resident Florida population, to *G. c. tabida* (Table 5).

Table 4. Subspecies pairwise fixation indices (ϕ_{ST})

	canadensis	*rowani*	*tabida*	*pratensis*
G. c. rowani	0.719**	–		
G. c. tabida	0.635**	−0.007	–	
G. c. pratensis	0.569*	0.159†	0.107†	–
G. c. pulla	0.621*	0.080†	0.068†	0.285‡

**$P < 0001$, *$P < 0.001$, ‡$P < 0.01$, †$P < 0.05$.

Genetic variation, population expansion and effective population size

Even though sample sizes for some taxa were small, estimates of genetic variability did not change systematically with sample size (Table 6). There was no evidence for reduced genetic variation in those subspecies considered Threatened or Endangered compared to those with robust populations. For instance, indices that reflect current levels of diversity, such as haplotype diversity (high for all subspecies), number of pairwise differences (k) and nucleotide diversity (π) did not differ significantly among subspecies (Table 6). Overall diversity at the nucleotide level (π), however, is low in all subspecies. If levels of variability are estimated taking geneological relationships into account, *G. c. canadensis* (lineage I) appears to be more diverse ($\theta_{MHMC} = 1.573$) than subspecies in lineage II (overall $\theta_{MHMC} = 0.339$; range from a low of 0.074 for *G. c. pulla* to a high of 0.356 for *G. c. tabida* (Table 6). These levels of variation translate to low estimates of female genetic effective population size N_{ef} relative to current census population estimates (Tables 1, 6). For instance, N_{ef} of *G. c. canadensis*, corrected for generation time, is only 18,500. If it is assumed that about 30% of a crane population is breeding females, then the population size of lesser sandhill cranes is estimated at only 61,600 compared to over 400,000 estimated from census data. The disparity between genetic population size and census estimates is similar though not as dramatic for all subspecies except *G. c. pulla*. In the case of the Mississippi population, genetic data imply a larger population size (approximately 2,900) than currently exists in the wild (∼150 individuals).

To test for recent population expansion as a possible explanation for current low levels of genetic diversity, mismatch distributions were estimated for haplotypes in lineages I and II. The small number of haplotypes in lineage I make it difficult to estimate the theoretical distribution of mismatches under the sudden population expansion model, but the observed pattern of mismatches is suggestive of a unimodal distribution (Figure 3a). For haplotypes in lineage II, the mismatch distribution was unimodal, suggesting an expected sudden population expansion in the recent past (Figure 3b). A significant negative value for Tajima's (1989) D statistic ($D = -1.92$, $P < 0.02$) for lineage II also supports the hypothesis of population growth (Rogers et al. 1996). A pattern of rapid population expansion also pertains to the combined *G. c. tabida* and *G. c. rowani*, reflecting the apparent lack of differentiation between them ($D = -1.68$, $P < 0.05$) (Figure 3c).

From the mismatch analysis, the timing of population expansion can be estimated at $t = \tau/(2u)$ generations ago. For our data, τ was estimated at 3.063 for lineage II, u averaged 3.4×10^{-6}/site for 650bp of control region sequence, or 2.21×10^{-3} (range: 1.50

Table 5. Maximum likelihood estimates of asymmetric migration ($2N_{ef}M_f$) among subspecies; migration rate estimated from ϕ_{ST} (no directionality implied) in brackets above the diagonal

To	From				
	canadensis	*rowani*	*pratensis*	*pulla*	*tabida*
G. c. canadensis	–	0.000003 (0.02)	0.000003 (0.03)	0.000012 (0.4)	2.0 (0.3)
G. c. rowani	0.000019	–	0.0 (5.8)	1.7 (2.6)	71.2 (∞)
G. c. pratensis	0.000001	0.4	–	7.2 (1.9)	0.4 (6.8)
G. c. pulla	0.000001	9.8	1.9	–	4.0 (4.1)
G. c. tabida	0.000008	8.6	38.9	0.9	–

Table 6. Measures of genetic diversity (± SD) summarized for sandhill crane lineages and subspecies. Female genetic effective population size (N_{ef}) estimated from θ_{MHMC}

Subspecies	(n)	Polymorphic sites (s)	Pairwise differences (k)	Haplotype diversity (h)	Nucleotide diversity[‡] (π)	θ_{MHMC}	N_{ef}	N_{ef}*
Lineage I								
canadensis	7	15	5.7 ± 3.1	1.00 ± 0.08	0.0087 ± 0.0055	1.5727 ± 1.0947	231,300	18,500
Lineage II[†]	66	46	4.2 ± 2.1	0.99 ± 0.01	0.0064 ± 0.0035	0.3386 ± 0.0331	49,800	4,000
tabida	15	20	4.5 ± 2.3	0.98 ± 0.03	0.0069 ± 0.0041	0.3565 ± 0.0972	52,400	4,200
rowani	35	33	4.2 ± 2.1	0.97 ± 0.02	0.0065 ± 0.0037	0.0976 ± 0.0129	14,350	1,150
[*tabida-rowani*]	50	37	4.3 ± 2.2	0.98 ± 0.03	0.0066 ± 0.0037	0.1535 ± 0.0187	22,600	1,800
pratensis	9	10	3.1 ± 1.8	0.97 ± 0.06	0.0048 ± 0.0031	0.0867 ± 0.0397	12,750	1,000
pulla	5	9	4.6 ± 2.7	1.00 ± 0.13	0.0071 ± 0.0049	0.0740 ± 0.0437	10,900	900

[‡]Analogous to $\theta = 2N_{ef}\mu$ (Watterson 1975; Tajima 1983).
*Corrected for generation time (12.5 years).
[†]Includes two *canadensis* haplotypes.

$\times 10^{-3}$ to 2.92×10^{-3}), and t is about 690 generations (range: 525 to 1020 generations).

Discussion

Phylogeny of sandhill crane haplotypes

Although six sandhill crane subspecies are currently recognized on the premise that clinal variation in size, slight variation in plumage coloration, and/or distribution of breeding populations are sufficient for diagnosis, phylogenetic analysis of mtDNA control region sequences indicate that only two distinct lineages of sandhill cranes have evolved. Lineage I is composed of arctic-nesting *G. c. canadensis* and lineage II, of all other subspecies. Control region data are not yet available for *G. c. nesiotes*, the Cuban sandhill crane, but analysis of 285 bp of the cytochrome-*b* gene amplified from two museum skins (USNM 172721 and USNM 211220) indicates that the Cuban subspecies is most similar to the one in Florida (C Krajewski, unpublished data). Once more samples are available, it will be interesting to see whether *G. c. nesiotes* also clusters with lineage II on the basis of control region data.

The deep split between *G. c. canadensis* in lineage I and *G. c. tabida* and *G. c. rowani* in lineage II is consistent with the fixed allelic difference in a pancreatic protein reported by Gaines and Warren (1984). Although lineage I consists only of *G. c. canadensis* haplotypes, two birds considered *G. c. canadensis* on the basis of morphology (one from the Akron Zoo and one from Rankin Inlet, NT) had haplotypes in lineage II. Mixed pairings between *G. c. canadensis* and *G. c. tabida*-*G. c. rowani* could well occur, as all three subspecies overlap in some regions during the winter and on spring migration (Tacha 1981). One possibility is that the split between lineages I and II may be degrading, due to secondary introgressive hybridization, resulting in transfer of haplotypes between *G. c. canadensis* and the other subspecies. Altern-

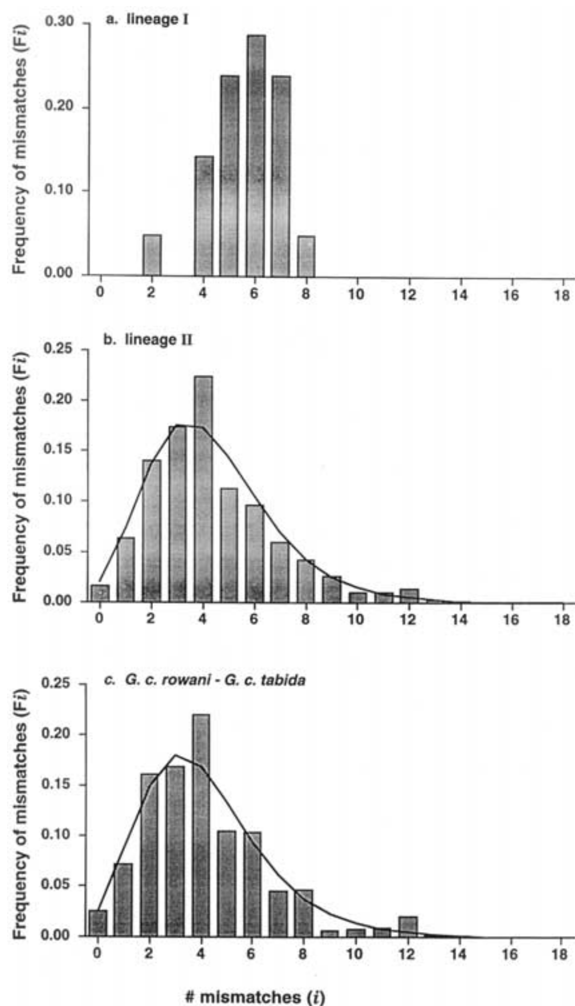

Figure 3. Pairwise nucleotide mismatch distributions for: a) haplotypes in lineage I; b) all haplotypes in lineage II; and c) *G. c. tabida* and *G. c. rowani* haplotypes. Solid lines indicate expected distributions under the sudden population expansion model of Rogers (1995).

atively, a more detailed sampling of lesser sandhill cranes across the arctic regions of North America could reveal a more extensive pattern of mixing of two highly divergent lineages, similar to that observed in snow geese (*Anser caerulescens*) (Quinn 1992). [Note that 11 lesser sandhill cranes sampled in 2000 from the Yukon-Kuskokwim Delta in western Alaska all belong to lineage I].

Divergence dates and biogeography

Divergences for control region domains I and II were obtained for sequences from all crane species (M Fain, unpublished data), and a rate of 3.4%/my was calculated relative to divergences from cytochrome-*b*. This was previously calibrated to dates estimated from the crane fossil record (Wetmore 1928) and is consistent with a late Miocene – early Pliocene gruine radiation (Krajewski and King 1996). Divergence of the two haplotype lineages for sandhill cranes was estimated at approximately 1.5 Mya (average of 5.3% between lineages). Examination of rates of change within each lineage revealed that lineage I substitutions accumulated approximately 1/3 faster than those in lineage II. Using the magnitude of this disparity to bracket the estimated average crane rate, we obtain a range from 2.3%/my to 4.5%/my resulting in dates of divergence from 1.2 to 2.3 Mya. This is consistent with a late-Pliocene – mid-Pleistocene split, on the order of that estimated for speciation between many North American avian species (Klicka and Zink 1997; Avise and Walker 1998). In other words, the level of genetic divergence between *G. c. canadensis* and the other subspecies of sandhill cranes would be consistent with that between full species.

Within lineages, coalescent times were estimated at approximately 256,000 (190,000–380,000) years ago in lineage I and 188,000 (140,000–280,000) years ago in lineage II. These coalescent times fall within the timeframe associated with Late Pleistocene glacial cycles, suggesting that fragmentation of widespread sandhill crane populations could have occurred in glacial refugia, followed by range expansion during interglacial periods (Klicka and Zink 1997). Lending support to this scenario is the concordant pattern of geographic partitions in the mtDNA gene tree of similarly distributed subspecies of Canada goose (*Branta canadensis*) (Van Wagner and Baker 1990; Baker and Marshall 1997). The Canada goose also exhibits intraspecific geographic variation in morphological variation, with small-bodied subspecies nesting in the arctic and large-bodied subspecies nesting farther south. Two distinct genetic lineages corresponding to small- and large-bodied subspecies were also found, along with paraphyly of one of the small-bodied subspecies which Baker and Marshall (1997) attribute to hybridization between subspecies. Suprisingly, divergence between Canada goose lineages was estimated to occur in a similar timeframe to that of sandhill cranes, approximately 1.2 Mya in the Pleistocene, suggesting a potential common cause. Baker and Marshall (1997) suggest that divergence in mtDNA sequence between the two lineages was probably promoted by geographic isolation between arctic-nesting birds and those breeding farther south.

The two highly divergent lineages in sandhill cranes and Canada geese could represent populations that were separated for a long period of time on either side of Beringia (Zink et al. 1995). Zink et al. (1995) documented similar levels of trans-Berigian intraspecific divergence for several avian taxa. Sandhill cranes still nest in Siberia, while Canada geese nest on islands in the Bering Sea and were thought to have nested in Siberia into the 19th century (Dement'ev et al. 1967). Siberian nesting populations could have subsequently expanded into arctic North America, resulting in the deep split between arctic and southern nesting birds observed today.

Morphological divergence between arctic and southern subspecies of Canada geese has been attributed to selection for small body size, rapid development and short fledging time in the arctic environment (Baker and Marshall 1997). Baldwin (1977) used similar reasoning to invoke selection for small body size in arctic-nesting *G. c. canadensis*. On the other hand, clinal variation in morphology among avian populations has also been shown to have a significant environmental component (James 1983). Considerable variation in environmental conditions between arctic and southern nesting areas could have contributed to geographic patterns of morphological variation in sandhill cranes.

Phylogeography and population structure

The shallow phylogenetic structure among putative subspecies in lineage II was initially a suprising result; we expected to find phylogeographic differentiation, at least between the migratory (*G. c. tabida-G. c. rowani*) and non-migratory (*G. c. pratensis* and *G. c. pulla*) subspecies. However, one haplotype (B, Table 2) is shared among birds of three different subspecies: *G. c. tabida* (western), *G. c. rowani*, and the critically endangered *G. c. pulla* in Mississippi.

Quite apart from the issue that insubstantial criteria are often used to define subspecies, lack of concordance between haplotype variation and even well-defined subspecies delineations based on morphology, is not uncommon for North American avian taxa (Ball and Avise 1992; Zink 1996, 1997; Baker and Marshall 1997). These authors suggest that relatively recent range expansions and increases in population size will result in a haplotype tree that is unstructured, with the average time to coalescence of haplotypes, such as those we observed within lineage II, being longer than the time since the population expanded. As a result, incomplete lineage sorting would make it difficult to elucidate recent subspecific splits with a mtDNA control region tree (Baker and Marshall 1997). Thus, lack of mtDNA sequence divergence among subspecies in lineage II may be the result of recent (post-Pleistocene) separation.

A relatively recent population expansion in an unstructured population can be inferred from an analysis of the distribution of pairwise genetic differences or mismatch distribution, if a plot of the frequency of mismatched sites between haplotypes is unimodal (Rogers and Harpending 1992; Rogers 1995; Rogers et al. 1996). We tabulated the mismatch distributions of haplotypes within lineages I and II and there is a reasonable fit to the theoretical distribution under this hypothesis (Figure 3), very similar to that observed for other avian taxa (Zink 1997). According to this analysis, the timing of population expansion occurred anywhere between 525 to 1020 generations ago. If a generation time of 12.5 years is assumed (Krajewski and Wood 1995), this translates to a rapid population expansion of lineage II sandhill cranes about 6500 to 12,750 years ago – consistent with a post-Pleistocene separation of *G. c. tabida-rowani*, *G. c. pratensis* and *G. c. pulla*. Wood and Krajewski (1996) attributed a similar pattern of mtDNA variation among Sarus crane (*Grus antigone*) subspecies to lack of longterm isolation.

To assess relatively recently evolved population genetic variation, we did an analysis of molecular variance, using variation within and among haplotypes of each subspecies. There are statistically significant differences among most subspecies not evident from the phylogenetic analysis. The one exception is the apparent panmixia of greater sandhill cranes *G. c. tabida* with the relatively recently delineated (Walkinshaw 1965) intermediate Canadian subspecies, *G. c. rowani*. The mismatch distribution of *G. c. tabida-rowani* was similar to the distribution for all lineage II haplotypes under a rapid population expansion scenario (Figures 3b, 3c).

Recent demographic history

Additional insight into current patterns of genetic variation can be gained by considering recent history of the sandhill crane breeding distribution in North America, migration patterns, population status, and reproductive strategies. Walkinshaw (1949) summarized what was known about sandhill crane breeding distributions pre- and post-1920. At that time, only *G.*

c. canadensis, *G. c. tabida*, *G. c. pratensis*, and *G. c. nesiotes* were recognized taxonomically (Figure 1a). Prior to 1920, *G. c. tabida* (greater sandhill cranes) bred across the mid-continent and as far south as Arizona and New Mexico, and the breeding distribution of *G. c. pratensis* extended from central Florida and southern Georgia, along the Gulf coast to east Texas (Figure 1a). By early in the 20th century, *G. c. tabida* had disappeared from most of its range. It was extirpated from many regions by the 1930s and 1940s, reduced to only 25 breeding pairs in Wisconsin (currently a stronghold of eastern greater sandhill cranes) and to only a few hundred birds in western regions (Meine and Archibald 1996). These populations have rebounded to about 30,000–40,000 birds in the east and to about 20,000–30,000 birds in the west, despite a reproductive strategy of delayed sexual maturity (average age at first successful reproduction is approximately five years, Tacha et al. 1989), long generation time (approximately 12.5 years) and low fecundity (lay two eggs but usually raise only one chick per year, Miller 1973). Such rapid population increases in eastern and western populations of *G. c. tabida* in only four to five generations suggest that, in addition to demographic factors, dispersal of birds from other regions may have occurred. Low estimates of population size based on N_{ef} compared to current census estimates, in conjunction with lack of differentiation of *G. c. tabida* from the putative *G. c. rowani*, lend support to this possibility.

Sandhill cranes from many breeding populations across North America and Siberia overlap on migration staging areas and wintering grounds where some pair formation takes place (Tacha et al. 1992). There is little information on levels of philopatry in migratory sandhill cranes, although one long-term field study of *G. c. tabida* in the western United States indicated that there is little or no male-biased gene flow and possibly very weak female gene flow (R Drewein, personal communication). If female gene flow is more extensive than perceived, as suggested by estimates of $N_{ef}m_f$ in this study, particularly between *G. c. tabida* and *G. c. rowani*, this could explain some of the lack of phylogeographic structure within lineage II.

Gene flow between migratory and non-migratory populations is also possible in that birds from eastern populations of *G. c. tabida* overlap during the winter with *G. c. pratensis* resident in Florida – asymmetric estimates of gene flow support this scenario. *G. c. tabida* also overlaps with *G. c. rowani* populations wintering on the Gulf coast where non-migratory populations once resided until the early 1900s (Walkinshaw 1949). Our observation that one *G. c. pulla* individual in the resident Mississippi population had the same haplotype as birds considered *G. c. tabida* or *G. c. rowani* lends support to this possibility. On the other hand, speculation that *G. c. pulla* may be a remnant population of *G. c. pratensis* appears unfounded, based on the analysis of molecular variance. Although the Florida subspecies was once considered to breed as far west as coastal Texas until 1900 and Louisiana until 1919 (Meine and Archibald 1996), the distribution was disjunct between populations in central Florida and southern Georgia and those in Alabama, Mississippi, Louisiana and Texas (Walkinshaw 1949). Another species with a geographic distribution similar to that once observed for resident populations of sandhill cranes, the mottled duck (*Anas fulvigula*), also has a clear phylogeographic split between resident populations in Florida and those in Louisiana and Texas (McCracken et al. 2001, in press).

Implications of taxonomic classification for management and conservation

For a species that varies clinally or has broadly intergrading subspecies, identifying the origin of migrating and wintering birds becomes a matter of probability (Storer 1983). Because of this, Tacha (1981) concluded that management (for hunting) of mid-continental sandhill cranes (*G. c. canadensis*, *G. c. rowani*, *G. c. tabida*) by subspecies is not realistic. Avise and Ball (1990) and Ball and Avise (1992) suggest that subspecies designations should be reserved for those intraspecific groups for which major historical separations are evident – lineage I (= *G. c. canadensis*) and lineage II (*G. c. tabida, G. c. rowani, G. c. pulla, G. c. pratensis*) in the case of sandhill cranes. In the strictest sense, only these two lineages would be considered Evolutionary Significant Units (ESUs) (*sensu* Moritz 1994) for long-term management and conservation, because they display historical isolation and "evolutionary potential".

It is possible that analysis of nuclear microsatellite loci would indicate that there are separate Management Units (MUs, *sensu* Moritz 1994) within lineage II, that coincide with current subspecies boundaries and would be useful for short-term management goals. On the other hand, Ball and Avise (1992) maintain that short-term population separations not be given taxonomic recognition as subspecies, in part, because

sensitive genetic markers, such as microsatellites, have the potential to elucidate significant structure down to the familial level. This makes it difficult to determine where to draw the line in terms of distinct taxonomic entities. Wenink et al. (1996) echo this sentiment. Although supportive of the idea of using faster evolving genetic markers such as microsatellites to delineate more subtle population subdivisions when phylogenetic structure of rapidly evolving mtDNA control region sequences are shallow, they point out that such markers are likely to produce relatively recently evolved allelic frequency differences that are not diagnostic. Avise (1995) predicted that little difference in mtDNA and nuclear DNA structure would occur among populations in species with no gene flow via the males (i.e. strong male philopatry), even if low levels of female gene flow occur.

Can sandhill crane subspecies in lineage II be considered separate Management Units given the available data? A subsequent analysis of molecular variance did elucidate some genetic differentiation among all subspecies in lineage II, except *G. c. tabida* and *G. c. rowani*. Molecular evidence suggests that female gene flow ($N_{ef}m_f$) between these two subspecies has been more extensive than perceived. In light of this, and the long and inconclusive debate over whether migratory sandhill cranes can be distinguished morphologically (Johnson and Stewart 1973; Tacha et al. 1985), we suggest that recognition of *G. c. rowani* be abandoned, that sandhill cranes breeding in the prairie provinces of Canada be allocated to *G. c. tabida*, and that more extensive field sampling be undertaken to identify the precise distributional limits of *G. c. canadensis* and *G. c. tabida* in Canada. Management of crane populations in central Canada and the coterminous United States would be better focussed on regional nesting areas and their link to different wintering areas – from our genetic analyses, it is unlikely that a genetic approach will be useful in delineating MUs for these birds. [Note, however, that a reanalysis of morphological characters is currently underway for the *G. c. canadensis-rowani-tabida* group by two of us, J Austin and DH Johnson with G. Krapu, unpublished data].

Although we recommend the abandonment of *G. c. rowani* as a separate subspecies, we are reluctant to make the same taxonomic recommendation for the two non-migratory subspecies in lineage II, *G. c. pratensis* in Florida and *G. c. pulla* in Mississippi. The same detailed morphological analysis has not been done for these taxa and we feel that these populations require more scrutiny of a larger sample of birds before any recommendations are made. The concept of a Distinct Population Segment (DPS) has been applied in two ways in the United States: (1) it corresponds to an ESU; or (2) it simply reflects a political boundary, which may be appropriate for management but does not necessarily delineate an ESU (National Research Council 1995). This latter intrepretation may have little or no scientific basis but has gained recent support for some taxa ("geopolitical species" of Karl and Bowen 1999). Obviously other characters, such as morphological and behavioral traits, can also reflect underlying genetic subdivisions (Lynch 1996), and their concordance with allelic frequency differences could help delineate geographically defined populations, such as those sandhill cranes resident in Mississippi and Florida. At this stage in the analysis, these non-migratory subspecies appear to be DPSs based on the analysis of molecular variance of their mtDNA haplotypes (somewhere between (1) and (2) above in terms of their evolutionary history), thus supporting their distinction as separate entities worthy of continued conservation efforts on their behalf.

Where microsatellite analysis could be especially beneficial would be in documenting possible genetic inbreeding in the Mississippi population, *G. c. pulla*. Based on our small sample (only five individuals), haplotype diversity remains high, but nuclear allelic diversity may be low due to genetic drift. At the same time, genetic drift in a small population such as *G. c. pulla*, could magnify the extent of its divergence from other subspecies. The Mississippi population was reduced to 40 birds by the 1960s and has rebounded to only about 150 individuals with the release of captive-bred birds (Aldrich 1972; Meine and Archibald 1996). Levels of genetic diversity within this small population could, therefore, be quite low depending on how skewed the breeding strategy has been in pairing captive individuals (Ballou and Lacy 1995). Although the link between loss of genetic diversity and inbreeding depression is difficult to substantiate (Caughley 1994), reproductive success of these released sandhill cranes has been poor and the population has consistently fallen below replacement levels (Meine and Archibald 1996). One possibility in the case of such demographic reduction is to supplement a captive breeding population with some individuals from other closely related populations (Avise and Nelson 1989). The presence of a widespread mtDNA haplotype in one Mississippi sandhill crane suggests a closer historical genetic connection to *G.*

c. tabida-rowani than to the non-migratory *G. c. pratensis* in Florida. This could prove important if future conservation plans include an expanded captive breeding effort to increase genetic diversity in this population.

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