The Auk 113(3):655–663, 1996

MITOCHONDRIAL DNA SEQUENCE VARIATION AMONG THE SUBSPECIES OF SARUS CRANE (*GRUS ANTIGONE*)

TIMOTHY C. WOOD[1] AND CAREY KRAJEWSKI[2]

Department of Zoology, Southern Illinois University, Carbondale, Illinois 62901, USA

ABSTRACT.—We examined DNA sequence variation in an 1,831 base-pair segment of the mitochondrial DNA genome from representatives of the three subspecies of Sarus Crane (*Grus antigone*). The sequences include the entire cytochrome-*b*, $tRNA^{Thr}$, $tRNA^{Pro}$, and ND6 genes, as well as three short intergenic spacer regions. Nine distinct haplotypes were identified in a sample of nine individuals, three each from the Indian (*G. a. antigone*), Burmese (*G. a. sharpei*), and Australian (*G. a. gillae*) subspecies. Phylogenetic analysis revealed that although Sarus Crane haplotypes form a monophyletic assemblage relative to Brolga (*G. rubicunda*) and White-naped Crane (*G. vipio*) outgroups, they cannot be resolved onto a dichotomously branching tree. A minimum-length network for the Sarus Crane haplotypes reveals at least one instance of direct ancestry and one hard polytomy, but shows no phylogeographic partitioning of haplotypes among subspecies. Net sequence divergence among subspecies is not significantly different from zero. Estimated sequence divergence times, neutral coalescent times, and data on the Quaternary geology of Australasia suggest that Sarus Cranes colonized Australia during the late Pleistocene. *Received 25 October 1995, accepted 10 December 1995.*

SARUS CRANES (*Grus antigone*) are among the largest members in the crane family (Gruidae), sometimes reaching an adult height of 1.8 m. They are nonmigratory and currently found in isolated areas of northwestern India, Southeast Asia, and Australia. Blyth and Tegetmeier (1881) classified the Indian and Burmese Sarus Cranes as distinct species on the basis of plumage and body-size characteristics (the larger Indian Sarus Crane has a bright white neck ring and white tertials), and this distinction persisted through the classification of Sharpe (1894). Blanford (1895), however, reduced the Indian and Burmese Sarus Cranes to subspecies (*G. a. antigone* and *G. a. sharpei*, respectively), and this convention endured through all subsequent revisions. Sarus Cranes were first observed in Australia in 1966, and were then considered members of *G. a. sharpei* (Gill 1969, Archibald 1981). Schodde (1988) designated the Australian Sarus Crane as a distinct subspecies (*G. a. gillae*) on the basis of its darker plumage and larger ear patch.

The range of the Sarus Crane has been dramatically reduced by human activity during the past century. Once widely distributed across India, Nepal, Bangladesh, and western Burma, the Indian Sarus Crane is now restricted to portions of northwestern India and the Terai lowlands of Nepal (Johnsgard 1983). Until the late 1940s, Burmese Sarus Cranes were found in southern China, eastern Burma, Philippines, the Malay Peninsula, Thailand, Cambodia, Laos, and Vietnam (Delacour and Mayr 1946, Walkinshaw 1973, Meine and Archibald unpubl. report) but have now been extirpated from all but the latter three areas (Medway and Wells 1976, Madsen 1981, Yang 1991). The Australian Sarus is currently found in northern Queensland from Normanton north to Aurukun, east to the Atherton Tableland, and west to Kunnunurra in Western Australia.

Although confidence in the anatomical and geographic distinctness of Sarus Crane subspecies has increased in recent years (Meine and Archibald unpubl. report), questions have arisen regarding their genetic distinctness and evolutionary history. Dessauer et al. (1992) documented a relatively low allozyme heterozygosity ($H = 0.024$) in a sample of nine Australian Sarus Cranes, but they did not assay the other subspecies. In contrast, Krajewski and Wood (1995) identified a distinct mitochondrial haplotype in each of three individuals representing each Sarus Crane subspecies, with sequence di-

[1] Present address: Laboratory of Medical Genetics, University of Alabama, Birmingham, Alabama 35294, USA.

[2] Address correspondence to this author. E-mail: careyk@siu.edu

TABLE 1. Specimen information for DNA samples.

Sample no.[a]	Donor institution[b]	Donor no.[c]
	Grus vipio	
1	ICF	ICF 10-2
	G. rubicunda	
8	ICF	ICF 9-8
247[d]	Museum of Victoria	MV 790
	G. antigone antigone	
77	ICF	ICF 8-45
222	St. Louis Zoo	A09023
252	Miami Metro Zoo	A00225
	G. antigone sharpei	
55	ICF	1[e]
56	ICF	2[e]
66	ICF	12[e]
	G. antigone gillae	
5	ICF	ICF 8-28
17	ICF	ICF 8-39
103	ICF	ICF 8-31

[a] DNA sample catalog numbers used by Krajewski laboratory at Southern Illinois University.

[b] ICF, International Crane Foundation, Baraboo, Wisconsin; Museum of Victoria, Melbourne, Australia; St. Louis Zoo, St. Louis, Missouri; Miami Metro Zoo, Miami, Florida.

[c] Specimen identification numbers employed by donor institutions.

[d] Partial cytochrome-*b* sequence from this specimen was reported by Leeton et al. (1995).

[e] Arbitrary numbers assigned to blood samples from captive birds in Thailand. No voucher data.

vergences of 0.7 to 1.5%. In this study, we examine mitochondrial DNA (mtDNA) sequence variation in a larger sample of individuals to assess the genetic and phylogeographic distinctness of the Sarus Crane subspecies.

METHODS

Experimental design.—Our previous study of sequence variation in cytochrome-*b*, $tRNA^{Thr}$, and $tRNA^{Pro}$ genes of Sarus Cranes revealed low levels of haplotype divergence (Krajewski and Wood 1995). We therefore extended our data set to include the adjacent NADH dehydrogenase subunit 6 (ND6) locus, suggested to be appropriate for recent divergences by Moum and Johansen (1992). Our intention was to identify variable sites in the sequences from two individuals of each subspecies, then assay as many of those sites as possible with restriction endonucleases for a larger sample of individuals. Preliminary analysis revealed, however, that divergences remained low even across the four-gene alignment, and that even an extensive restriction-site survey would detect less than one-half of the variable sites and fail to diagnose haplotypes. We therefore obtained the complete, four-locus sequence from a third individual of each subspecies. We employed sequence from two Brolgas (*G. rubicunda*) and one White-naped Crane (*G. vipio*) as outgroups; previous systematic studies (Archibald 1976, Krajewski 1989, Krajewski and Fetzner 1994) have shown that these species are the closest relatives of the Sarus Cranes. Table 1 provides specimen information for the samples we employed.

Technical protocols.—DNA was extracted from blood samples following the method outlined by Sambrook et al. (1989). Blood cells were diluted 1:10 with lysis buffer (10 mM Tris, 100 mM NaCl, 100 mM EDTA, 0.5% SDS), digested with proteinase K, and extracted with phenol, phenol-chloroform, and chloroform-isoamyl alcohol. Each sample was digested with RNAse A, redigested with proteinase, and re-extracted with phenol-chloroform and chloroform-isoamyl alcohol. Purified DNA was precipitated with cold, 95% ethanol, washed with 70% ethanol, and rehydrated in a storage buffer (10 mM Tris, 0.1 mM EDTA).

Portions of the cytochrome-*b*/ND6 region were amplified via the polymerase chain reaction (PCR; Innis and Gelfand 1990) using combinations of the primers shown in Figure 1. PCR reactions were performed in 100 μL volumes using 1.5 mM $MgCl_2$, 5 μM concentrations of each primer, 200 μM dNTPs, one unit of *Taq* polymerase, and 10–100 ng of template DNA. Thermal cycling began with a three-minute denaturation at 94°C, followed by 35 cycles of denaturation (94°C, 1 min), annealing (50–55°C, 1 min), and primer extension (72°C, 1 min). A final extension for 7 min at 72°C was included to minimize the number of partial PCR products. Most PCR products were sequenced directly following asymmetric re-amplification as described by Krajewski and Fetzner (1994). The 5′ portion of ND6, however, includes regions of secondary structure that prevented extension by the T7 polymerase. For these areas, we employed the CircumVent cycle sequencing system (New England Biolabs).

Data analysis.—Sequences were aligned manually using the chicken mitochondrial genome (Desjardins and Morais 1990) as a reference. Basic sequence manipulations (reverse complementation, conceptual translation, secondary structure analysis) were performed with Hitachi's DNASIS software. Coding sequences of cytochrome *b* and $tRNA^{Thr}$ are on the heavy (H) strand, those of $tRNA^{Pro}$ and ND6 are on the light (L) strand.

To explore relative rates of change among genes, divergences among haplotypes were computed separately for cytochrome-*b*, spacer regions and tRNAs, and ND6, using the proportion of mismatched bases (the *p*-value of Nei 1987) as a metric. We also tallied the numbers of transitions and transversions for each region using the MEGA 1.0 package (Kumar et al. 1993). To estimate multilocus divergences, we designated seven rate categories of sites: first, second, and third codon-positions in each of cytochrome-*b* and ND6 sequences, and tRNA sites (including spac-

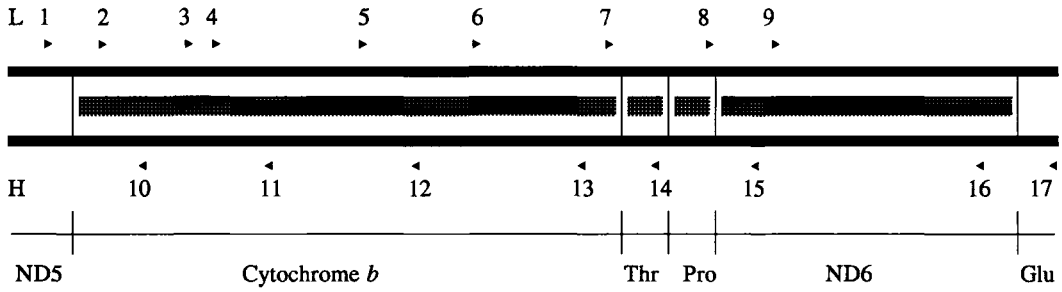

Fig. 1. Map of genes and primer locations. Scale is approximate. L and H refer to light and heavy strands, respectively, of mtDNA molecule. Regions sequenced in this study are shaded. Primers and sources are: (1) L14851 (Kornegay et al. 1993); (2) L14841 (Kocher et al. 1989); (3) L15087 (Edwards et al. 1991); (4) L15136 (Krajewski et al. 1992); (5) L15418 (Krajewski and Fetzner 1994); (6) L15615 (Krajewski and Fetzner 1994); (7) L16022 (Krajewski and Wood 1995); (8) L16152 (Krajewski and Wood 1995); (9) L16303 (5'-AACCCCACATGAATAAAACA-3', this study); (10) H14954 (Thomas et al. 1989); (11) H15149 (Kocher et al. 1989); (12) H15498 (Krajewski et al. 1992); (13) H15767 (Edwards et al. 1991); (14) H15915 (Edwards et al. 1991); (15) H16208 (5'-AGCTTGTACGAGGGTTGT-3', this study); (16) H16621 (5'-ATCCTTCACCGTACTATGGG-3', this study); (17) H16744 (Krajewski and Wood 1995).

ers). Relative rates for these categories were estimated as their relative proportions of variable bases, standardized to the lowest value (cytochrome-*b* second positions, see below). Pairwise distances (d_{MLC}) were computed using Felsenstein's (1992) DNAML model in the DNADIST program of PHYLIP 3.5, with the expected transition/transversion ratio set to 6 (the observed mean value for cytochrome-*b* and ND6 comparisons) and empirical base frequencies employed. This strategy for distance estimation was prompted by the results of Krajewski and King (1996), who showed that transition bias, codon-position rates, and nonuniform base composition were the major departures from random substitution in crane cytochrome-*b* sequences.

Gene trees were estimated using additive distance, parsimony, and maximum-likelihood methods. Distance analyses employed the weighted least-squares (Fitch and Margoliash 1967) and neighbor-joining (Saitou and Nei 1987) methods on d_{MLC} values. Resolution in distance trees was assayed by bootstrap resampling of sites. Because rate categories cannot be resampled with currently available software, bootstrapped distance analyses used DNAML distances computed without rate categories (d_{ML}). Krajewski and King (1996) showed that inclusion of rate categories did not alter the precision of distance trees for crane cytochrome-*b* sequences. Distance trees were obtained with the FITCH and NEIGHBOR programs of PHYLIP 3.5. Parsimony analysis employed equally weighted sites and substitutions with the branch-and-bound search algorithm DNAPENNY in PHYLIP 3.5. Given the small divergences among haplotypes, we did not employ differential weighting of positions or transversions (Krajewski and King 1996). Resolution in parsimony trees was assayed by bootstrap resampling using the DNAPARS program of PHYLIP 3.5. A maximum-likelihood estimate of haplotype relationships was obtained with the DNAML program of PHYLIP 3.5, using the transition and base-composition parameters described above (site-specific rate categories cannot be employed in this version of the program). Resolution on the DNAML tree is approximated by parametric confidence intervals on internodal branch lengths. Gene trees were estimated first separately from cytochrome-*b*, tRNA, and ND6 sequences to check for congruence of resolved nodes, then from a complete alignment of the multilocus data set.

All the phylogeny-estimation methods described above assume a strictly bifurcating tree, but such a tree may not apply to recently diverged haplotypes. In particular, the persistence of an ancestral sequence along with multiple descendant sequences derived from it will result in an unresolvable (i.e. "hard") polytomy. To assess the influence of this phenomenon, we manually constructed an unrooted, minimum-length network (Lansman et al. 1983) from parsimony-informative sites for Sarus Crane haplotypes. On such a network, haplotypes may occupy internal nodes, and polytomous branches may occur.

We estimated the net number of nucleotide differences (d_A) between subspecies as

$$d_A = d_{XY} - (d_X + d_Y)/2 \quad (1)$$

where d_X, d_Y, and d_{XY} are the average numbers of nucleotide substitutions for a randomly chosen pair of haplotypes from subspecies X, Y, and X and Y, respectively (Nei 1987:276–279). Variances of each d-value were computed from Nei's (1987) equations 10.23–10.26; d_A values within one standard deviation of zero were considered nonsignificant.

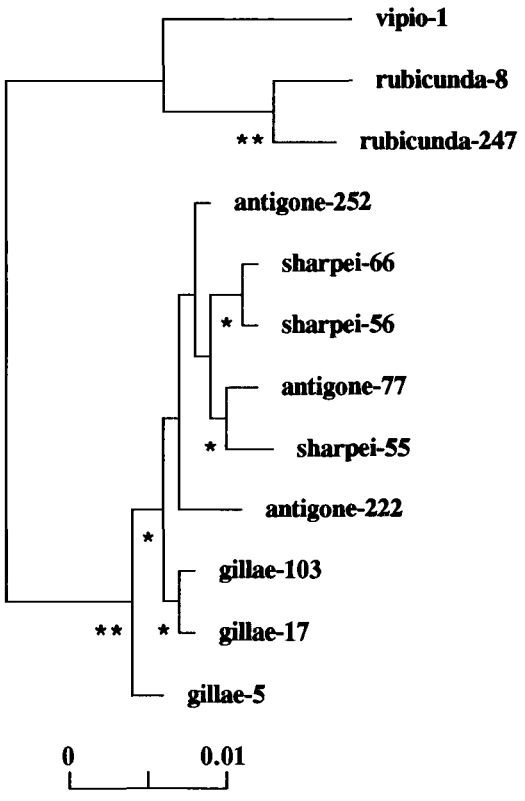

Fig. 2. Tree recovered by FITCH and NEIGHBOR algorithms for d_{MLC} distances from combined cytochrome-*b*, spacer, tRNA, and ND6 sequences. Branch length scale is substitution per site from FITCH analysis. Asterisks below branches indicate bootstrap resolution of >50% (*) and >90% (**), based on 200 resamplings.

RESULTS

Sequences.—The complete sequence alignment is 1,831 nucleotides, with specific regions as follows (5′ to 3′ order on the L strand): cytochrome *b* (1,143 bases), spacer (4 bases), $tRNA^{Thr}$ (70 bases), spacer (15–16 bases), $tRNA^{Pro}$ (70 bases), spacer (6 bases), and ND6 (522 bases). The spacer region between tRNA loci was not reported in the chicken (*Gallus gallus*) sequence of Desjardins and Morais (1990), but is found in all cranes and some other gruiforms (Krajewski unpubl. data). All sequences have been deposited in GenBank under accession numbers U11060 to U11065, U13622, and U43614 to U43625; a complete alignment is available from the authors. GenBank files contain corrections to previously published sequences. Relative rates for site categories were 2.6, 1.0, and 14.4 for cytochrome-*b* codon positions; 6.0 for tRNA and spacer positions; and 4.6, 2.3, and 18.4 for ND6 codon positions.

Haplotype variation.—Each of the 12 individuals examined in this survey possessed a distinct multilocus haplotype, although several had identical single-locus sequences. We designate haplotypes by specific or subspecific epithet and individual number (e.g. gillae-5 is the haplotype from *G. a. gillae* individual 5; see Table 1). Two pairs of haplotypes (sharpei-56 and sharpei-66, gillae-17 and gillae-103) are identical at all cytochrome-*b* sites, several have identical tRNA sequences, and one pair (antigone-222 and antigone-252) is identical for all ND6 sites.

Single-locus patterns.—Cytochrome-*b* distances (d_{MLC}) among Sarus Crane haplotypes are 0 to 0.011, and those among species are 0.034 to 0.050; there are 36 informative sites for parsimony analysis. Only two nodes, those showing monophyly of Brolga and Sarus Crane haplotypes, are recovered with high resolution (>90% bootstrap) on cytochrome-*b* trees (not shown). For tRNA and spacer regions, divergences among Sarus Crane haplotypes are 0 to 0.032, and those between species are 0.018 to 0.052; there are six parsimony-informative sites. Resolution is extremely limited on tRNA trees (not shown), but again monophyletic Brolga and Sarus Crane groups are recovered. ND6 divergences are 0 to 0.01 among Sarus Crane haplotypes, and 0.049 to 0.064 among species; there are 25 parsimony-informative sites. ND6 trees (not shown) again resolve Brolga and Sarus Crane groups, but reveal little structure among Sarus Crane haplotypes. There are no discordant patterns among single-locus trees for well-resolved groups. For weakly resolved groups (50–90% bootstrap), only sharpei-55 and sharpei-66 show different affinities with different genes.

Multilocus trees.—All phylogenetic analyses of the 1,831 base-pair alignment show the expected monophyly of Sarus Crane and Brolga haplotypes. The multilocus distance tree (Fig. 2; based on d_{MLC} values in Table 2) reveals somewhat more structure among Sarus Crane haplotypes than that from single loci, but suggests similar groupings. Gillae-5 appears as an early branch, and there are consistent pairings of gillae-17 with gillae-103, sharpei-55 with antigone-77, and sharpei-56 with sharpei-66. Parsimony analysis of all 67 informative sites (Table 3) yields three trees of 84 steps (consistency index = 0.80), the strict consensus of which is concordant with the distance trees at all nodes

TABLE 2. Sequence distances among haplotypes. Values above diagonal are mismatches per site (p-distances); values below diagonal are substitutions per site (d_{MLC}).

	1	2	3	4	5	6	7	8	9	10	11	12
1 vipio-1	—	0.037	0.038	0.038	0.040	0.040	0.040	0.040	0.040	0.038	0.042	0.038
2 rubicunda-8	0.043	—	0.009	0.034	0.036	0.036	0.038	0.038	0.037	0.036	0.039	0.035
3 rubicunda-247	0.044	0.010	—	0.035	0.035	0.034	0.038	0.034	0.036	0.037	0.038	0.034
4 gillae-5	0.045	0.040	0.041	—	0.004	0.004	0.007	0.006	0.006	0.006	0.007	0.003
5 gillae-17	0.048	0.042	0.041	0.005	—	0.002	0.007	0.004	0.004	0.006	0.005	0.002
6 gillae-103	0.048	0.042	0.040	0.004	0.002	—	0.007	0.003	0.003	0.005	0.004	0.002
7 sharpei-55	0.048	0.044	0.044	0.008	0.008	0.007	—	0.006	0.006	0.006	0.009	0.005
8 sharpei-56	0.048	0.045	0.040	0.007	0.004	0.004	0.006	—	0.002	0.006	0.006	0.003
9 sharpei-66	0.048	0.043	0.042	0.007	0.004	0.004	0.006	0.002	—	0.005	0.006	0.003
10 antigone-77	0.045	0.042	0.044	0.006	0.006	0.005	0.006	0.006	0.005	—	0.007	0.003
11 antigone-222	0.050	0.046	0.044	0.008	0.005	0.005	0.010	0.006	0.006	0.008	—	0.004
12 antigone-252	0.045	0.041	0.039	0.004	0.002	0.002	0.005	0.003	0.003	0.004	0.004	—

showing >50% boostrap values (Fig. 3). The DNAML tree (Fig. 4) has terminal clusters very similar to those on distance and parsimony trees, but shows a basal polytomy among Sarus haplotypes.

Parsimony network.—Only 7 of the 31 variable sites among Sarus Crane haplotypes are informative for parsimony analysis. These sites can be placed on a haplotype network with a minimum of 11 steps (Fig. 5). This network reveals a potential hard polytomy corresponding to nodes not resolvable by phylogenetic analysis. Moreover, antigone-252 is placed on an internal node directly ancestral to at least one other lineage. If this network reflects the actual mutation history of Sarus Crane haplotypes, it suggests an almost complete absence of phylogeographic structure. None of the possible rootings would result in all haplotypes from any subspecies forming a single lineage. However, a network on which all Australian or Burmese haplotypes are proximal to one another requires only one additional step (12 total), and a network with all three subspecies haplotypes in proximal groups requires only three additional steps (14 total). These topologies are not significantly longer than the minimum-length network according to Templeton's (1983) test.

Net divergence.—Because each individual sampled possessed a unique DNA sequence, we used one third as the within-population frequency of all haplotypes (Nei 1987:276). All estimated values of net divergences (d_A) among subspecies (Table 4) are less than one standard deviation from zero. Thus, assuming that the nine haplotypes are representative of each subspecies and that they occur in roughly equal frequencies, there is no evidence for genetic differentiation of subspecies at these loci.

DISCUSSION

Divergence rates of cytochrome b *and ND6.*—Few studies since that of Moum and Johansen (1992) have examined divergence of the ND6 sequence in birds, in contrast to the increasing

TABLE 3. Parsimony-informative sites for haplotypes examined. Dots indicate a match to vipio-1 sequence. Asterisks indicate sites occurring in spacer regions.

Haplotype	Cytochrome *b*	tRNA	ND6
		** *	
vipio-1	ACCATTCAGATCCTTGCATTTTACACTCTTGCTTTT	CTGCAG	CCCAAATCCTGGCACACACCCATAC
rubicunda-8	.....TG...TT..ATG....GT..C.C.AA..C.	.G..G.	T.T...CTT.AATG.G..T...CGG
rubicunda-247	...TC.TG...TT..ATG....G...C.C.AAC.C.	.G..G.	T..G..CTT.AATG.G..T...CGG
gillae-5	.TT.C...AGC..CC...CCCC.TGT.T.CAACC.C	T.AT..	.T..C....C.A..T.TG.TTG..A
gillae-17	CTTTCC..AGC..CC...CCCC.TGT.T.CAACC.C	T.AT..	.T..C....C.A..T.TG.TTGC.A
gillae-103	CTTTCC..AGC..CC...CCCC.TGT.T.CAACC.C	T.AT..	.T..C....C.A..T.TG.TTGC.A
sharpei-55	.TT.CC..AGC..CC...CCCC..GT.T.C.ACC.C	T.AT..	.TT.CC...C.A..T.TG.TTGC.A
sharpei-56	CTTTCC..AGC..CC...CCCC..GT.T.C.ACC.C	T.AT.T	.T.GCC...C.A..T.TG.TTGC.A
sharpei-66	CTTTCC..AGC..CC...CCCC..GT.T.C.ACC.C	T.AT.T	.TT.C....C.A..T.TG.TTGC.A
antigone-77	.TT.CC..AGC..CC...CCCC.TGT.T.C..CC.C	T.AT..	.TT.CT...C....T.TG.TTGC.A
antigone-222	CTTTCC..AGC..CC...CCCC.TGT.T.C.ACC.C	T.AT..	.T..C....C.A..T.TG.TTGC.A
antigone-252	.TTTCC..AGC..CC...CCCC.TGT.T.C.ACC.C	T.AT..	.T..C....C.A..T.TG.TTGC.A

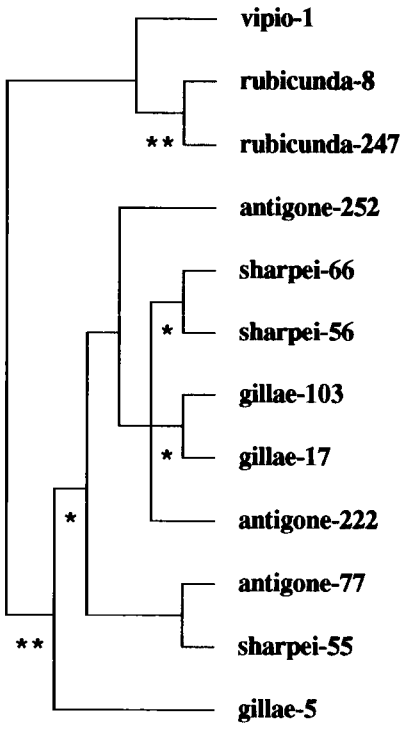

Fig. 3. Strict consensus of three equally parsimonious trees for combined cytochrome-*b*, spacer, tRNA, and ND6 sequences. Each requires 84 steps for 67 characters. Labeling conventions as for Figure 2.

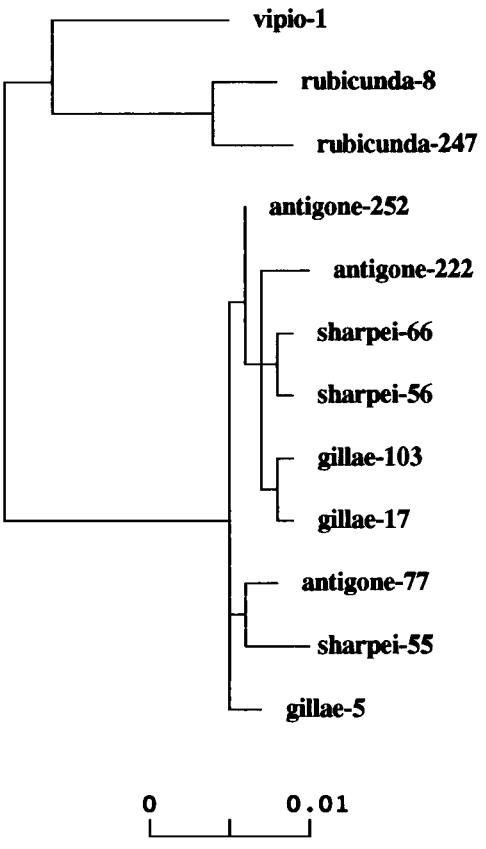

Fig. 4. Tree recovered by DNAML for combined cytochrome-*b*, spacer, tRNA, and ND6 sequences. Branch length scale is substitutions per site.

popularity of cytochrome *b* among molecular systematists. Moum and Johansen (1992) suggested that ND6 incorporates substitutions at a sufficiently rapid rate to be useful for intraspecific studies. Our results are consistent with this, but also reveal an interesting pattern relative to cytochrome *b*. Within species, divergence levels are comparable for both genes (approximately 0–1%). Among species, however, ND6 is distinctly more divergent (4.9–6.4%) than cytochrome *b* (3.4–5.0%). This pattern might reflect different evolutionary dynamics in the two genes, with extremely labile sites mutating at roughly equal rates early in divergence, followed by a more constrained rate of substitution in cytochrome *b*. Several authors (e.g. Irwin 1991, Krajewski and King 1996) have identified constraints on cytochrome *b* evolution, but no comparable analysis is available for ND6. Our results suggest that ND6 and cytochrome *b* are of similar utility for assaying intraspecific variation.

Sarus Crane phylogeography.—The apparent absence of phylogeographic structure in Sarus Crane haplotype trees implies that there has been no long-term geographic isolation of subspecies. Several caveats are attached to this con-

TABLE 4. Within-population (d_x, diagonal) and net between-population (d_A, below diagonal) divergences (±SD) among Sarus Crane subspecies based on mtDNA haplotypes.

	G. a. antigone	*G. a. sharpei*	*G. a. gillae*
G. a. antigone	0.00236 ± 0.00155		
G. a. sharpei	0.00159 ± 0.00510	0.00220 ± 0.00200	
G. a. gillae	0.00098 ± 0.00398	0.00330 ± 0.00377	0.00162 ± 0.00106

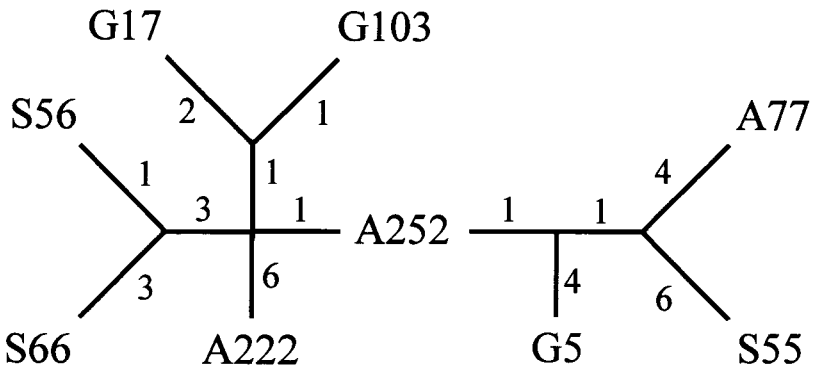

Fig. 5. Minimum-length network for Sarus haplotypes. Haplotypes are indicated by single-letter codes corresponding to subspecies of origin (A = *G. a. antigone*; G = *G. a. gillae*; S = *G. a. sharpei*). Numbers along branches represent nucleotide substitutions. Network requires 11 steps for 7 informative sites; autapomorphic changes are also shown to distinguish haplotypes.

clusion. Foremost is the problem of resolution. Given the small divergences and relatively high levels of homoplasy among Sarus Crane haplotypes, the gene tree cannot be estimated with high precision. Nevertheless, subspecies monophyly is not the best estimate of relationships for any optimality criterion employed (additive distance, parsimony, and maximum likelihood) or for any single-locus partition of the data. Another issue is sample size. It is unlikely that we have identified all Sarus Crane haplotypes from a sample of nine birds. Unsampled haplotypes might provide more structure to the mtDNA gene tree, perhaps even revealing clusters of closely related genes within one or more subspecies. Nonetheless, the relationships of the nine haplotypes assayed here, to the extent that they are accurate, preclude a pattern of reciprocal monophyly or paraphyly (Avise 1994) for subspecies mtDNAs. Unless new haplotypes substantially alter the topology of Figure 5, at least one haplotype within each subspecies will remain most closely related to one or more haplotypes from a different subspecies. A third caveat is that mtDNA is a single linkage group that reveals only matrilineal relationships. It is possible that other loci (e.g. from the nuclear genome) would reveal a stronger geographic pattern. Although the genetic basis for morphological differences among Sarus Crane subspecies is unknown, variation in genes influencing these traits may have a geographic component. Thus, our conclusion is not that the subspecies are "invalid," but only that they are not distinct on the basis of mtDNA variation.

Lack of long-term isolation is consistent with the historical distributions of Indian and Burmese Sarus Cranes. Until the mid-1900s, these subspecies had parapatric distributions bounded by the Godavari River in Bangladesh (Johnsgard 1983). Although nothing is known about the extent of gene flow across this boundary, allopatry is a very recent phenomenon. The duration of Sarus Crane isolation in Australia is much less clear. There are no described fossils of Australian Sarus Cranes and no recorded sightings prior to 1966. Archibald (1981) speculated that Sarus Cranes arrived in Australia as recently as the 1960s, a view seemingly at odds with the evolution of diagnostic morphological features.

An estimate of the time frame of Sarus Crane haplotype differentiation may be derived from estimates of coalescent times and times to reciprocal monophyly. There are currently no more than about 23,000 Sarus Cranes in the wild (Meine and Archibald unpubl. report). Applying demographic data from Whooping Cranes (Mirande et al. 1991) to Sarus Cranes, approximately 30% of all birds are breeding females (N_f), so N_f = 6,900. The mean coalescent time for neutral mtDNA haplotypes is $2N_f$ generations (Birky 1991), that is 2 × 6,900, or 13,800 generations in Sarus Cranes. The average generation time in gruine cranes is 12.5 years (Krajewski and Wood 1995), giving a coalescent time for Sarus Crane haplotypes of 13,800 × 12.5, or 172,500 years. The largest uncertainty in this calculation involves using the current Sarus Crane census as an estimate of long-term population size. An alternative approach to dating the coalescent is to root the network in Figure

5 at its midpoint (i.e. either the node occupied by antigone-252 or the node at which gillae-5 joins the network) and compute the mean genetic distance between all pairs of haplotypes at tips on opposite sides of the root. This value is 0.007 ± 0.002 (d_{MLC}, n = 15). Krajewski and King (1996) suggested that the maximum rate of cytochrome-*b* divergence in cranes is about 1.7% per million years (my). Because ND6 appears to evolve slightly faster than cytochrome *b*, this rate may be applied to the Sarus Crane divergences. Thus, the elapsed time since the common ancestor of all Sarus Crane haplotypes would be 0.7% divided by 1.7%/my, or 411,765 years. This suggests that the population size in the coalescent calculation is too small by a factor of 2.4.

A similar approach may be used estimate the duration of Sarus Crane isolation in Australia. Avise et al. (1984) showed that populations have polyphyletic mtDNA haplotypes when they have been isolated for fewer than N_f generations (assuming neutral haplotypes and a Poisson female fecundity model with mean 1.0). The current census size of Australian Sarus Cranes is no more than 10,000 birds and is possibly increasing (Meine and Archibald unpubl. report). Avise et al. (1984) demonstrated that in expanding populations the final value of N_f is a stronger determinant of lineage survivorship than the number of founders. It follows that Sarus Cranes have been isolated in Australia for fewer than

$$N_f = (0.3)(10{,}000) = 3{,}000 \text{ generations},$$

or 37,500 years. This time frame is consistent with Mayr's (1944) scenario for the origin of Australian-endemic avian subspecies. Mayr (1944) postulated that lowered sea levels created grasslands and marshy areas between islands of the Malay Archipelago and provided a dispersal route for Southeast Asian birds into Australia via Timor. Morley and Flenley (1987) provided geological evidence that lowered sea levels associated with worldwide glaciation during the last 100,000 years allowed land bridges to form between the Malay Peninsula and Borneo, with the last such disappearing some 18,000 years ago.

ACKNOWLEDGMENTS

We thank George Archibald, Larry Buckley, Jim Fetzner, and Claire Mirande for their assistance in various phases of this research, as well as the donor institutions in Table 1. Our study was supported by NSF grants BSR-9102482 and DEB-9419907 to C.K.

LITERATURE CITED

ARCHIBALD, G.W. 1976. Crane taxonomy as revealed by the unison call. Pages 225–251 *in* Crane research around the world (J. C. Lewis and H. Masatomi, Eds.). International Crane Foundation, Baraboo, Wisconsin.

ARCHIBALD, G. W. 1981. Introducing the Sarloga. Pages 213–215 *in* Crane research around the world (J. C. Lewis and H. Masatomi, Eds.). International Crane Foundation, Baraboo, Wisconsin.

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

AVISE, J. C., J. C. NEIGEL, AND J. ARNOLD. 1984. Demographic influences on mitochondrial DNA lineage survivorship in animal populations. Journal of Molecular Evolution 20:99–105.

BIRKY, C. W., JR. 1991. Evolution and population genetics of organelle genes: Mechanisms and models. Pages 112–134 *in* Evolution at the molecular level (R. K. Selander, A. G. Clark, and T. S. Whittam, Eds). Sinauer, Sunderland, Massachusetts.

BLANFORD, W. 1895. *Grus (antigone) sharpei.* Bulletin of the British Ornithologists' Club 5:7.

BLYTH, E., AND W. G. TEGETMEIER. 1881. The natural history of the cranes. Horace Cox, London.

DELACOUR, J., AND E. MAYR. 1946. Birds of the Philippines. Macmillan, New York.

DESJARDINS, P., AND R. MORAIS. 1990. Sequence and gene order of the chicken mitochondrial genome: A novel gene order in higher vertebrates. Journal of Molecular Biology 212:599–634.

DESSAUER, H.C., G. F. GEE, AND J. S. ROGERS. 1992. Allozyme evidence for crane systematics and polymorphisms within populations of Sandhill, Sarus, Siberian, and Whooping cranes. Molecular Phylogenetics and Evolution 1:279–288.

EDWARDS, S. V., P. ARCTANDER, AND A. C. WILSON. 1991. Mitochondrial resolution of a deep branch in the genealogical tree for perching birds. Proceedings of the Royal Society of London Series B 243:99–107.

FELESENSTEIN, J. 1992. PHYLIP (phylogeny inference package), version 3.5. Distributed by the author. Department of Genetics, University of Washington, Seattle.

FITCH, W. M., AND E. MARGOLIASH. 1967. Construction of phylogenetic trees. Science 155: 279–284.

GILL, H. B. 1969. First record of the Sarus Crane in Australia. Emu 69:49–52.

INNIS, M. A., AND D. H. GELFAND. 1990. Optimization of PCRs. Pages 3–12 *in* PCR protocols: A guide to methods and applications (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.). Academic Press, San Diego.

IRWIN, D. M., T. D. KOCHER, AND A. C. WILSON. 1991. Evolution of the cytochrome *b* gene of mammals. Journal of Molecular Evolution 32:128-144.

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

KOCHER, T. D., W. K. THOMAS, A. MEYER, S. V. EDWARDS, S. PÄÄBO, F. X. VILLABLANCA, AND A. C. WILSON. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences USA 86:6196-6200.

KORNEGAY, J. R., T. D. KOCHER, L. A. WILLIAMS, AND A. C. WILSON. 1993. Pathways of lysozyme evolution inferred from the sequences of cytochrome *b* in birds. Journal of Molecular Evolution 37:367-379.

KRAJEWSKI, C. 1989. Phylogenetic relationships among cranes (Gruiformes: Gruidae) based on DNA hybridization. Auk 106:603-618.

KRAJEWSKI, C., A. C. DRISKELL, P. R. BAVERSTOCK, AND M. J. BRAUN 1992. Phylogenetic relationships of the thylacine (Marsupialia: Thylacinidae) among dasyuroid marsupials: Evidence from cytochrome *b* DNA sequences. Proceedings of the Royal Society of London Series B 250:19-27.

KRAJEWSKI, C., AND J. W. FETZNER, JR. 1994. Phylogeny of cranes (Gruiformes: Gruidae) based on cytochrome-*b* DNA sequences. Auk 111:351-365.

KRAJEWSKI, C., AND D. G. KING. 1996. Molecular divergence and phylogeny: Rates and patterns of cytochrome *b* evolution in cranes. Molecular Biology and Evolution 13:21-30.

KRAJEWSKI, C., AND T. C. WOOD. 1995. Phylogenetic relationships within the Sarus Crane species group (Gruiformes: Gruidae) based on mitochondrial DNA sequences. Emu 95:99-105.

KUMAR, S., K. TAMURA, AND M. NEI. 1993. MEGA: Molecular evolutionary genetics analysis, version 1.01. Pennsylvania State University, University Park.

LANSMAN, R. A., J. C. AVISE, C. F. AQUADRO, J. F. SHAPIRA, AND S. W. DANIEL. 1983. Extensive genetic variation in mitochondrial DNA's among geographic populations of the deer mouse, *Peromyscus maniculatus*. Evolution 37:1-16.

LEETON, P. R. J., L. CHRISTIDIS, M. WESTERMAN, AND W. E. BOLES. 1995. Molecular phylogenetic affinities of the Night Parrot (*Geospittacus occidentalis*) and the Ground Parrot (*Pezoporus wallicus*). Auk 111:833-843.

MADSEN, K. K. 1981. Search for the Eastern Sarus Crane on Luzon, Philippines. Pages 116-118 *in* Crane research around the world (J. C. Lewis and H. Masatomi, Eds.). International Crane Foundation, Baraboo, Wisconsin.

MAYR, E. 1944. Timor and the colonization of Australia by birds. Emu 46:113-130.

MEDWAY, L., AND D. R. WELLS. 1976. The birds of the Malay Peninsula. H. F. and G. Witherby, London.

MIRANDE, C., R. LACY, AND U. SEAL (Eds.). 1991. Whooping Crane conservation viability assessment. Captive Breeding Specialist Group, Apple Valley, Minnesota.

MORLEY, R. J., AND J. R. FLENLEY. 1987. Late Cainozoic vegetational and environmental changes in the Malay Archipelago. Pages 50-59 *in* Biogeographical evolution of the Malay Archipelago (T. C. Whitmore, Ed.). Clarendon Press, Oxford.

MOUM, T., AND S. JOHANSEN. 1992. The mitochondrial NADH dehydrogenase subunit 6 (ND6) gene in murres: Relevance to phylogenetic and population studies among birds. Genome 35:903-906.

NEI, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

SAITOU, N., AND M. NEI. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

SAMBROOK, F., E. F. FRITSCH, AND T. MANIATIS. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.

SCHODDE, R. 1988. New subspecies of Australian birds. Canberra Bird Notes 13:1.

SHARPE, R. B. 1894. Catalogue of birds in the British Museum, vol. 23. British Museum of Natural History, London.

TEMPLETON, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37:221-244.

THOMAS, R. H., W. SCHAFFNER, A. C. WILSON, AND S. PÄÄBO. 1989. DNA phylogeny of the extinct marsupial wolf. Nature 340:465-467.

WALKINSHAW, L. W. 1973. Cranes of the world. Winchester Press, New York.

YANG, L. 1991. The distribution and habitat of the cranes in Yunnan Province. Pages 127-129 *in* Proceedings of the 1987 crane workshop (J. Harris, Ed.). International Crane Foundation, Baraboo, Wisconsin.