PHYLOGENY OF THE CRANES (AVES: GRUIDAE) AS DEDUCED FROM DNA-DNA HYBRIDIZATION AND ALBUMIN MICRO-COMPLEMENT FIXATION ANALYSES

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ABSTRACT.—Reassociation kinetic analysis of nuclear DNAs from *Balearica pavonina* (Black-crowned Crane) and *Grus leucogeranus* (Siberian Crane) shows that the nuclear genome of each contains at least 75% single-copy sequences. The kinetic data show the haploid genome of each species to be 1.0–1.5 pg.

Neither DNA-DNA hybridization of single-copy nuclear sequences under conditions of reduced stringency nor albumin micro-complement fixation (MC'F) separated the three crane genera *Grus, Anthropoides,* or *Bugeranus* in our experiments. These three genera were separable from *Balearica* by both techniques. The DNA and MC'F results compare favorably with a parallel electrophoretic study. We conclude that *Balearica* and *Grus* separated between 10.0 and 6.2 MYBP (million years before present), as determined from MC'F and electrophoretic data, which is consistent with fossil evidence for cranes. *Received 7 November 1988, accepted 3 May 1989.*

THE CRANES (Aves: Gruidae) are a widely distributed group of 15 species in four genera (Johnsgard 1983). Interest in the cranes has increased as many natural populations have become endangered. Recent systematic investigations examined the unison call as a taxonomic character (Archibald 1976a, b), a variety of external morphological and skeletal characters (Wood 1979), and proteins using starch gel electrophoresis (Ingold et al. 1987a, b).

Comparison of Wood's and Archibald's taxonomic schemes indicates that the Siberian Crane (*Grus leucogeranus*) clusters with the genus *Bugeranus* and not with *Grus*. Wood (1979) found the Siberian Crane to be very similar to the Whooping Crane (*G. americana*) in external features but more similar to the Wattled Crane (*Bugeranus carunculatus*) in skeletal characters. In contrast, the Siberian Crane is more similar electrophoretically to the Whooping Crane than it is to the Wattled Crane (Ingold et al. 1987a). Wood (1979) attributed the similarity between the Siberian and Whooping cranes to convergence that resulted from selection in similar ecological niches. The proper taxonomic position of the Siberian Crane is of intense interest (Archibald pers. comm.) because it and the Whooping Crane are endangered in the wild. Cross-fostering and possible hybridization between species make information on relationships important.

There has also been controversy regarding composition of the genus *Balearica* (crowned cranes). Some investigators (White 1965, Snow 1978) have suggested that *Balearica* represents one species with four well-defined subspecies, whereas Walkinshaw (1964) maintained that there are two species, each of which consists of two well-defined subspecies. The studies of Archibald (1976a), Wood (1979), and Ingold et al. (1987b) provide strong support for Walkinshaw's (1964) contention that two species of *Balearica* are distinct and not subspecies.

The study of this family offers an exceptional opportunity for the comparison and evaluation of various molecular techniques commonly utilized in studying phylogenetic relationships. We present data from DNA-DNA hybridization and albumin micro-complement fixation (MC'F) to measure genetic divergence among 14 species of cranes and compare our data with results derived by electrophoretic techniques (Ingold et al. 1987a). We find that all three of these

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diverse experimental approaches produce congruent patterns of evolutionary relationships.

METHODS

Materials.—We studied 9 species of *Grus*, 1 species of *Bugeranus*, and 2 species each of *Balearica* and *Anthropoides* (Appendix). Most were captive individuals housed at the International Crane Foundation, Baraboo, Wisconsin; the specimens of *G. americana*, however, were from the captive flock at the U.S. Fish and Wildlife Patuxent Research Center, Laurel, Maryland. House Sparrows (*Passer domesticus*) and Common Yellowthroats (*Geothlypis trichas*), used as outgroups for the DNA-DNA hybridization and MC'F, respectively, were collected near Oxford, Butler County, Ohio.

DNA isolation.—Blood was collected from two individuals in each of 15 species (Table 1). High molecular weight DNA was obtained from the combined erythrocyte nuclei of both individuals using a phenol extraction procedure (Vaughn et al. 1982), which included RNAase and pronase digestions. The purified DNA was sonicated to obtain short fragments, which were sized by agarose gel electrophoresis using pBR322 DNA cut with *Hae*III as a standard (Southern 1979). This procedure yielded fragments averaging 380 nucleotide pairs in length (range: 250–500 nucleotide pairs). Sample purity was determined by the 260/230 absorption ratio. A sample with a ratio that is greater than or equal to 2/1 is considered to be pure. The purity of our samples ranged from 2.16/1 to 2.45/1.

DNA reassociation kinetics.—Kinetics of reassociation were determined for genomic DNA of the two species to be used as probes, *B. pavonina* and *G. leucogeranus*, utilizing standard techniques (Britten et al. 1974, Laird and McCarthy 1969). We have previously described in detail our modifications of these methods, and our second-order reaction kinetic calculation procedures (Vaughn 1975). *Balearica* and *Grus* were selected because they represent two different subfamilies of cranes (Brodkorb 1967); *G. leucogeranus* was used in anticipation of clarifying its questionable phylogenetic position within the family.

DNA labeling and preparation of single-copy component.—We labeled sonicated total nuclear DNA of *B. pavonina* and *G. leucogeranus* with ^{3}H-dCTP (23 Ci/mmole, 1 mCi/ml; New England Nuclear) by the nick translation technique (Rigby et al. 1977) to a specific activity of 1,200 cpm/ng and 630 cpm/ng, respectively, as determined by liquid scintillation counting in Scinti-verse II cocktail (Fisher). Single-copy nuclear DNA probes for the two species were isolated by denaturing and renaturing the labeled DNAs of each species to a C_0t of 200. Prior kinetic analysis showed this to be sufficient to permit renaturation of virtually all repeated sequences but very little of the single-copy component (Fig. 1). Because the labeled DNAs were in small quantity, DNA fractionation was carried out on 0.3 g hydroxylapatite (HAP, Bio-Rad) columns which had previously been treated with 100 μg of sonicated, denatured salmon sperm DNA to block all irreversible DNA binding sites. Single-stranded single-copy DNAs were eluted with 0.12 M sodium phosphate buffer (PB) at 60°C, after a 0.03 M PB wash step. The single-copy probes were examined for possible contamination with repetitive or snap-back sequences (Galau et al. 1976) and for their ability to form stable duplexes, by hybridization of probes to unlabeled homoduplex nuclear DNAs in excess (driver DNA) at C_0t values of ca. 50–5,000 at a driver/probe ratio of about 1000:1.

Hybridization and DNA fractionation by thermal hydroxylapatite chromatography.—Homoduplex and heteroduplex hybrids were formed by mixing labeled and unlabeled DNA at a driver/probe ratio of 1,000:1. Two-hybridization reactions were performed for each species pair comparison (30 comparisons). We were limited to small sample sizes because of the small amounts of DNA available from these endangered birds. Reaction mixtures were sealed in glass capillary tubes, denatured for 3 min in a boiling water bath, incubated in 0.50 M PB at 55°C to a driver C_0t of 5,000 and frozen quickly at −20°C until samples could be fractionated on HAP columns. The 55°C incubation temperature represents a condition of lowered stringency and was used to detect the more divergent cross-reactive DNA sequences expected to be present in heteroduplex reactions (Rice 1972), and permitted comparison of a greater percentage of each genome. Samples were adjusted to 0.03 M PB prior to loading DNAs onto water-jacketed columns equilibrated to 0.03 M PB and 55°C. The 0.3 g HAP bed was determined previously to be more than sufficient to bind all the DNA contained in each reaction tube. Very short DNA fragments incapable of binding to HAP were eluted with 0.03 M PB; the column was equilibrated to 0.12 M PB prior to thermal elution.

Column temperature was raised in 5°C increments to a final temperature of 95°C. Once each new temperature was reached, the column was allowed to equilibrate for 2 min before the fractions were eluted. Column temperature was monitored with a thermistor probe (Bailey) rated to be linearly sensitive to the nearest 0.1°C. At each temperature, two 2.5-ml fractions were collected manually by elution with 0.12 M PB. After the 95°C elution step, the column was washed with three 1.2-ml fractions of 0.50 M PB to remove any remaining double-stranded DNA. Each fraction was mixed with 15 ml of scintillation cocktail (Scinti-verse II, Fisher) and counted in a liquid scintillation counter to an error of <10%. We determined that, under these conditions, quenching was proportional for all samples.

DNA data analysis.—Hybridization data were analyzed by the $T_{50}H$ statistic (Kohne 1970, Bonner et al. 1981). $T_{50}H$ and T_m values were obtained by plotting the cumulative counts per minute eluted vs. temper-

ature on normal probability paper (Knittel et al. 1968) and fitting a linear regression line to the data points that made up the major DNA component. This technique accommodates those DNA sequences that have diverged to such an extent that they no longer hybridize between distantly related species (Sibley and Ahlquist 1983), and the results are expected to be more nearly linear with true genealogical distance constructions than analysis by ΔT_m values.

$\Delta T_{50}H$ values were corrected by averaging the reciprocal heterologous hybridization values obtained between *Balearica pavonina* and *Grus leucogeranus*. When *B. pavonina* was the labeled taxon, the uncorrected $\Delta T_{50}H$ was 5.0, and when *G. leucogeranus* was the labeled taxon, the uncorrected $\Delta T_{50}H$ was 4.5; the corrected $\Delta T_{50}H$ was 4.8. The remaining values were corrected as follows: When *B. pavonina* is the labeled taxon, 4.8/5.0 = 0.96. The uncorrected $\Delta T_{50}H$ for *B. pavonina* vs. *G. americana* is 4.7. We calculated the corrected $\Delta T_{50}H$ value as 4.7 × 0.96 = 4.5. All other $\Delta T_{50}H$ values derived from the hybridization of labeled *B. pavonina* DNA were similarly adjusted. When *G. leucogeranus* is the labeled taxon, 4.8/4.5 = 1.07, so all $\Delta T_{50}H$ values derived from labeled *G. leucogeranus* DNA were corrected by multiplying those values by 1.07.

Micro-complement fixation.—Antisera were prepared to serum albumin purified from 1 ml each of plasma from *Bugeranus carunculatus* from Africa and *Grus leucogeranus* from Asia. Each antiserum was made in two female New Zealand white rabbits by established procedures (Maxson and Szymura 1979) and individual rabbit antisera were pooled in inverse proportion to their MC'F titers. A total of 4–8 mg of albumin was administered to each rabbit over the 13-week immunization period. All rabbit antisera were directed primarily against albumin as evidenced by immunoelectrophoresis with whole plasma (Prager et al. 1974). Additional MC'F tests with pure albumin and whole plasma gave identical results. The titers and slopes of the pooled antisera were 5,000 and 400 in both cases.

Micro-complement fixation analyses were performed at standard conditions (Champion et al. 1974, Maxson et al. 1979) and reported as immunological distance units (IDU). For albumin, it has been estimated that 1 unit of ID is roughly equivalent to 1 amino acid difference between the albumins compared (Maxson and Wilson 1974, Maxson and Maxson 1986). Sequence evolution in albumin can be translated into a divergence time for when the lineages compared diverged. In birds, 10 IDU stochastically accumulate every 16–17 million years (Prager et al. 1974, Prager and Wilson 1975) of lineage independence.

Results

DNA reassociation kinetics of nuclear DNAs.—DNA reassociation curves for various avian species (Shields and Straus 1975, Epplen et al. 1978, Burr and Schmike 1980, Wagenmann et al. 1981) reveal three more-or-less distinct kinetic components that correspond to the highly repetitive, moderately repetitive, and single-copy DNA fractions. The moderately repetitive component, which was not critical in our study, is usually difficult to resolve clearly from the single-copy component. These three components were not all clearly distinguishable in reassociation curves for crane DNAs (Fig. 1), and we suspect that an unresolved, moderately repetitive component is present. This fraction would not, however, be expected to have contaminated the single-copy fraction used as a probe since genomic DNAs were reassociated

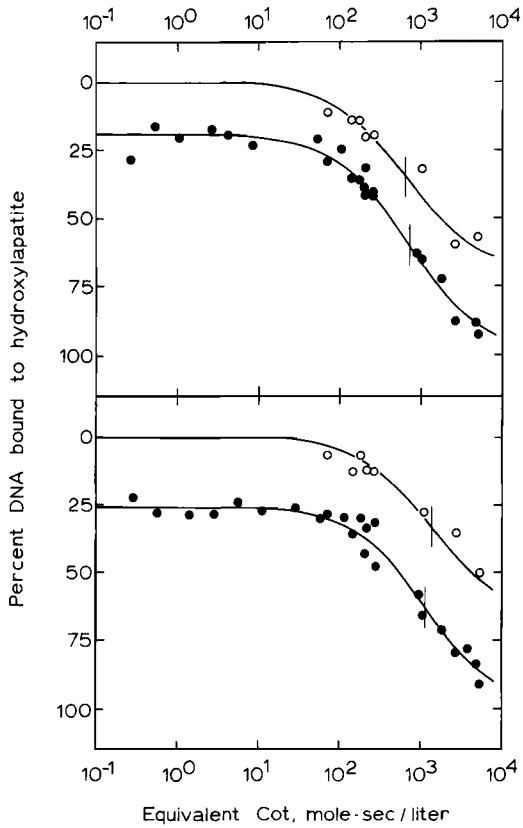

Fig. 1. Genomic renaturation kinetic curves (—●—) for the cranes *B. pavonina* (Top) and *G. leucogeranus* (Bottom). The upper curve in each panel (—○—) shows the results of hybridization of the labeled single-copy probe to the driver genomic DNA. The C_0t 1/2 for the DNA component is indicated by a vertical bar for each curve. Each curve is the calculated theoretical second-order reaction kinetic plot that best fits the data points.

TABLE 1. Thermal stability (°C) of hybrids formed between ^{3}H-single-copy DNA and total genomic DNAs and average single-copy sequence divergence in cranes.

	ΔT_m	$T_{50}H$	$\Delta T_{50}H$		ΔT_m	$T_{50}H$	$\Delta T_{50}H$
^{3}H-single-copy *Balearica pavonina* vs.				^{3}H-single-copy *Grus leucogeranus* vs.			
Balearica pavonina	0.0	79.7	0.0	*Grus leucogeranus*	0.0	78.4	0.0
B. regulorum	2.3	77.4	2.3	*G. americana*	0.8	77.6	0.9
Grus americana	4.7	75.0	4.7	*G. grus*	2.3	76.1	2.5
G. leucogeranus	4.2	74.7	4.8	*G. canadensis*	2.5	75.9	2.7
G. grus	4.4	74.2	5.3	*G. japonensis*	2.9	75.5	3.1
G. canadensis	5.7	74.0	5.5	*Anthropoides virgo*	2.6	75.3	3.3
G. japonensis	6.0	73.7	5.8	*Grus rubicunda*	2.7	75.2	3.4
G. monacha	4.1	73.3	6.1	*G. vipio*	2.7	75.1	3.5
G. antigone	6.7	73.0	6.4	*G. monacha*	3.3	74.9	3.7
Anthropoides paradisea	6.0	72.9	6.5	*Bugeranus carunculatus*	3.4	74.9	3.7
A. virgo	5.3	72.8	6.6	*Anthropoides paradisea*	2.5	74.5	4.2
Bugeranus carunculatus	5.9	72.7	6.7	*Grus antigone*	4.1	74.3	4.4
Grus rubicunda	5.5	72.5	6.9	*Balearica pavonina*	3.7	73.9	4.8
G. vipio	5.5	72.3	7.1	*B. regulorum*	4.8	72.1	6.7
Passer domesticus	20.1	36.5	41.5	*Passer domesticus*	16.8	41.1	39.8

to C_0t 200, which is sufficient to renature any presumptive moderately repetitive component. Britten and Kohne (1968) state that the central two-thirds of a C_0t curve should be a straight line. The slope of this line can be evaluated by determining the ratio of the values of C_0t at the ends of the straight line, with the ratio being ca. 100. The two C_0t curves (Fig. 1) show this approximation. The test for purity of the isolated single-copy probes (Fig. 1) affords confidence that our probe contained primarily single-copy DNA. The C_0t 1/2 is virtually identical to that of the presumptive single-copy component within the genomic DNA of each species. Both crane species contain up to 75% single-copy DNA, which is sharply resolved from the other kinetic components and comparable to other avian species (Epplen et al. 1978).

The haploid genome size is determined to be ca. 1.0–1.5 pg for both crane species examined, based on the observed C_0t 1/2 values of ca. 1×10^3 for their single-copy component (Laird 1971, Crain et al. 1976). This estimate is only approximate because an internal standard was not used and it is not possible to make meaningful comparisons of this type between different laboratories. The small difference in C_0t 1/2 between the two species is not significant. Our estimates of genomic size values are comparable to the 1.4–1.7 pg values determined for other cranes

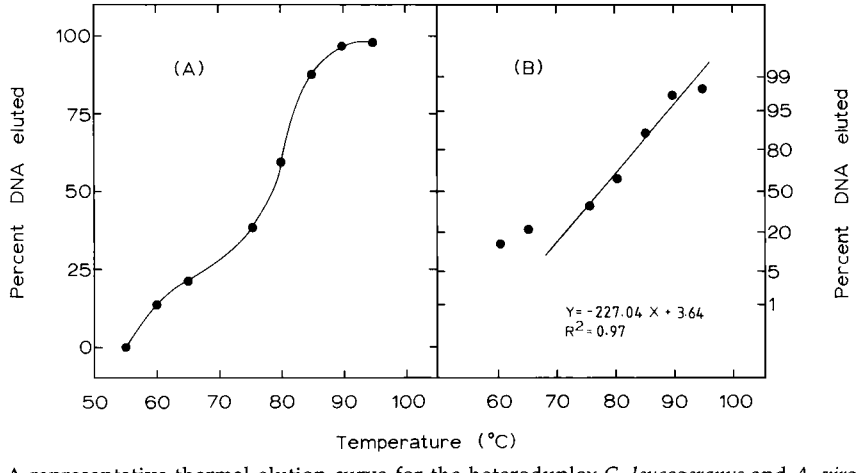

Fig. 2. A representative thermal elution curve for the heteroduplex *G. leucogeranus* and *A. virgo* reaction: (A) traditional curve and (B) regression line for major components plotted on normal probability paper.

(Rasch and Kurtin 1975, Biederman et al. 1982) and other birds (Shields and Straus 1975, Epplen et al. 1978, Wagenmann et al. 1981).

Hybridization of single-copy DNAs with nuclear DNAs.—The hybridization and thermal stability determinations are expressed as $T_{50}H$ and $\Delta T_{50}H$ (Table 1). A representative thermal elution curve, for the hybrid pair *G. leucogeranus*/*A. virgo*, is presented in Fig. 2. Within the family, the $\Delta T_{50}H$ values range from 0.9°C to 7.0°C. With *Grus* as the reference, the two *Balearica* species are most divergent ($\bar{x} = 5.8$), and *G. americana* is the most similar (0.9). When *Balearica* is used as a reference, the other three genera are at a mean distance of 6.0 and the two *Balearica* species have a $\Delta T_{50}H$ of 2.3°C.

Micro-complement fixation.—Albumin immunological distance values for cranes are presented in Table 2. MC'F curves for the reciprocal homologous comparisons are presented in Fig. 3. In both experiments the homologous antiserum concentration was used for each antigen. The difference in fixation observed with the *Bugeranus* antiserum reflects an immunological distance of 4 units. The reciprocal distance of 0 is evidenced by identical peak heights obtained with the *Grus* antiserum. The values for comparisons between the species of *Grus*,

TABLE 2. Albumin immunological distances between *Grus leucogeranus*, *Bugeranus carunculatus* and the other 12 species of cranes and *Geothlypis trichas*.

Species compared	ID to *G. leucogernaus*	ID to *B. carunculatus*
Grus grus	0	2
G. monacha	0	2
G. canadensis	1	0
G. japonensis	1	2
G. americana	0	2
G. vipio	0	3
G. antigone	1	6
G. rubicunda	0	2
G. leucogeranus	0	4
Bugeranus carunculatus	0	0
Anthropoides paradisea	0	2
A. virgo	0	5
Balearica pavonina	9	10
B. regulorum	11	10
Geothlyphis trichas	76	76

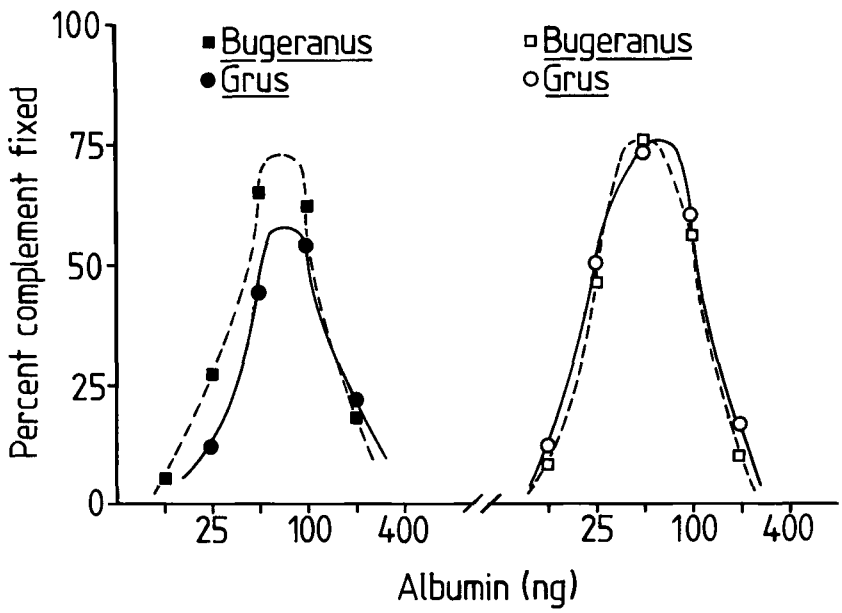

Fig. 3. Micro-complement fixation curves obtained with antiserum to *B. carunculatus* albumin (—■—, homologous reaction; —●—, heterologous reaction) and antiserum to albumin of *G. leucogeranus* (—□—, homologous reaction; —○—, heterologous reaction). In all four curves, the antiserum dilution was 1:5000 of the antiserum pool. The concentration of albumin was estimated from average concentrations of albumin in avian plasma. Using *Bugeranus* albumin antiserum and *Grus* albumin as antigen, there is 15% less complement fixation than in the homologous reaction. This corresponds to an immunological distance (ID) of 4 units. Using antiserum to to *Grus*, the same amount of complement fixation is obtained with *Bugeranus* albumin as with the homologous albumin, indicating a distance of 0 ID. Thus the average reciprocal distance is 2 IDU.

Anthropoides, and *Bugeranus* range between 0 and 6 immunological distance units (IDU), whereas the two species of *Balearica* are an average of 10 IDU from the other species.

Discussion

Published DNA-DNA hybridization studies for nonpasserine birds are becoming more common (Sibley and Ahlquist 1983, 1985; Sibley et al. 1988; Sheldon 1987a, b; Madsen et al. 1988). DNA-DNA hybridization data for cranes are limited [but see Krajewski, this issue—ed.]. Sibley and Ahlquist (1985) obtained a $\Delta T_{50}H$ between *Grus* and *Anthropoides* of only 0.7. Because such low values are subject to a relatively high percentage of error, we chose to use conditions of lower stringency to detect the more divergent cross-reactive sequences. This is a useful alternative because birds have been shown to have a slower rate of protein evolution (Prager et al. 1974) and, as a result, have a high potential for interspecific hybridization (Prager and Wilson 1975). The lower stringency gives greater $\Delta T_{50}H$ values.

Micro-complement fixation data for other nonpasserine serum albumins are similar to our crane data (Prager et al. 1974; Prager and Wilson 1975, 1976). In their comparisons of ducks in the genus *Anas*, Prager and Wilson (1975) obtained serum albumin IDUs of 0.0 and 2.0 while intergeneric values within the order ranged from 8 to 13. Intra-ordinal serum albumin IDUs >13 have been reported for the Galliformes (Prager and Wilson 1975). When nonpasserine birds are compared with passerines, serum albumin IDUs typically range from 42 to 93 ($\bar{x}$ = 68; Prager and Wilson 1976); an average value of 76 IDU was obtained in a comparison of the cranes to *Geothylpis trichas* (Parulinae).

When the DNA-DNA hybridization (Table 1) and MC'F data sets (Table 2) are compared with the electrophoretic data (Ingold et al. 1987a), *Grus*, *Anthropoides*, and *Bugeranus* show very little genetic differentiation whereas the genus *Balearica* is relatively distinct. The close relationship among *Grus*, *Anthropoides*, and *Bugeranus* is more obvious when we compare their genetic distances (Nei's [1978] *D* ranged 0.00–0.12) to those of other nonpasserines. Barrowclough et al. (1981) obtained genetic distance values of 0.11–0.61 in 7 species (each in a different genus) in the family Procellariidae. Ingold et al. (1984) obtained values of 0.39 and 0.53 when comparing *Zenaida macroura* (Mourning Dove) with *Columba livia* (Rock Dove) and *C. f. fasciata* (Band-tailed Pigeon), respectively.

Behavioral (Archibald 1976a, b) and morphological (Wood 1979) data on evolutionary relationships within the cranes differ somewhat from the biochemical data presented here. The most consistent relationship from all data sets is the separation of *Balearica* from the other three genera. *Balearica* is usually placed in the subfamily Balearicinae while the remaining genera are included in the subfamily Gruinae (Brodkorb 1967). The DNA-DNA hybridization data (Table 1) are consistent with the idea that there are two distinct species of crowned cranes. It is not possible, however, to separate *Bugeranus* and *Anthropoides* from *Grus* using the present biochemical data.

Both Archibald (1976a, b) and Wood (1979) suggest that the Siberian Crane should be considered congeneric with *Bugeranus* and placed in that genus. DNA-DNA hybridization and electrophoretic data show the Siberian Crane is most closely related to the Whooping Crane. These two species are very similar in their external features, breed in similar habitats, and have breeding ranges separated only by the Bering Strait (Johnsgard 1983). Archibald (1976a, b) showed that these two species have very dissimilar unison calls and therefore are not closely related. We believe, however, that the unison call, which is part of the courtship display, is not a good character for determining relationships. Based on the genetic distance, the Siberian and Whooping cranes most likely split very recently, and one would expect their courtship behaviors to diverge rapidly if we are to accept the function of pre-mating reproductive isolating mechanisms.

We believe that *Bugeranus* and *Anthropoides* might be considered congeneric with *Grus*. Sibley and Ahlquist (1982) proposed that birds are oversplit at the supraspecific level, which may be the case for the cranes. Systematic considerations based on DNA-DNA hybridization will require larger sample sizes and a more complete study. Even though we lack a complete matrix of DNA-DNA hybridization values, we feel that we can comment on relationships among the above species as all three data sets give similar results. Reciprocal pairwise comparisons were made only between two species and no outgroup was used as a probe. Therefore, we have

no information on the extent to which $\Delta T_{50}H$ values within subfamilies truly represent genetic distance or are instead artifacts of differences in rates of evolution, genome size, sample purity, fragment length, or conditions between experimental trials. Crane systematics may be further enhanced by analysis of mitochondrial DNA which has been shown to evolve 5-10 times faster than nuclear DNA (Wilson et al. 1985, Avise 1986).

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APPENDIX. Species used in this study.

Scientific name	Common name
Grus grus	Common Crane
G. monacha	Hooded Crane
G. canadensis	Sandhill Crane
G. japonensis	Red-crowned Crane
G. americana	Whooping Crane
G. vipio	White-naped Crane
G. antigone	Sarus Crane
G. rubicunda	Brolga
G. leucogeranus	Siberian Crane
Bugeranus carunculatus	Wattled Crane
Anthropoides virgo	Demoiselle Crane
A. paradisea	Stanley Crane
Balearica pavonina	Black-crowned Crane
B. regulorum	Gray Crowned Crane
Geothlypis trichas	Common Yellowthroat
Passer domesticus	House Sparrow

[a] Common names for cranes are those used by the International Crane Foundation (Archibald pers. comm.).