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Molecules vs. morphology in avian evolution: The case of the "pelecaniform" birds

(DNA·DNA hybridization/mtDNA sequences/phylogeny/systematics)

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ABSTRACT **The traditional avian Order Pelecaniformes is composed of birds with all four toes connected by a web. This "totipalmate" condition is found in ca. 66 living species: 8 pelicans (*Pelecanus*), 9 boobies and gannets (*Sula, Papasula, Morus*), ca. 37 cormorants (*Phalacrocorax*), 4 anhingas or darters (*Anhinga*), 5 frigatebirds (*Fregata*), and 3 tropicbirds (*Phaethon*). Several additional characters are shared by these genera, and their monophyly has been assumed since the beginning of modern zoological nomenclature. Most ornithologists classify these genera as an order, although tropicbirds have been viewed as related to terns, and frigatebirds as relatives of the petrels and albatrosses. DNA·DNA hybridization data indicated that the pelicans are most closely related to the Shoebill (*Balaeniceps rex*), a stork-like bird that lives in the swamps of central Africa; the boobies, gannets, cormorants, and anhingas form a closely related cluster; the tropicbirds are not closely related to the other taxa; and the frigatebirds are closest to the penguins, loons, petrels, shearwaters, and albatrosses (Procellarioidea). Most of these results are corroborated by DNA sequences of the 12S and 16S rRNA mitochondrial genes, and they provide another example of incongruence between classifications derived from morphological versus genetic traits.**

Until recently, morphological characters have been the principal, and virtually the only, source of evidence for organizing species into larger categories in the construction of classifications. The traditional Order Pelecaniformes (pelicans, cormorants, anhingas and darters, boobies and gannets, frigatebirds, and tropicbirds) has been defined by the totipalmate foot, in which the hallux is turned forward and connected by a web to digit II, in addition to the webs between digits II and III and between III and IV. In other birds with webbed feet only the three front toes (digits II, III, IV) are connected by webs, and the hallux is free or absent; no bird has five toes. Of the totipalmate birds all but the tropicbirds have an obvious gular pouch; the frigatebird's gular pouch is inflatable and used in display, thus differing from those of the other species. The combination of totipalmate feet and gular pouch has seemed so unlikely to evolve more than once that the monophyly of the group has been widely accepted since Linnaeus (1) placed them in the genera *Pelecanus* (pelicans, cormorants, boobies, anhingas, and frigatebirds) and *Phaethon* (tropicbirds). They also share the location of the salt-excreting gland within the orbit instead of in a supraorbital groove, and they lack an incubation patch which is present in all other seabirds and waterbirds. However, they vary in pelvic musculature, carotid artery arrangement, and several other anatomical characters. The similarities argue for monophyly; the differences raise the possibility of polyphyly. The palmate avian foot with two webs between the three front toes has evolved in groups with separate origins—e.g., ducks, gulls, flamingos, and albatrosses. Could the totipalmate condition, which occurs in fewer species, also have multiple origins? Sibley and Ahlquist (2) reviewed the literature from 1758 to 1990.

There have been many morphological studies of the pelecaniforms; those of Lanham (3), Saiff (4), and Cracraft (5) are among the most recent. Lanham (3) recognized their diversity but concluded that the totipalmate birds form a natural order. He assigned *Phaethon* and *Fregata* to separate suborders, the other genera to the suborder Pelecani, and suggested that the nearest relatives of the pelecaniforms are the procellariiforms (shearwaters, petrels, and albatrosses). Saiff (4) studied the middle ear region of the pelecaniforms and of the herons and storks. He concluded that the Shoebill (*Balaeniceps rex*) (Fig. 1), which lives in the swamps of central Africa, is more closely related to the pelecaniforms than to the herons and storks, and that *Phaethon* resembles the procellariiforms but is quite distant from them. Cracraft (5) conducted a "phylogenetic analysis . . . to evaluate the monophyly of the Pelecaniformes and to determine interfamilial relationships within the order." He analyzed 52 characters with "numerical cladistic" methods and concluded that "Pelecaniform monophyly was highly corroborated, with 12 postulated synapomorphies supporting the hypothesis." (Fig. 2 *Upper*).

Sibley and Ahlquist (2) compared the genomes of representative species of pelecaniforms and other major groups of living birds with the technique of DNA·DNA hybridization. Most of the results agreed with classifications based on morphological characters, but several disagreed (Fig. 2 *Lower*). Among the most surprising departures was evidence that the pelicans are more closely related to the Shoebill (Fig. 1) than to the other totipalmate birds. However, this was not the first suggestion of a close relationship between pelicans and the Shoebill. In addition to the study by Saiff (4), noted above, Cottam (8) made an osteological study of the Shoebill, herons, storks, and pelecaniforms and concluded that the Shoebill "could occupy a monotypic family in the Order Pelecaniformes, possibly near the Pelecanidae." J. M. Lowenstein, from radioimmunoassay comparisons of proteins, concluded that *Pelecanus* is not closely related to the other totipalmate taxa. Lowenstein was so surprised by his results that he assumed that the material must have been degraded and did not publish his data, but he gave Sibley and Ahlquist permission to do so (ref. 2, pp. 500–502).

Sibley and Ahlquist (ref. 2, pp. 502–503) concluded that "This group may present the most complex and controversial questions in the avian phylogeny. Is it possible that the totipalmate foot, lack of an incubation patch, the intraorbital salt gland, and other shared characters either have evolved more than once or are primitive characters that have been lost in the other lineages of the Ciconiides? This explanation will be rejected as improbable by most morphologists, but it should be considered as an alternative hypothesis to the

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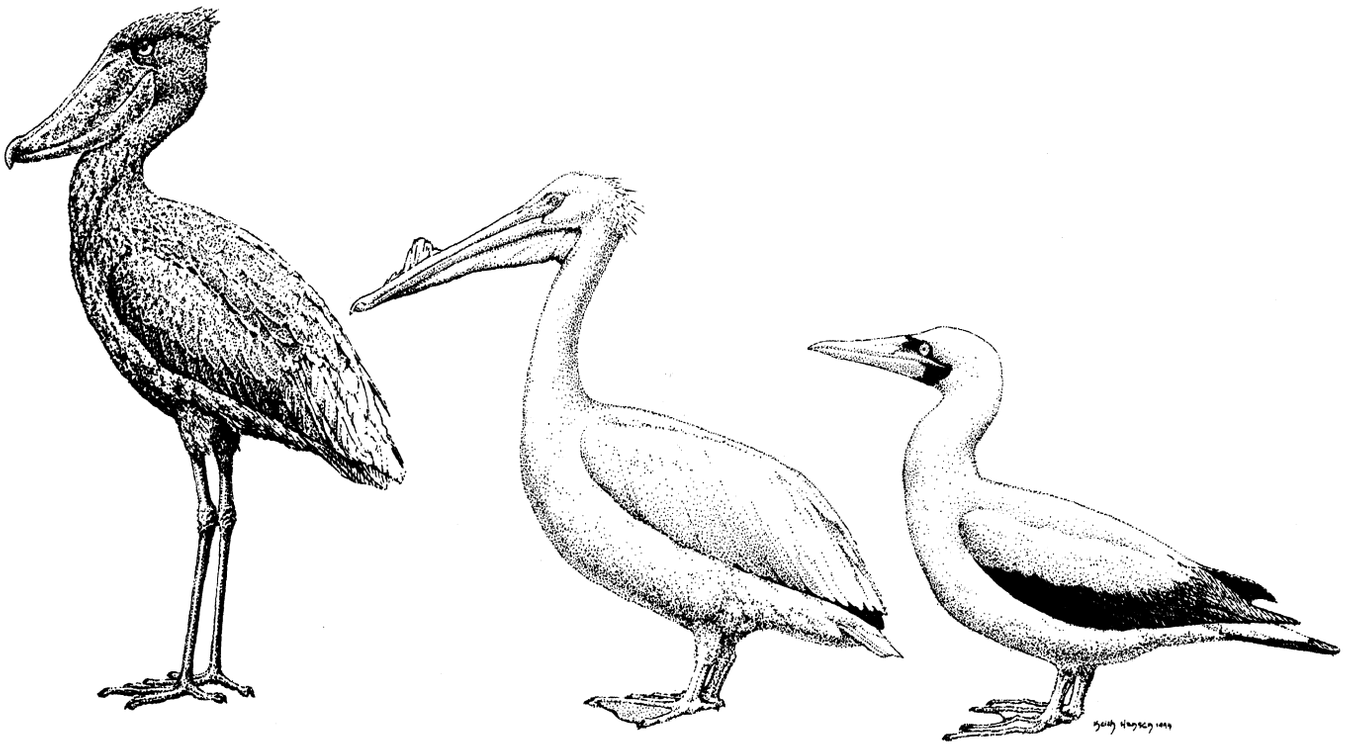

FIG. 1. (*Left*) Shoebill. (*Center*) American White Pelican. (*Right*) Masked Booby.

monophyly of the totipalmate birds. We urge that the possibility of polyphyly be tested by independent studies of both molecules and morphology. Whatever the correct answer may be, it will be instructive."

In this paper we present the results of comparisons among the DNA sequences of the mitochondrial 12S rRNA and 16S rRNA genes‡ of 16 avian taxa, including the totipalmate genera. These results provide additional evidence that the traditional Order Pelecaniformes is polyphyletic.

MATERIALS AND METHODS

DNA sequences of portions of two mitochondrial genes (12S rRNA and 16S rRNA), totalling 1.7 kb of aligned sequence, were obtained from each of the following 15 species of birds: Egyptian Vulture (*Neophron percnopterus*), Horned Grebe (*Podiceps auritus*), Red-billed Tropicbird (*Phaethon aethereus*), Blue-footed Booby (*Sula nebouxii*), Neotropic Cormorant (*Phalacrocorax brasilianus*), Black Stork (*Ciconia nigra*), California Condor (*Gymnogyps californianus*), Andean Condor (*Vultur gryphus*), Magnificent Frigatebird (*Fregata magnificens*), Shoebill (*Balaeniceps rex*), Brown Pelican (*Pelecanus occidentalis*), Adelie Penguin (*Pygoscelis adeliae*), Common Loon (*Gavia immer*), Red-throated Loon (*Gavia stellata*), and Great Shearwater (*Puffinus gravis*). Corresponding sequences from a Domestic Fowl (*Gallus gallus*; accession no. X52392) and American Alligator (*Alligator mississippiensis*; accession no. L28074) were obtained from the data bases for comparison. The sequenced regions correspond to sites 1760–2128 (12S rRNA) and 2797–3995 (16S rRNA) in the published sequence of *Gallus gallus* (9). Latin and English names of birds are from ref. 10.

DNA was amplified (PCR) with the use of primers made to conserved regions among vertebrates, and these same primers were used for dideoxynucleotide sequencing of both complementary strands. The 10 primers (five pairs) used were 12L5/12H4, 16L2a/16H10, 16L9/16H3, 16L1/16H13, and 16L4/16H12 (11); those not previously described are 16H12 (TTA GGG AGA GGA TTT GAA CCT CTG) and 16H13 (CCG GTC TGA ACT CAG ATC ACG TA). The 12S rRNA primer pair results in an approximately 400-bp fragment that corresponds to the region described in ref. 12. The four 16S rRNA primer pairs produce overlapping fragments that, when joined, correspond approximately to 1.2 kb of that 1.6-kb gene.

Double-stranded DNA was amplified (Perkin–Elmer GeneAmp PCR System 9600) in 25–30 cycles (94°C for 15 s, 50°C for 15 s, 72°C for 45 s), and single-stranded DNA in 30–35 cycles (55–60°C annealing temperature). The double-stranded DNA was gel purified and used as template for single-stranded amplifications, with one of the two primers as limiting (1:100). Single-stranded DNA was filtered with 30-kDa cut-off filters (Millipore) prior to sequencing with *Taq* DNA polymerase.

Sequence alignment was performed with ESEE (13), and the two sequenced regions were combined for all analyses. The aligned sequences were analyzed with MEGA (14). Neighbor-joining (15) analyses, except as noted, were performed with the Jukes–Cantor (16) distance, and statistical confidence of the nodes on the trees was inferred by a *t* test for significance of the difference between branch length and zero, expressed as the complement of the probability or confidence probability (CP; refs. 14 and 17). Sites containing gaps and ambiguities were not included in the distance analyses. Parsimony analysis was performed with MEGA and PAUP (7), and sites containing gaps and ambiguities were included.

RESULTS

All Taxa. Combined sequences from the two mitochondrial genes totalled 1699 aligned sites (12S rRNA, 388 bp; 16S rRNA, 1311 bp), including 832 variable and 516 parsimony sites. Phylogenetic analysis of 16 avian taxa (including *Gallus*) with *Alligator* as the root resulted in a tree (Fig. 3) that does not support the monophyly of the "Pelecaniformes" but agrees in many respects with the evidence from DNA hybridization. Most notably, the pelican clusters with the Shoebill, not with the booby and cormorant; the New World vultures are closer to the storks and pelicans than to the Old World vultures; and the loons are closer to the shearwater than to the grebe. A group containing the penguins, storks,

‡The sequences discussed in this paper have been deposited in the GenBank data base (accession nos. L33368-L33397).

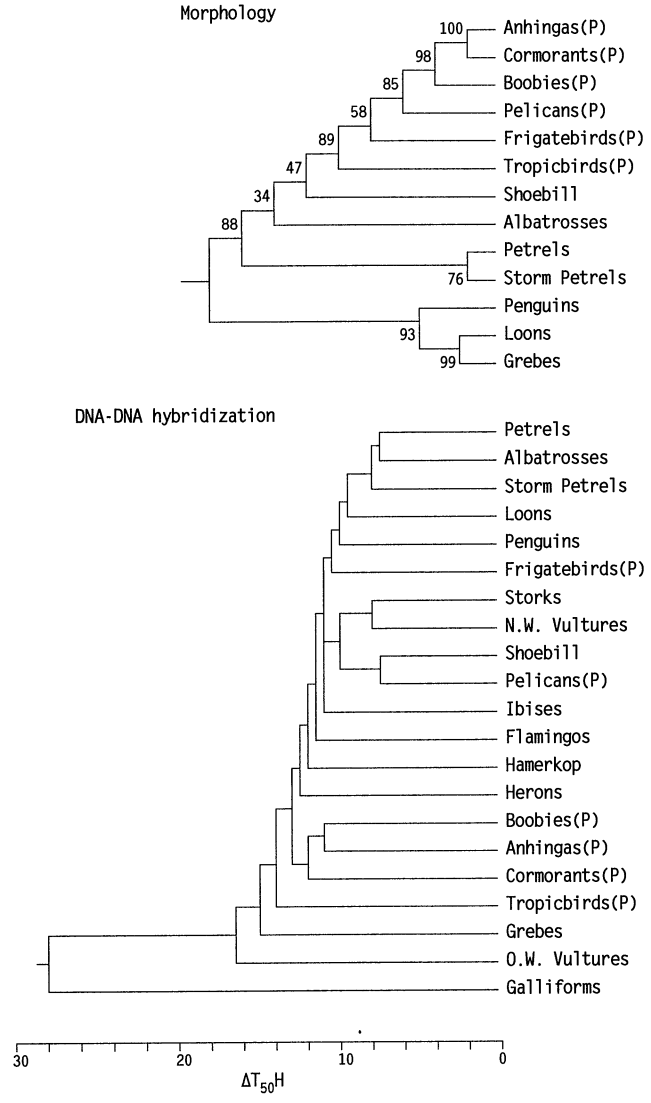

FIG. 2. Conflicting hypotheses of the phylogenetic relationships of "pelecaniform" birds. (*Upper*) Maximum parsimony tree based on 52 morphological characters, with bootstrap *P* values (6) based on 2000 replications; PAUP (7) reanalysis of data in table 1 of ref. 5, unordered characters. (*Lower*) Unweighted pair-group method of averages (UPGMA) tree based on DNA hybridization (after ref. 2). Taxa previously placed in the order Pelecaniformes are indicated (P). N.W., New World; O.W., Old World; $\Delta T_{50}H$, temperature difference (°C) for the midpoints of the melting curves for hybrid and native DNAs.

New World vultures, frigatebirds, shearwaters, pelicans, Shoebill, and loons is strongly supported (CP = 98%) and agrees with Sibley and Ahlquist (2), who brought them together in the Parvorder Ciconiida of the Order Ciconiiformes. Thus, the total DNA evidence indicates that these groups of birds shared a relatively recent common ancestry, although their morphological diversity suggested greater phylogenetic differences.

Some of the results from the DNA sequence analysis do not agree with the DNA hybridization evidence. The condors cluster with the frigatebird (CP = 98%) rather than with the stork. An alliance between New World vultures and storks has support from anatomy (18) and an analysis of mitochondrial cytochrome *b* sequences (19), although those two studies did not include comparisons with a frigatebird. Condors and frigatebirds seem to bear little resemblance to each other and have not been suggested as close relatives. Additional data are required to clarify this unexpected alliance.

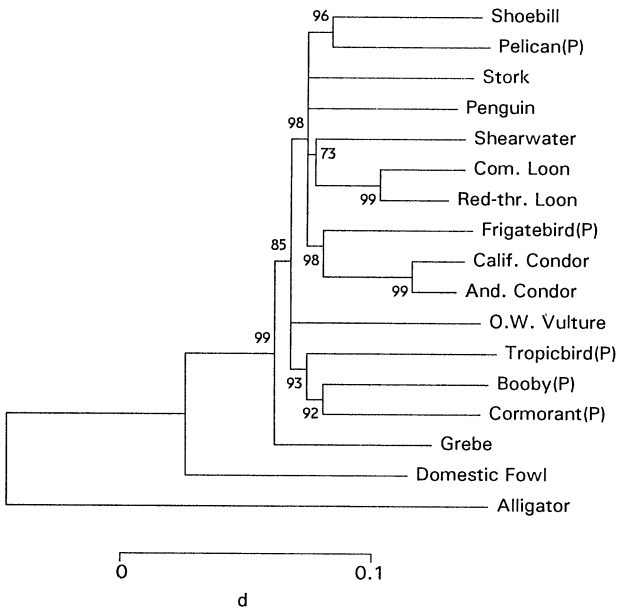

FIG. 3. Phylogenetic relationships of "pelecaniform" birds and relatives inferred from DNA sequences of the mitochondrial 12S rRNA and 16S rRNA genes (1.7 kb). The tree was constructed by the neighbor-joining method with Jukes–Cantor distance (*d*); confidence probability (CP) values are indicated on the nodes. For comparison with Fig. 2, note that condors are New World vultures and the shearwater is a petrel.

The tropicbird clusters (CP = 93%) with two other totipalmate birds (booby and cormorant), although in the DNA hybridization tree (Fig. 2 *Lower*) it appeared as the most divergent taxon of the "pelecaniforms." A smaller portion of the 16S rRNA region (1.1 kb) was sequenced (S.B.H., unpublished results) in another totipalmate, the African Darter (*Anhinga rufa*), and in a restricted analysis of that region in all taxa (not shown) it clustered with the booby–cormorant cluster, as expected. The morphological and DNA hybridization evidence (Fig. 2) also place the boobies, cormorants, and darters (also called anhingas) in a monophyletic cluster.

A striking feature of the DNA sequence phylogeny (Fig. 3) is the short length of many internal branches. This also is evident in the DNA hybridization tree (Fig. 2 *Lower*) and in an analysis of cytochrome *b* sequences (19), suggesting that many of the divergences in this group occurred during a relatively short period of time. Rapid radiations require long DNA sequences to resolve all nodes, thus, although some questions are answered by these data, more sites will be needed for a complete resolution of this phylogeny. The use of other distance corrections (Kimura, Tajima-Nei, Tamura, Tamura–Nei, and γ; γ parameter = 0.80, calculated from data) with neighbor-joining yielded nearly identical results, with only minor rearrangement among nodes showing poor resolution in Fig. 3.

Parsimony analysis without weighting and with transversions weighted 10 × transitions each resulted in a different single most-parsimonious tree. In both cases, the pelican clustered with the Shoebill, the New World vultures were closer to the storks and pelicans than to the Old World vultures, and the loons were closer to the shearwater than to the grebe. The weighted parsimony tree is nearly identical to the neighbor-joining tree, except that the Old World vulture (*Neophron*) clusters with the tropicbird and in regions where the branching order is poorly resolved in Fig. 3. The statistical limitations of weighted parsimony analysis have been discussed elsewhere (20, 21).

Subsets of Taxa. Phylogenetic resolution may be increased when subsets of taxa are analyzed. In this case, the conflict between morphology and molecules in "pelecaniform" birds and their relatives can be reduced to questions that require only small subsets of taxa. In each subset, the galliform (*Gallus*) was included, the tree was rooted with *Alligator*, and each of the eight ciconiidian taxa was analyzed.

The affinities of the pelican were tested by comparing only the pelican, booby, cormorant, and one other taxon. The highest statistical confidence (99%) for clustering of the pelican with some other taxon was obtained with the Shoebill (Fig. 4*A*). In other subsets, the pelican clustered with the penguin (94%), Common Loon (94%), Red-throated Loon (86%), shearwater (86%), and stork (74%). The pelican was a sister taxon to the booby–cormorant group when the frigatebird (86%), Andean Condor (76%), or California Condor (74%) was included.

An alliance between loons and grebes often has been proposed, but Stolpe (22) found substantial differences between them in the hind limb musculature and skeletal anatomy. Many ornithologists accepted convergence as the explanation for the similarities between these two groups of diving birds, but Cracraft (23) dismissed Stolpe's work as irrelevant and concluded that loons and grebes are close relatives. However, a loon–grebe alliance is not supported by DNA hybridization (Fig. 2). This question was tested in the present study by including only the two loons, the grebe, and another taxon. In all cases, the loon–grebe alliance was broken and the loons clustered with the added taxon (Fig. 4*B*): shearwater (98%), pelican (98%), California Condor (98%), stork (98%), Andean Condor (97%), Shoebill (96%), frigatebird (93%), and penguin (86%).

The monophyly of the diurnal birds of prey (falconiforms) was tested by including only the Old World vulture, the two condors, and one other taxon. In all cases, the falconiforms were found to be polyphyletic (Fig. 4*C*) and the condors clustered with the added taxon: Shoebill (99%), shearwater (99%), frigatebird (99%), Common Loon (97%), stork (96%), penguin (94%), Red-throated Loon (79%), and pelican (73%).

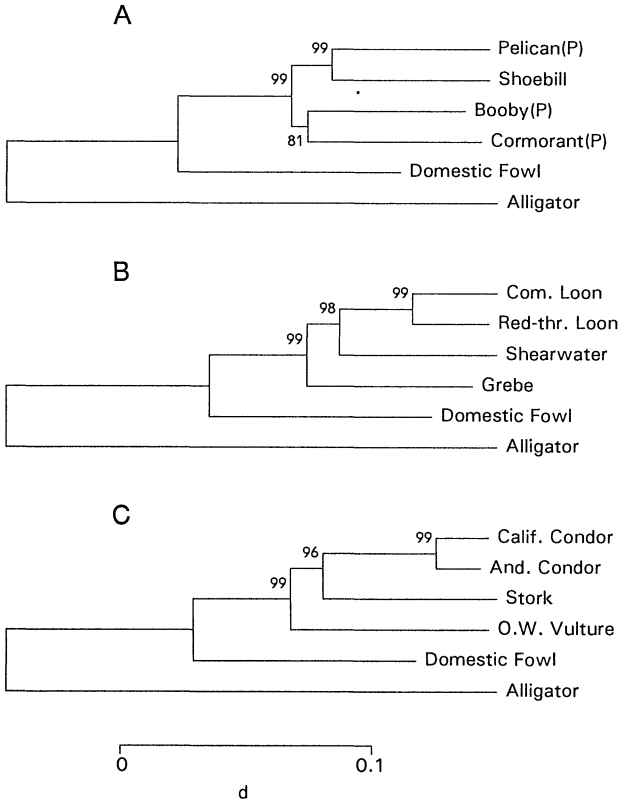

FIG. 4. Phylogenetic relationships of selected subsets of taxa (data and methods of analysis as in Fig. 3). (*A*) Test of the pelican–booby morphological grouping. (*B*) Test of the loon–grebe morphological grouping. (*C*) Test of the monophyly of vultures.

Correlations Among Methods. The technique of DNA·DNA hybridization measures the degree of sequence similarity between complete genomes (2). Although the DNA sequences in the present study examined only a small portion (1.7 kb) of the genomes of these birds, it is possible to compare the quantitative estimates of genetic divergence produced by these two methods. For the DNA sequence measure we used the Kimura distance (transversions only, to avoid transition bias), and for DNA hybridization the $\Delta T_{50}H$ values were taken from the averages in ref. 2, figures 357–368. The two methods are highly correlated (Fig. 5*A*; r^2 = 0.90; r^2 = 0.72 when a smaller subset of pairwise $\Delta T_{50}H$ values are used).

The morphological data set for these birds (table 1 of ref. 5) was converted into a pairwise matrix of differences to compare morphological with molecular divergence. As expected from the phylogenetic results, the correlation is poor [with DNA sequence, r^2 = 0.01 (Fig. 5*B*); with DNA hybrid-

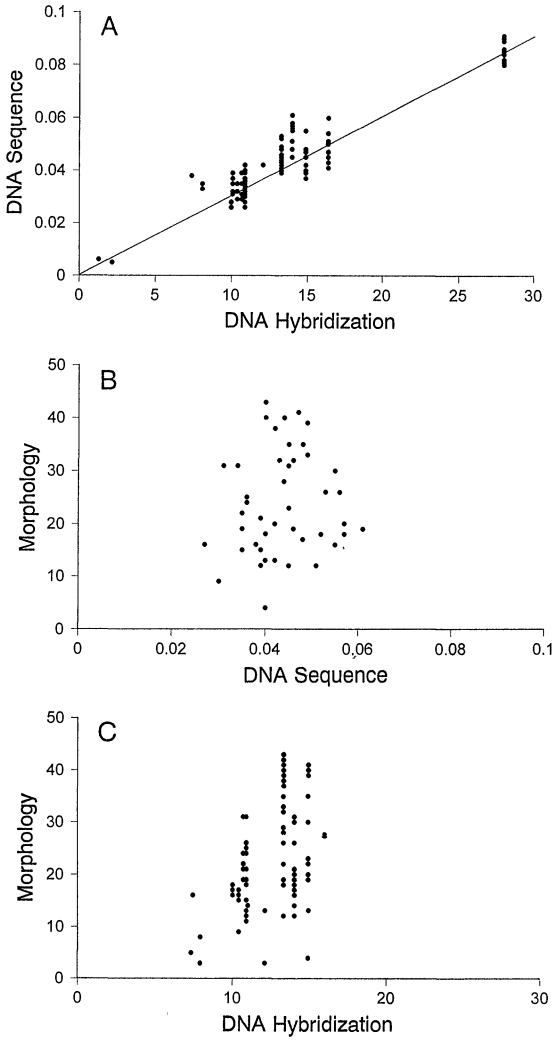

FIG. 5. Relationships between pairs of analyses. (*A*) DNA sequence divergence (Kimura *d*, transversions only; this study) and DNA hybridization ($\Delta T_{50}H$; ref. 2) (r^2 = 0.90). (*B*) Morphological divergence (character differences; table 1 of ref. 5) and DNA sequence divergence (r^2 = 0.01). (*C*) Morphological divergence and DNA hybridization (r^2 = 0.23).

ization, $r^2 = 0.23$ (Fig. 5C)]. Both neighbor-joining and parsimony analyses are able to account for some rate differences, therefore a poor correlation between morphological and molecular distance does not necessarily indicate that either is providing a poor (or different) estimate of phylogeny. However, in this case, the estimates of phylogeny differ and there is better agreement between the two molecular techniques than between either and morphology.

DISCUSSION

The DNA sequence data support earlier results from DNA hybridization (2) that indicated that the totipalmate foot, gular pouch, and several other morphological characters diagnostic of the traditional Order Pelecaniformes are not useful for inferring phylogeny. Instead, the pelicans and frigatebirds are found to be more closely related to birds with diverse morphologies, such as the Shoebill, condors, penguins, storks, loons, and shearwaters. An enlarged Order Ciconiiformes, which combined eight previously recognized orders, was proposed (2, 24) to reflect the results of the DNA hybridization study. The DNA sequence data include taxa representing seven of the eight orders and support this major change in the classification of birds. Additional examples that support the classification of Sibley *et al.* are noted in ref. 25.

Two approaches, "total evidence" and "taxonomic congruence," have been suggested to resolve conflicts between molecular and morphological estimates of phylogeny. Advocates of the total evidence method suggest combining all morphological and molecular data into one analysis to produce a single estimate of phylogeny (26). In addition to the statistical problems involved in combining DNA sequence data with morphological characters, this method suffers from the fact that the answer is determined less by the true phylogeny than by the data set with the most characters, although character weighting has been suggested (27). Advocates of taxonomic congruence suggest searching among conflicting phylogenies for regions of agreement to obtain a consensus (28). This avoids the size problem, but it imposes unequal weighting of characters and results in a lack of resolution if there is serious disagreement. Some have proposed combining elements of both approaches (29, 30).

When molecular data produce a robust phylogeny that conflicts with morphology, we suggest a third approach: reevaluate the morphological evidence. In such cases it is probable that morphological characters presumed to be shared-derived are shared-primitive or convergent. Unlike morphology, a considerable proportion of nucleotide variation is selectively neutral (e.g., synonymous sites, pseudogenes, and many noncoding and some coding regions) so that significant convergence at the molecular level is unlikely. Morphological convergence is widespread and well documented (e.g., see ref. 31), and we should be delighted to encounter additional interesting examples.

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1. Linnaeus, C. (1758) *Systema Naturae per Regna Tria Naturae* (Laurentii, Stockholm), 10th Ed.
2. Sibley, C. G. & Ahlquist, J. E. (1990) *Phylogeny and Classification of Birds: A Study in Molecular Evolution* (Yale Univ. Press, New Haven, CT).
3. Lanham, U. N. (1947) *Auk* **64,** 65–70.
4. Saiff, E. (1978) *Zool. J. Linnaean Soc.* **63,** 315–370.
5. Cracraft, J. (1985) *Auk* **102,** 834–853.
6. Felsenstein, J. (1985) *Evolution* **39,** 783–791.
7. Swofford, D. L. (1993) PAUP, Phylogenetic Analysis Using Parsimony (Illinois Nat. Hist. Surv., Champaign, IL), Version 3.1.1.
8. Cottam, P. A. (1957) *Bull. Br. Mus. (Nat. Hist.) Zool.* **5,** 49–72.
9. Desjardins, P. & Morais, R. (1990) *J. Mol. Evol.* **32,** 153–161.
10. Monroe, B. L., Jr., & Sibley, C. G. (1993) *A World Checklist of Birds* (Yale Univ. Press, New Haven, CT).
11. Hedges, S. B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2621–2624.
12. Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. & Wilson, A. C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6196–6200.
13. Cabot, E. L. & Beckenbach, A. T. (1989) *Comput. Appl. Biosci.* **5,** 233–244.
14. Kumar, S., Tamura, K. & Nei, M. (1994) *Comput. Appl. Biosci.* **10,** 189–191.
15. Saitou, N. & Nei, M. (1987) *Mol. Biol. Evol.* **4,** 406–425.
16. Jukes, T. H. & Cantor, C. R. (1969) in *Mammalian Protein Metabolism,* ed. Munroe, H. N. (Academic, New York), pp. 21–132.
17. Rzhetsky, A. & Nei, M. (1992) *Mol. Biol. Evol.* **9,** 945–967.
18. Ligon, J. D. (1967) *Occas. Pap. Univ. Mich. Mus. Zool.* No. 651.
19. Avise, J. C., Nelson, W. S. & Sibley, C. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5173–5177.
20. Hedges, S. B. & Maxson, L. R. (1992) *Mol. Biol. Evol.* **9,** 374–377.
21. Hedges, S. B. & Bezy, R. L. (1993) *Mol. Phylogenet. Evol.* **2,** 76–87.
22. Stolpe, M. (1935) *J. Ornithol.* **83,** 115–128.
23. Cracraft, J. (1982) *Syst. Zool.* **31,** 35–56.
24. Sibley, C. G., Ahlquist, J. E. & Monroe, B. L., Jr. (1988) *Auk* **105,** 409–423.
25. Sibley, C. G. (1994) *J. Avian Biol.* (formerly *Ornis Scand.*) **25,** 87–92.
26. Kluge, A. G. (1989) *Syst. Zool.* **38,** 7–25.
27. Chippindale, P. T. & Wiens, J. J. (1994) *Syst. Biol.* **43,** 278–287.
28. Mickevich, M. F. (1978) *Syst. Zool.* **27,** 143–158.
29. de Queiroz, A. (1993) *Syst. Biol.* **42,** 368–372.
30. Huelsenbeck, J. P., Swofford, D. L., Cunningham, C. W., Bull, J. J. & Waddell, P. J. (1994) *Syst. Biol.* **43,** 288–291.
31. Darwin, C. (1859) *On the Origin of Species by Means of Natural Selection* (Murray, London).