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### Journal

Proceedings of the National Academy of Sciences of the United States of America, 91(11)

### ISSN

0027-8424

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### Publication Date

1994-05-24

### DOI

10.1073/pnas.91.11.5173

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# DNA sequence support for a close phylogenetic relationship between some storks and New World vultures

(phylogeny/convergent evolution/mitochondrial DNA/birds)

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Contributed by John C. Avise and Charles G. Sibley, February 10, 1994

**ABSTRACT** Nucleotide sequences from the mitochondrial cytochrome *b* gene were used to address a controversial suggestion that New World vultures are related more closely to storks than to Old World vultures. Phylogenetic analyses of 1-kb sequences from 18 relevant avian species indicate that the similarities in morphology and behavior between New World and Old World vultures probably manifest convergent adaptations associated with carrion-feeding, rather than propinquity of descent. Direct sequence evidence for a close phylogenetic alliance between at least some New World vultures and storks lends support to conclusions reached previously from DNA-DNA hybridization methods and detailed morphology-based appraisals, and it illustrates how mistaken assumptions of homology for organismal adaptations can compromise biological classifications. However, there was a lack of significant resolution for most other branches in the cytochrome *b* phylogenetic reconstructions. This irresolution is most likely attributable to a close temporal clustering of nodes, rather than to ceiling effects (mutational saturation) producing an inappropriate window of resolution for the cytochrome *b* sequences.

Vultures are large, carrion-eating birds with hooked bills, featherless heads, and soaring food-search behavior. Similarities in external appearance and lifestyle between New World ("cathartid") and Old World ("accipitrid") vulture species (Fig. 1) usually have been interpreted to reflect a close phylogenetic relationship, such that both groups traditionally have been included in the Falconiformes (diurnal birds of prey; Table 1). However, Sibley and Ahlquist (3) interpreted results of DNA-DNA hybridization studies to indicate that New World vultures are related more closely to storks and allies (Ciconiidae) than to Old World vultures. Earlier studies of anatomical characters also supported a common ancestry of New World vultures and storks (4, 5), but these conclusions have been ignored by most avian systematists (review in ref. 1). Here we employ 1-kb sequences from mitochondrial DNA (mtDNA) to examine the phylogenetic relationships among the New World and Old World vultures and the storks and their allies. If New World vultures prove to be related to the ciconiiforms rather than to the traditional falconiforms, the morphological and behavioral adaptations for carrion-feeding shared by New World and Old World vultures would represent "one of the more striking examples of evolutionary convergence to be found in the class Aves" (5).

## MATERIALS AND METHODS

Mitochondrial DNA fragments containing sequences of the cytochrome *b* (cyt *b*) gene were amplified by the PCR using primers numbered 3, 5, 7, 8, and 10 as described in ref. 6, and

three additional primers constructed in our laboratory (that of J.C.A.): CB2H (5'-TGA GGC CAA ATA TCA TTC TGA GGG-3'; this is the reverse of primer 5 in ref. 6); CBINT (5'-GGT TGT TTG AGC CGG ATT C-3'; located between the primers 5 and 7 in ref. 6; and CBEND (5'-GTT GAG TAT TTT GTT TTC-3'; located between primers 9 and 10 in ref. 6). Both the heavy and light strands of the amplified products were sequenced directly, in our laboratory by using dideoxynucleotide chain termination with T7 DNA polymerase and <sup>35</sup>S labeling and/or in the Molecular Genetics Instrumentation Facility at the University of Georgia by using fluorescent-dye sequencing. Sequences totaling 1009 bp in length (88% of the 1143-bp cyt *b* gene) were gathered for each of 18 species of vultures, storks, and other taxa listed in Table 1.† A previously published sequence from the Domestic Fowl (chicken; *Gallus gallus*; Galliformes, Phasianidae) (7) was included as an outgroup. All sequences began at position 14,993 (as numbered in the Domestic Fowl), terminated at position 16,001, and could be aligned unambiguously.

Using the computer programs PAUP (8) and PHYLIP (9), we used 12 distance-based and character-based approaches to estimate phylogenetic relationships among the sequences. No phylogenetic method is without controversy, so we prefer to include a wide spectrum of philosophical and operational approaches (in our experience, results from these methods usually tend to agree quite well—see also ref. 10). Three algorithms—(i) phenetic clustering by the unweighted pair-group method with arithmetic means (UPGMA) (11), (ii) neighbor-joining (N-J) (12), and (iii) maximum parsimony (8)—were applied to each of four partially overlapping data classes: (i) original nucleotide sequences, (ii) first and second positions of codons only, (iii) transversions only, and (iv) translated amino acid sequences deduced by using the mitochondrial genetic code for vertebrates. Our approach is conservative, in the sense that we emphasize phylogenetic conclusions that are statistically well supported in particular data analyses, and consistent across most or all of the data classes and analytical methods employed. The first two algorithms were applied to genetic difference matrices reflecting the counted numbers of the relevant substitutions between all pairs of sequences (data classes *ii–iv* above), or to a distance matrix corrected for multiple substitutions at a nucleotide position by using Kimura's two-parameter method (13) with an assumed 5:1 ratio of transitions to transversions (data class *i*). Parsimony reconstructions, applied to each qualitative data class, were evaluated by bootstrapping in heuristic searches across 100 replicates.

## RESULTS

**Total Cytochrome *b* Evidence.** Sequence comparisons against the Domestic Fowl provided the largest genetic

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Abbreviations: cyt *b*, cytochrome *b*; UPGMA, unweighted pair-group method with arithmetic means; N-J, neighbor-joining.

†The sequences discussed in this paper have been deposited in the GenBank data base (accession nos. U08934–U08951).

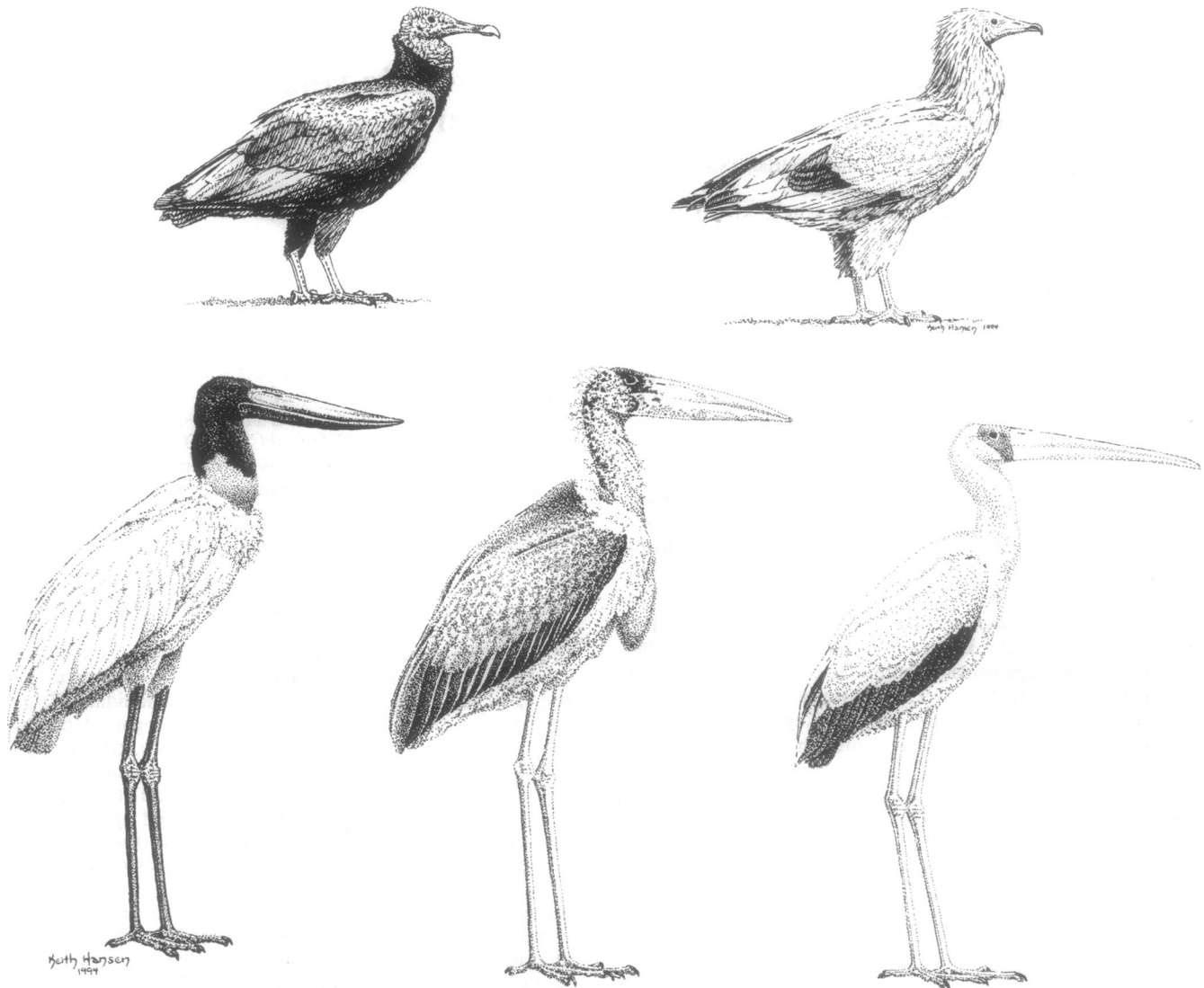


FIG. 1. Line drawings of the Black Vulture (New World) and the Egyptian Vulture (Old World) (*Upper Left and Right*, respectively) and three storks (Jabiru, Marabou, and Yellow-billed) (*Lower Left, Center, and Right*, respectively).

distances observed in the study (mean sequence divergence of the Domestic Fowl to ingroup taxa, corrected for multiple hits, was  $\approx 23.6\%$ , whereas the *largest* mean genetic distance among the other species was  $20.4\%$ ). A N-J tree for the 18 ingroup species, rooted by using the Domestic Fowl as outgroup, is presented in Fig. 2. A UPGMA dendrogram based on the same data showed many of the same structural features (particularly for "shallower" groupings), but it differed as follows: (i) the two flamingo species joined the cluster consisting of the Wood Stork and the Marabou Stork first, and then these collectively joined the same group of six New World vulture and stork species as identified in the N-J tree; and (ii) the Shoebill and the Lappet-faced Vulture dropped from their respective clusters in the N-J tree to more basal positions in the dendrogram. However, in the N-J and UPGMA summaries, many nodes (particularly those deeper in the trees) were distinguished by small genetic distances, so it is not surprising that these branching orders were labile. This interpretation is supported by the results of the parsimony analyses (Fig. 2 *Right*), where many deeper nodes remain unresolved according to bootstrapping criteria. In summary, the most robust assemblages recognized in parsimony and distance-based analyses of the total *cyt b* data were as follows: (i) at least two and perhaps all three of the Old World vultures assayed; (ii) the Andean Condor with the

Lesser Yellow-headed Vulture; (iii) the Black Vulture with the Jabiru Stork, and these two species with the California Condor and the Yellow-billed Stork; (iv) the Wood Stork with the Marabou Stork; (v) the Puna Ibis with the African Spoonbill; and (vi) the two flamingo species.

**Potentially Informative Subsets of Data.** Analyses of various subsets of the *cyt b* data (first and second positions of codons, transversions, and translated amino acid sequences) weight strongly for conservative classes of character state change that might in principle be especially informative phylogenetically. However, these analyses exclude large numbers of character state conversions (third-position changes, transitions, and synonymous substitutions, respectively) that probably contribute to phylogenetic signal (as well as to homoplasy) in the total data set (14). Does a greater resolution of older phylogenetic branching order for the vultures and allies occur when the analyses are confined to these more conservative classes of character state change? And, is resolution of the shallower clades compromised due to the exclusion of rapidly evolving characters? For the present data, the empirical answer to both of these questions appears to be "No."

For example, several different branching orders were observed among dendrograms produced by application of the two distance-based algorithms to the three restricted data

Table 1. New World and Old World vultures, storks, and other species examined in this report, and their traditional taxonomic placements in relevant portions of a conventional classification (1) for the Falconiformes, Ciconiiformes, and other orders

Order Falconiformes (diurnal birds of prey)	
Family Cathartidae (New World vultures)	
<i>Vultur gryphus</i> (Andean Condor)	
<i>Gymnogyps californianus</i> (California Condor)	
<i>Cathartes burrovianus</i> (Lesser Yellow-headed Vulture)	
<i>Coragyps atratus</i> (Black Vulture)	
Family Accipitridae (Old World vultures, hawks, eagles)	
<i>Torgos tracheliotus</i> (Lappet-faced Vulture)	
<i>Neophron percnopterus</i> (Egyptian Vulture)	
<i>Gypaetus barbatus</i> (Lammergeier)	
Order Ciconiiformes	
Family Ciconiidae (storks)	
<i>Mycteria americana</i> [Wood Stork (New World)]	
<i>Mycteria ibis</i> [Yellow-billed Stork (Old World)]	
<i>Jabiru mycteria</i> [Jabiru Stork (New World)]	
<i>Leptoptilos crumeniferus</i> [Marabou Stork (Old World)]	
Family Scopidae*	
<i>Scopus umbretta</i> [Hamerkop (Old World)]	
Family Balaenicipitidae*	
<i>Balaeniceps rex</i> [Shoebill (Old World)]	
Family Threskiornithidae (ibises, spoonbills)	
<i>Plegadis ridgwayi</i> (Puna Ibis)	
<i>Platalea alba</i> (African Spoonbill)	
Order Phoenicopteriformes (flamingos)	
Family Phoenicopteridae	
<i>Phoenicopus andinus</i> (Andean Flamingo)	
<i>Phoenicopus ruber</i> (Greater Flamingo)	
Order Pelecaniformes	
Family Pelecanidae	
<i>Pelecanus erythrorhynchos</i> (American White Pelican)	

\*These Old World families are not included in the American Ornithologists' Union checklist; their placement here reflects the classification of ref. 2.

bases. Among the six such trees, the only groups to consistently emerge (i.e., appear in at least four of the six treatments) were those described earlier based on the total *cyt b* evidence: namely, the "clades" *i-vi*. In other respects, the dendrograms (one example of which is presented in Fig. 3) usually differed in branching order from one another and also from the branching order presented earlier (Fig. 2 *Left*). In the parsimony analyses of the three restricted data sets, the message remains much the same (Table 2; Fig. 3). Again, there was consistent resolution of the six groups described above, and no others.

Table 2. The six "clades" recognized most consistently across the 12 data treatments and analyses of *cyt b* sequences from 18 species of storks, vultures, and allies

Clade*	Total <i>cyt b</i> evidence			Transversions only			First and second codon positions			Amino acid sequences		
	N-J	UPG	Pars.	N-J	UPG	Pars.	N-J	UPG	Pars.	N-J	UPG	Pars.
<i>i</i> <sup>†</sup>	Y	Y (2)	Y	Y	Y	Y	Y (2)	—	Y (2)	Y (2)	—	—
<i>ii</i>	Y	Y	Y	—	Y	—	Y	Y	—	Y	Y	Y
<i>iii</i>	Y	Y	Y	Y <sup>‡</sup>	Y <sup>‡</sup>	Y <sup>‡</sup>	Y	Y	Y	Y	Y	Y
<i>iv</i>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
<i>v</i>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
<i>vi</i>	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

The body of the table answers whether the indicated clade was identified (Y, yes; —, no) in a given treatment. No other putative groupings appeared in more than, at most, four such treatments. The three algorithms summarized are the face-value N-J and UPGMA (UPG) dendrograms, and 50% majority-rule consensus trees from bootstrapped parsimony (Pars.) reconstructions.

\**(i)* Lappet-faced Vulture, Egyptian Vulture, Lammergeier; *(ii)* Andean Condor, Yellow-headed Vulture; *(iii)* Black Vulture, Jabiru Stork, and these joining with California Condor and Yellow-billed Stork; *(iv)* Wood Stork, Marabou Stork; *(v)* Puna Ibis, African Spoonbill; and *(vi)* Andean Flamingo, Greater Flamingo.

<sup>†</sup>In the cases indicated, only two of the three Old World Vultures assayed were grouped.

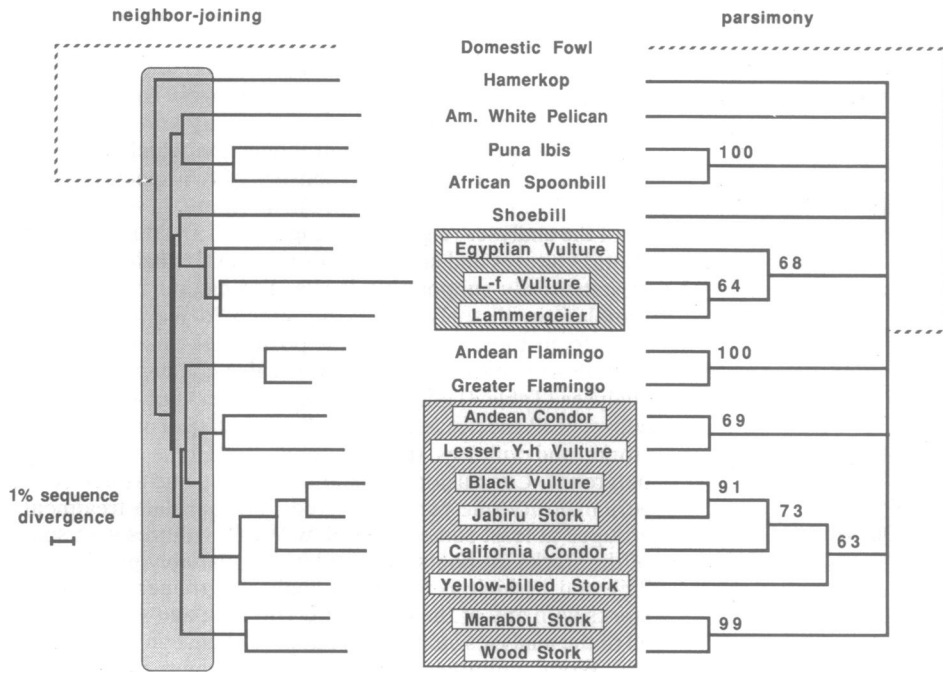
<sup>‡</sup>Some but not all of the four species in this putative clade were grouped.

**Storks and Vultures.** Most of the six clades identified above were expected. For example, ibises and spoonbills are treated as close relatives in nearly all classifications (e.g., Table 1), as are the two species of flamingos. However, one group in the *cyt b* data was not necessarily anticipated, and it is highly relevant to the central question which motivated this study. Clade *iii* (Table 2) consists of two species of New World vultures and two storks. Furthermore, in some but not all of the analyses (e.g., *Left* of Figs. 2 and 3), additional New World vultures and stork species joined this clade, albeit not at statistically supportable levels. Far distant from this assemblage in all analyses are the three Old World vultures.

To focus more closely on the postulated alliance between New World vultures and storks, we also conducted phenetic analyses (Table 3) and parsimony analyses (not shown) on a subset of 12 species, excluding all assayed taxa other than the storks and vultures (but retaining the Domestic Fowl as outgroup). Conclusions remain much the same as before. In 50% majority-rule consensus trees, all four data treatments grouped at least some of the New World vultures with some of the storks (at bootstrapping levels invariably greater than 75% and as high as 98%); no analyses grouped any of the storks with any of the Old World vultures; and some but not all of the analyses identified a putative clade consisting of the Old World vultures alone. All else in these phylogenetic reconstructions (with the exception of the consistent Marabou Stork/Wood Stork clade) remained unresolved.

## DISCUSSION

**Lack of Resolution of Deeper Branching Orders.** One sobering result of this study is the lack of significant resolution of most older nodes in the *cyt b* phylogenies. Several explanations might be advanced. First, perhaps there is an inherent bias in the clustering procedures or phylogenetic algorithms that favors resolution of more recent nodes. This seems unlikely, but if such bias does exist, it must apply to both distance-based and parsimony algorithms, and to treatments of conservative subsets of sequence data as well as those based on the total *cyt b* evidence. Second, ceiling effects on levels of sequence divergence might be indicated (i.e., the *cyt b* molecules may have approached saturation with respect to acceptable nucleotide substitutions over longer periods of evolutionary separation). We doubt that this is the case here for at least two reasons: *(i)* the lack of resolution of deeper nodes appeared not only in the total *cyt b* evidence but also in analyses of the various (but partially overlapping) classes of conservative characters; and *(ii)* the magnitudes of genetic divergence in each of the four data sets were lower than those



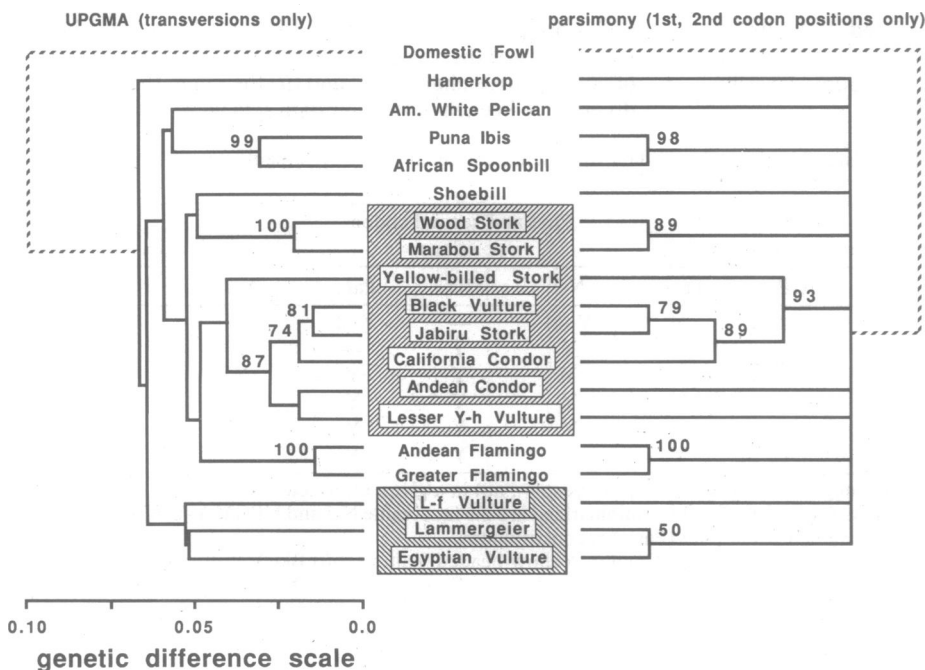
**FIG. 2.** Examples of phylogenetic analyses based on total evidence from the *cyt b* gene. (Left) N-J network (with branch lengths drawn according to scale, as indicated) derived from a matrix consisting of genetic distances calculated according to Kimura's two-parameter model (13) with an assumed transition-to-transversion ratio of 5:1. (Right) A 50% majority-rule consensus tree (consistency index 0.487) derived from heuristic parsimony searches of the qualitative data base (numbers indicate levels of statistical support across 100 bootstrap replicates). The boxes in the center highlight the Old World vultures (left hatching), and the New World vultures and storks (right hatching) and are included to facilitate visual inspection of the trees (and not necessarily to imply clades). The shaded vertical box encompasses deeper nodes that were not well resolved in the total *cyt b* data (or, indeed, in analyses of the other data classes—Table 2).

in some other avian groups similarly assayed. For example, in *cyt b* sequences from the Cuculiformes and Galliformes, pronounced ceiling effects on genetic distances appear not to have been encountered much below the following levels of sequence difference: total sequences, 0.20; transversal substitutions, 0.10; first and second positions of codons, 0.09; amino acid sequences, 0.14 (14). In the current study, approximate divergence levels for unresolved deeper nodes in the tree were 0.15 for total sequences, 0.06 for transversions (e.g., Fig. 3 Left), 0.05 for first and second codon positions, and 0.09 for amino acid sequences. Thus, there is considerable scope for further avian *cyt b* differentiation beyond levels observed within the vultures and presumed allies. This conclusion is also indicated by the larger mean *cyt b* distances of ingroup members to the Domestic Fowl (e.g., Figs. 2 and 3).

A third possible explanation for the lack of phylogenetic resolution is that the deeper nodes in this study may truly be

rather tightly clustered temporally. Under this hypothesis, at deeper phylogenetic levels, shallow slopes in the regression lines relating avian *cyt b* sequence divergence to time make it unlikely that sequences of the length monitored here could in principle resolve closely spaced nodes (14). These problems related to short stretches of DNA sequence no doubt are confounded further by the extensive homoplasy in sequence data (as indicated, for example, by low consistency indices—Figs. 2 and 3), as well as by the fact that even in the absence of sampling error and homoplasy, gene genealogies can differ from the organismal phylogeny (when nodes are temporally close) due to stochastic lineage-sorting from polymorphic ancestral taxa (15–17).

Is it plausible that the lineages leading to such morphologically distinctive groups as pelicans, flamingos, spoonbills, storks, and vultures separated close in time and relatively recently in avian evolution? The traditional classification (Table 1) would seem to suggest not. However, on the basis



**FIG. 3.** Examples of phylogenetic analyses based on potentially informative subsets of *cyt b* sequence. (Left) UPGMA dendrogram derived from a distance matrix reflecting transversions only (the dendrogram is drawn such that the axis at the bottom refers to the joint distances, rather than individual branch lengths, between nodes or taxa). Also indicated are bootstrap values for "clades" supported at >70% level in parsimony analyses of a purine vs. pyrimidine data matrix (which thus also reflects transversal substitutions only). (Right) A 50% majority-rule consensus tree (consistency index 0.601) derived from heuristic parsimony searches of the qualitative data base of first and second codon positions only. As in Fig. 2, boxes in the center highlight the Old World vultures (left hatching) and the New World vultures and storks (right hatching) and are included to facilitate visual inspection of the trees.

Table 3. Means (and ranges) of genetic distance in *cyt b* sequences between various species of New World (N.W.) vultures, Old World (O.W.) vultures, and storks

Comparison	Genetic distance* × 100			
	Total sequences	Transversions only	First and second codon positions	Amino acid sequences
Two N.W. vultures vs. two storks within clade <i>iii</i> —see text	7.4 (4.3–9.8)	2.6 (1.3–3.9)	2.4 (1.3–3.6)	3.9 (2.4–5.4)
Clade <i>iii</i> vultures vs. O.W. vultures	16.5 (14.8–18.4)	7.0 (6.7–7.2)	6.5 (5.4–8.2)	11.1 (10.4–12.5)
Clade <i>iii</i> storks vs. O.W. vultures	15.3 (13.0–17.1)	6.5 (6.2–6.8)	6.1 (5.1–8.0)	11.0 (9.5–12.5)
All N.W. vultures vs. all storks	12.1 (4.3–15.3)	4.1 (1.3–5.8)	4.3 (1.3–6.1)	6.2 (2.4–9.2)
All N.W. vultures vs. O.W. vultures	15.7 (13.0–18.4)	6.5 (5.8–7.2)	5.9 (4.3–8.2)	10.2 (7.1–12.5)
All O.W. vultures vs. all storks	15.0 (12.3–17.1)	6.3 (5.8–6.8)	9.6 (4.0–8.0)	10.3 (8.3–12.5)

\*"Face-value" differences, uncorrected for multiple hits.

of DNA hybridization studies, Sibley and Ahlquist (3) proposed that all of the above-mentioned taxa (plus several others such as penguins and loons) are allied more closely to one another than to other avian taxa, and should be classified together in a revised and enlarged order Ciconiiformes. Although our current analyses of *cyt b* sequences cannot be taken as corroboration of this possibility (comparisons with many additional "outgroup" taxa based on this and other genes will be required before firm conclusions are drawn), neither are the present data inconsistent with the Sibley–Ahlquist suggestion.

**An Alliance Between New World Vultures and Storks.** Notwithstanding the limited phylogenetic information in short DNA sequences, several putative clades were supported (Table 2), and one of these proved relevant to the central question of this study. Overall, the *cyt b* data provide support for a closer phylogenetic relationship between at least some storks and some New World vultures than between any assayed representatives of these groups with the Old World vultures. In this respect, the data parallel previous conclusions from the DNA hybridization approach, as well as those based on detailed inspections of morphological characters.

Nonetheless, the agreement with conclusions from the Sibley–Ahlquist studies should not be overstated. First, in the DNA hybridization assays, no direct comparisons between storks and Old World vultures were accomplished. The inference of a closer relationship between storks and New World vultures stemmed from lower  $\Delta T_{\text{mode}}$  values between Old World vultures (including hawks and eagles) and New World vultures (mean value 8.5) than between the latter and storks (mean 7.6). Second, there was no indication from DNA hybridization that storks and New World vultures were phylogenetically intermixed, as a literal interpretation of the *cyt b* sequence information might suggest. Thus,  $\Delta T_{\text{mode}}$  values among various species of storks (range 0.2–3.8) and among various species of New World vultures (0.7–3.7) were much lower than in comparisons between assayed representatives of these two groups (6.7–8.6). In other words, the *cyt b* sequences suggest an even closer phylogenetic association between certain storks and New World vultures than was implied by the DNA hybridization data.

In any event, the *cyt b* sequence data bolster the view that the carrion-feeding lifestyles and the associated morphologies shared by New World and Old World vultures do not represent synapomorphic conditions linking these groups. Two possibilities remain. (i) Carrion-feeding was the ancestral condition for the entire assemblage, in which case the carrion-feeding adaptations could be symplesiomorphic for New World and Old World vultures. This seems unlikely, given that several other taxonomic groups such as diurnal raptors, flamingos, ibises, and many others may be included in an extended ciconiiform clade. (ii) More plausibly, carrion-feeding has evolved independently at least twice in vulture lineages.

Drawings were by Keith Hansen. We thank Ned Johnson and Paul DeBenedictis for useful comments on the manuscript. Work was supported by funds provided by the University of Georgia.

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