PHYLOGENY OF HERONS ESTIMATED FROM DNA-DNA HYBRIDIZATION DATA

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ABSTRACT.—Genetic distances derived by hybridizing single-copy DNAs of 31 heron species (or subspecies) and 1 ibis species are summarized as ΔTm values. From these distances, a phylogeny is estimated and distributional properties of DNA hybridization data are computed. I found that the distinction between night and day herons is primarily adaptive, not genealogical; *Syrigma* is closely related to *Egretta*; *Bubulcus* and *Casmerodius* are closely related to *Ardea*, but *Egretta* is not; bitterns are the sister taxon of the day and night herons; *Cochlearius* and *Tigrisoma* are each others' closest relatives and together form the sister group of the rest of the ardeids; and the rate of single-copy DNA evolution differs in different heron lineages. *Received 18 April 1986, accepted 3 September 1986.*

MOST taxonomists agree that herons belong in a family of their own, the Ardeidae, but there is considerable disagreement concerning the intrafamilial relationships of these birds. Over the last 100 years, the number of recognized species in the Ardeidae has varied from 60 to 93 and the number of genera from 15 to 35 (Sharpe 1898, Reichenow 1913). Although herons are usually divided into four groups (day herons, night herons, tiger herons, and bitterns), species are moved back and forth among these groups with each revision of the classification. A fifth group is sometimes considered necessary to accommodate the enigmatic Boat-billed Heron (*Cochlearius cochlearius*). The continual problems of determining most heron relationships derive primarily from the fact that adaptive changes within the limits of the ardeids' wading-piscivorous *Bauplan* are difficult to interpret. Herons are constrained to have long bills, legs, and necks, and this constraint has induced a family history rife with parallel and convergent evolution.

To develop a hypothesis of heron phylogeny without having to interpret tracked morphological or behavioral characters, I used DNA-DNA hybridization to compare taxa. The logic of DNA hybridization has been reviewed by Sibley and Ahlquist (e.g. 1983), Benveniste (1985), and others. The technique operates under the assumption that the genetic relatedness of organisms is reflected in the similarity of their DNA base pair sequences. This similarity can be measured by hybridizing strands of DNA from different species and measuring the bonding strength of these hybrids. The poorer the bonding strength, the more distantly related the organisms. The advantages of DNA hybridization are that it is objective and it accounts for historically informative characteristics encoded in the DNA that are not necessarily expressed physically. Such previously unmeasurable genetic features include pseudogenes (e.g. the obsolete genes coding for tooth structure in birds; Kollar and Fisher 1980) and regulatory genes.

Estimating and testing phylogenies.—DNA hybridization produces distance data, and the most appropriate method for clustering such data is least-squares regression (Sheldon in press). Templeton (1985) pointed out that tree-building algorithms based on procedures like least squares simply provide estimates of phylogenies. Alternative estimates require testing by statistical methods before one phylogeny can be accepted as better than others.

Unfortunately, statistical methods for testing alternative phylogenetic hypotheses have not been established. Templeton (1985), for example, introduced the delta Q-test, but Saitou (1986) argued that this test is inadequate for differentiating topologies, and Fitch (1986) argued that it assumes evolutionary rate constancy. Although statistical procedures can be used to test for different evolutionary rates in different lineages (e.g. Felsenstein 1984) and, in special situations, can resolve multifurcations

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TABLE 1. List of the taxa used in DNA hybridization comparisons and the number of separate purifications made of the DNAs of those taxa. Asterisks indicate taxa that were radio-labeled.

Species	No. of preparations
Syrigma sibilatrix (Whistling Heron)*	1
Ardea herodias (Great Blue Heron)*	5
A. cocoi (Cocoi Heron)	2
A. pacifica (White-necked Heron)	1
A. melanocephala (Black-headed Heron)	2
A. sumatrana (Great-billed Heron)	1
Casmerodius albus egretta [Great Egret (North America)]*	6
C. a. modestus [Great Egret (Australasia)]	1
Bubulcus ibis (Cattle Egret)*	4
Egretta vinaceigula (Slatey Egret)	1
E. tricolor (Tricolored Heron)	4
E. intermedia (Intermediate Egret)	2
E. novaehollandiae (White-faced Heron)	2
E. caerulea (Little Blue Heron)*	4
E. thula (Snowy Egret)*	5
E. garzetta (Little Egret)	2
E. sacra (Eastern Reef Egret)	1
Ardeola grayii (Indian Pond Heron)	1
Butorides striatus virescens [Green-backed Heron (North America)]*	5
B. s. striatus [Green-backed Heron (South America)]	1
B. s. javanicus [Green-backed Heron (Southeast Asia)]	2
Nycticorax violaceus (Yellow-crowned Night-Heron)*	3
N. nycticorax (Black-crowned Night-Heron)*	10
N. caledonicus (Nankeen Night-Heron)	1
Cochlearius cochlearius (Boat-billed Heron)*	5
Tigrisoma lineatum (Rufescent Tiger-Heron)*	3
Ixobrychus exilis (Least Bittern)*	1
I. minutus (Little Bittern)	1
I. cinnamomeus (Cinnamon Bittern)	3
Botaurus lentiginosus (American Bittern)*	5
B. stellaris (Palearctic Bittern)	1
Plegadis falcinellus (Glossy Ibis)*	1

(e.g. Fitch 1986), none so far is useful in evaluating alternative phylogenies consisting of more than four taxa. The best way to evaluate different phylogenies is by the consensus tree method, which seeks corroboration among alternative phylogenies. Lanyon's (1985) Jackknife Strict-Consensus Tree (JST) algorithm was chosen to evaluate trees derived from subsets of the data in this study. The JST method has one additional feature. By comparing subsets of a single distance matrix, it provides an intuitive indication of the additivity and independence of the data.

MATERIALS AND METHODS

Biochemistry.—The methods used to prepare hybrids were essentially those of Sibley and Ahlquist (1981). Further detail is provided in Sheldon (1986).

Briefly, high molecular weight DNAs were extracted from bird erythrocytes and tissues and analyzed spectrophotometrically for protein contamination. The DNAs were then sheared by sonification, yielding fragments with an average length of 400–500 base pairs, as determined by agarose gel electrophoresis. Single-copy DNA fragments were recovered by hydroxyapatite chromatography and radioactively labeled with ^{125}I. The labeled DNAs were mixed with unlabeled driver DNAs in a ratio of 1:400, boiled, and incubated at 60°C to a Cot exceeding 15,000. These conditions permitted hybrids to form between DNA sequences differing in base pair complementarity by a maximum of 25–30%. The hybrids were then fractionated thermally at 2.5°C increments in lots of 25, from 55° to 95°C. Each 25-hybrid lot (= 1 experiment), contained at least one homoduplex control hybrid, comprising label and driver DNAs prepared from the same sample of purified DNA. The radioactivity eluted at each of the 17 fractionation temperatures, representing the amount of DNA that had dissociated to single-stranded form at that temperature, was counted in a gamma spectrometer and constituted a raw datum.

Reciprocal comparisons, involving ca. 940 hybrids, were made among 13 species of heron and 1 species of ibis (Table 1). About 300 one-way comparisons were made using labeled DNAs from the same 13 heron species and driver DNAs from 18 additional heron taxa. Another ca. 130 hybrids were produced to determine genetic distances within species. In planning the reciprocal comparisons, an effort was made to hybridize the DNA of each of the 14 labeled species at least 5 times with the driver DNA of each of those species to produce 10 hybrids per pair. This was not always possible, however, because of their availability and supply.

Data analysis.—The 23,000 heron raw data are available to any person who sends six formatted, IBM-PC disks in a self-addressed, stamped container.

Methods of data analysis differed from those of Sibley and Ahlquist (e.g. 1981, 1983) in that ΔTm was used as the measure of genetic distance, instead of ΔT50H, and clustering was performed by least squares. The logic behind the use of ΔTm is discussed in the Results and Discussion and that of data correction in Sheldon (in press).

To calculate Tm, the count recorded at each temperature from 62.5° to 95°C was normalized to a percentage of the total counts in that range, and a cumulative frequency distribution was constructed. Tm

equaled the temperature at which 50% of the counts was recorded, extrapolated by linear regression. Genetic distances (ΔTm's) were calculated by subtracting heteroduplex Tm values from the homoduplex Tm of the same experiment.

Interspecific distances were summarized in lists (Tables 2–15). The average distances between the 14 labeled taxa were calculated by multiplying the values in the lists by sample size, adding reciprocal products, and dividing the sum by the total number of observations. [A matrix of average distances was published by Sheldon (in press).] From these average distances, trees were drawn using the least-squares option of the programs "Fitch" and "Kitsch" in J. Felsenstein's phylogenetic computer package, PHYLIP (version 2.8). The relative quality of the fit of these trees was judged from the residual sum of squares (RSS).

A Jackknife Strict-Consensus Tree was developed using the program of Lanyon (1985). For the 14 labeled taxa, 13 13-taxa pseudoreplicate trees were constructed using PHYLIP. The nodes where the 13 trees disagreed were combined to form multifurcations.

TABLE 2. Delta Tm values for taxa compared with labeled *Syrigma sibilatrix*.

Species	$\bar{x}$	n	SD	Range
Syrigma sibilatrix	0.0	3		
Egretta thula	2.5	3	0.31	0.6
Ardea herodias	3.3	3	0.05	0.1
Bubulcus ibis	3.3	3	0.24	0.4
Nycticorax nycticorax	3.4	3	0.12	0.2
Butorides striatus virescens	3.5	3	0.06	0.1
Ixobrychus exilis	5.2	3	0.26	0.6
Botaurus lentiginosus	5.7	3	0.20	0.3
Tigrisoma lineatum	5.8	3	0.04	0.1
Plegadis falcinellus	9.7	2		0.5

RESULTS AND DISCUSSION

DATA CHARACTERISTICS

Reproducibility.—The average standard deviation (ASD) for all the heron ΔTm values was 0.20 ± 0.12 SD (n = 221, range = 0.01–0.69). This degree of reproducibility did not change with genetic distance (r = 0.086, n = 196, $P >$ 0.10). The ASD for hybrids made with more than one individual of another species was 0.21 ± 0.12 SD (n = 132, range = 0.03–0.69). The ASD for hybrids made from only one individual of another species was 0.19 ± 0.10 SD (n = 74, range = 0.01–0.65). These numbers indicate that individual variation has a minor effect on ΔTm variance. The ASD for ΔT50H values among birds is ±0.35 for $n > 5$ (Sibley and Ahlquist pers. comm.). For ΔTmR distances among mammals, ASD is ±0.2 when ΔTmR is less than 5 and ±0.5 when ΔTmR is greater than 5 (O'Brien et al. 1985).

Delta Tm is more reproducible than the commonly used statistics ΔT50H and ΔTmR because, unlike these measures, ΔTm does not take into account normalized percent hybridization (NPH), which has a large variance, especially when closely related organisms are compared (Bledsoe 1984, Caccone 1986, Sheldon in press). The factoring of NPH into distance measures also increases relative genetic distances. (The same is true of corrections made for multiple substitutions at single base sites.) These greater distances permit better resolution of fine branching patterns, but are based on additional assumptions about DNA evolution, notably that all DNA sequences have homologs and can potentially hybridize. Thus, of the DNA hybridization distance measures, ΔTm is the least variable and the most conservative.

However, ΔTm is applicable only in studies of closely related groups, because distance measures greater than Δ10 become compressed by the effects of DNA reassociation criteria and sequence divergence (Sibley and Ahlquist 1983). Delta Tm also loses its usefulness if NPH is less than 80–90%. When NPH is low, ΔTm estimates the similarity of a smaller, more conserved part of the genome (Zwiebel et al. 1982, Templeton 1986). For herons, the NPH averages greater than 90% (Sheldon in press); therefore, ΔTm summarizes the similarity of more than 90% of the sequences.

Reciprocity.—Reciprocal discrepancy occurs when the distance measured from labeled taxon A to driver taxon B differs from the distance of labeled B to driver A. For herons, the average discrepancy of mean reciprocal ΔTm values was only 0.29 ± 0.21 (n = 74, range = 0–0.9). Such high reciprocity is expected when ΔTm is used as the distance measure, regardless of any disparity in genome size, because the number of sequences that can hybridize is dictated by the species with the smaller single-copy genome. When nonreciprocity occurs, it is most likely to be the result of experimental error. Caccone (1986) found, for example, that almost all of her nonreciprocity resulted from variation in DNA fragment lengths. Compensation for this problem can be achieved either by cor-

TABLE 3. Delta Tm values for taxa compared with labeled *Ardea herodias*.

Species	$\bar{x}$	*n*	SD	Range
Ardea herodias (homo)[a]	0.0	13		
Ardea herodias (het)	0.4	6	0.38	1.0
Ardea cocoi	0.5	16	0.19	0.7
Ardea sumatrana	1.2	6	0.27	0.8
Ardea melanocephala	1.4	10	0.25	0.8
Ardea pacifica	1.4	6	0.21	0.5
Casmerodius albus egretta	1.4	17	0.33	1.3
Casmerodius a. modestus	1.8	5	0.22	0.5
Egretta intermedia	1.8	4	0.14	0.3
Bubulcus ibis	2.0	10	0.34	1.2
Nycticorax caledonicus	3.3	1		
Butorides striatus virescens	3.5	12	0.13	0.5
Egretta vinaceigula	3.5	1		
Egretta novaehollandiae	3.5	2		0.1
Nycticorax violaceus	3.6	9	0.22	0.7
Nycticorax nycticorax	3.6	15	0.13	0.5
Butorides s. striatus	3.8	2		0.0
Egretta caerulea	3.8	4	0.18	0.4
Egretta thula	3.9	10	0.13	0.4
Egretta tricolor	3.9	3	0.47	0.9
Syrigma sibilatrix	4.0	7	0.15	0.4
Cochlearius cochlearius	5.2	1		
Ixobrychus minutus	5.7	8	0.19	0.5
Ixobrychus exilis	5.9	7	0.22	0.7
Ixobrychus cinnamomeus	6.0	2		0.2
Botaurus lentiginosus	6.1	12	0.22	0.8
Tigrisoma lineatum	6.2	10	0.16	0.4
Plegadis falcinellus	9.9	3	0.65	0.9

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

TABLE 4. Delta Tm values for taxa compared with labeled *Casmerodius albus egretta*.

Species	$\bar{x}$	*n*	SD	Range
Casmerodius albus egretta (homo)[a]	0.0	5		
Casmerodius a. egretta (het)	0.2	8	0.17	0.5
Egretta intermedia	0.7	4	0.19	0.4
Casmerodius a. modestus	0.8	6	0.23	0.5
Ardea pacifica	0.9	6	0.23	0.7
Ardea melanocephala	1.1	4	0.11	0.2
Ardea sumatrana	1.3	6	0.19	0.5
Bubulcus ibis	1.3	5	0.30	0.7
Ardea cocoi	1.7	3	0.26	0.5
Ardea herodias	1.8	6	0.69	1.6
Egretta vinaceigula	3.1	1		
Butorides s. virescens	3.2	5	0.22	0.6
Egretta tricolor	3.2	2		0.7
Egretta novaehollandiae	3.3	2		0.7
Nycticorax violaceus	3.3	1		
Nycticorax nycticorax	3.3	2		0.2
Egretta caerulea	3.4	3	0.33	0.6
Egretta thula	3.5	4	0.29	0.6
Syrigma sibilatrix	3.7	2		0.2
Ixobrychus exilis	5.5	1		
Botaurus lentiginosus	5.8	1		
Tigrisoma lineatum	5.8	1		

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

recting data for differences in experimental conditions or by making a series of replicate hybrids prepared from different samples of DNA to form a more representative distribution of distances. The latter was done in this study. Those species with the greatest average reciprocal disparity were, as expected, those for which the fewest replicates were prepared, viz. *Syrigma sibilatrix* and *Egretta caerulea*. The effects that the data of these reciprocally discrepant species have on tree-building are discussed in the Phylogeny section below.

Distinguishing closely related species.—Intrasubspecific distances were measured when DNAs from more than one individual of a labeled species were available. These distances provide an approximate baseline for determining whether intertaxon delta values are significant. For example, the distances between *Ardea herodias*, *Bubulcus ibis*, and *Casmerodius albus* are small at ΔTm 1.5–1.8. Nevertheless, these distances are significantly greater than the distances of any of these species to themselves, ΔTm 0.2–0.4 ($P < 0.001$).

Subjective judgment is required in weighing the importance of some intra- vs. interspecific DNA hybrid values. This is especially true for one-way comparisons, but also for a few reciprocal comparisons. For example, the distance from *Egretta thula* to *E. caerulea*, ΔTm 1.2, was significantly greater than from *thula* to itself, ΔTm 0.6 ($P < 0.001$); but *caerulea* to *thula*, ΔTm 1.6, was not significantly different from *caerulea* to itself, ΔTm 1.1 ($P = 0.134$). When such disagreement occurs, one can decide whether two taxa are genetically distinct if it can be shown that the data of one species are more trustworthy than those of the other. For *thula* and *caerulea*, the decision is easy. *Egretta thula* had better average reciprocity than *caerulea* (ΔTm 0.2 vs. 0.5), a larger sample size of intraspecific comparisons ($n = 5$ vs. 2), and an average intraspecific value closer to that of all herons (0.38 ± 0.34 SD, $n = 55$). It is safe to assume, therefore, that *thula* and *caerulea* are genetically distinct by DNA hybridization standards.

TABLE 5. Delta Tm values for taxa compared with labeled *Bubulcus ibis*.

Species	$\bar{x}$	*n*	SD	Range
Bubulcus ibis (homo)[a]	0.0	9		
Bubulcus ibis (het)	0.4	4	0.40	0.8
Ardea herodias	1.5	5	0.08	0.3
Ardea sumatrana	1.5	4	0.06	0.2
Ardea cocoi	1.6	4	0.05	0.2
Ardea melanocephala	1.6	6	0.22	0.6
Ardea pacifica	1.7	4	0.10	0.2
Casmerodius albus egretta	1.7	5	0.12	0.4
Egretta intermedia	1.7	3	0.16	0.3
Butorides striatus striatus	3.2	1		
Butorides s. virescens	3.2	8	0.19	0.5
Nycticorax nycticorax	3.2	5	0.08	0.2
Nycticorax violaceus	3.3	5	0.08	0.2
Egretta caerulea	3.6	2		0.1
Egretta tricolor	3.6	2		0.1
Syrigma sibilatrix	3.7	5	0.17	0.3
Egretta thula	3.8	6	0.15	0.4
Egretta vinaceigula	3.8	2		0.1
Egretta garzetta	3.9	1		
Egretta novaehollandiae	3.9	2		0.0
Ixobrychus exilis	5.7	9	0.30	1.1
Botaurus lentiginosus	5.8	8	0.22	0.8
Tigrisoma lineatum	5.9	6	0.11	0.2
Plegadis falcinellus	10.0	3	0.16	0.3

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

TABLE 6. Delta Tm values for taxa compared with labeled *Egretta caerulea*.

Species	$\bar{x}$	*n*	SD	Range
Egretta caerulea (homo)[a]	0.0	2		
Egretta caerulea (het)	1.1	2		0.5
Egretta novaehollandiae	1.1	4	0.13	0.3
Egretta garzetta	1.2	3	0.23	0.5
Egretta vinaceigula	1.2	3	0.34	0.7
Egretta tricolor	1.3	3	0.26	0.5
Egretta sacra	1.6	4	0.20	0.5
Egretta thula	1.6	4	0.28	0.7
Ardea pacifica	2.6	1		
Syrigma sibilatrix	2.8	3	0.10	0.2
Egretta intermedia	3.1	1		
Ardea herodias	3.2	2		0.4
Casmerodius albus modestus	3.3	1		
Nycticorax violaceus	3.7	2		0.2
Nycticorax nycticorax	3.7	3	0.28	0.5
Butorides striatus virescens	3.8	2		0.2
Camerodius a. egretta	4.2	1		
Bubulcus ibis	4.4	1		
Ixobrychus exilis	5.6	1		

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

PHYLOGENY

When distances among the 14 labeled taxa were clustered by least squares using PHYLIP so that negative branches were not allowed and sister branches were not required to be equal in length (i.e. a molecular clock was not assumed), the best tree had a residual sum of squares (RSS) of 2.97 (Fig. 1). When negative branches were allowed, PHYLIP produced a slightly better tree (RSS = 2.95). The latter tree had two short negative branches, one of −0.09 at the node where *A. herodias*, *Casmerodius*, and *Bubulcus* emanated (forming the "*Ardea* clade"), and one of −0.05 where *Nycticorax nycticorax*, *N. violaceus*, *Butorides striatus*, and the *Ardea* clade derived.

Rates of evolution.—The differences in sister branch lengths (Fig. 1) suggest that different rates of DNA evolution have occurred in different heron lineages. The long branch comprising *Botaurus lentiginosus* and *Ixobrychus exilis* indicates that the average rate of bittern single-copy DNA evolution was about 25% faster than that of day and night herons (hereafter called the "typical herons"). *Cochlearius cochlearius* and *Tigrisoma lineatum* DNA appears to have evolved about 19% slower than that of the typical herons.

The statistical significance of these rate differences was confirmed (Sheldon in press) by using the method suggested by Felsenstein (1984). RSS values of trees computed with and without the assumption of a molecular clock were compared by *F*-test. Trees built without the clock assumption always provided significantly better fits to the data, even when the ingroup was comprised solely of typical herons.

It might be thought that differential rates would negate the effectiveness of DNA hybridization data in reconstructing phylogeny. Trees can be constructed from DNA hybridization data, however, regardless of differential rates, because in a pairwise DNA hybrid comparison only the sum of autapomorphic base pair changes is measured. Symplesiomorphy and synapomorphy are constants defined at the ancestor of the two taxa. If autapomorphic changes are greater in one lineage than in the other (i.e. the rate of evolution is faster in one lineage), the difference will be detected by outgroup comparisons with those two lineages (Sheldon 1986, in press).

Evaluating estimated phylogenies.—A second

TABLE 7. Delta Tm values for taxa compared with labeled *Egretta thula*.

Species	$\bar{x}$	*n*	SD	Range
Egretta thula (homo)[a]	0.0	5		
Egretta garzetta	0.5	6	0.08	0.2
Egretta thula (het)	0.6	5	0.16	0.4
Egretta vinaceigula	0.8	6	0.16	0.4
Egretta caerulea	1.2	8	0.12	0.3
Egretta novaehollandiae	1.2	6	0.13	0.4
Egretta tricolor	1.2	6	0.12	0.3
Syrigma sibilatrix	3.1	5	0.21	0.6
Ardea cocoi	3.6	5	0.12	0.3
Ardea melanocephala	3.6	5	0.09	0.3
Egretta intermedia	3.6	6	0.21	0.5
Ardea herodias	3.7	6	0.05	0.1
Ardeola grayii	3.7	4	0.17	0.4
Casmerodius albus egretta	3.7	8	0.19	0.5
Nycticorax nycticorax	3.7	5	0.10	0.2
Nycticorax violaceus	3.8	5	0.28	0.7
Butorides striatus virescens	3.9	6	0.21	0.6
Bubulcus ibis	4.0	5	0.49	1.0
Butorides s. striatus	4.2	3	0.15	0.3
Ixobrychus cinnamomeus	5.4	1		
Cochlearius cochlearius	5.7	8	0.23	0.7
Botaurus lentiginosus	5.9	7	0.36	1.1
Ixobrychus exilis	6.0	6	0.10	0.2
Tigrisoma lineatum	6.1	8	0.12	0.3
Plegadis falcinellus	10.5	1		

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

TABLE 8. Delta Tm values for taxa compared with labeled *Butorides striatus virescens*.

Species	$\bar{x}$	*n*	SD	Range
Butorides striatus virescens (homo)[a]	0.0	5		
Butorides s. virescens (het)	0.3	12	0.12	0.5
Butorides s. javanicus	0.7	14	0.12	0.4
Butorides s. striatus	0.9	6	0.07	0.3
Ardea herodias	3.4	5	0.10	0.3
Ardea melanocephala	3.4	1		
Casmerodius albus egretta	3.4	5	0.17	0.5
Bubulcus ibis	3.5	5	0.06	0.2
Egretta intermedia	3.5	4	0.19	0.4
Nycticorax violaceus	3.7	5	0.10	0.3
Nycticorax nycticorax	3.8	4	0.20	0.4
Egretta caerulea	4.0	2		0.3
Egretta tricolor	4.0	1		
Egretta thula	4.1	5	0.11	0.3
Egretta novaehollandiae	4.1	4	0.10	0.2
Syrigma sibilatrix	4.2	5	0.10	0.2
Botaurus lentiginosus	6.0	6	0.10	0.3
Ixobrychus exilis	6.0	5	0.12	0.2
Tigrisoma lineatum	6.1	5	0.24	0.7

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

feature of the 14-taxa trees computed by PHYLIP is that all of the negative branches and several of the positive branches separating typical heron nodes are short (i.e. less than ±0.1). The question arises, therefore, whether these branches are significant or whether multifurcations explain parts of the phylogeny equally well.

The Jackknife Strict-Consensus Tree, built to pinpoint areas of weakness in the DNA-hybridization tree, revealed that some of the 13-taxa (pseudoreplicate) trees contradict one another at the point where *N. nycticorax, N. violaceus, Butorides,* and the *Ardea* clade come together. Hence, a single multifurcating node best represents the origin of these four branches. The consensus tree did not indicate a problem with the short branch that separates the egret clade from the rest of the typical herons nor with the short branch that separates *Bubulcus* from *A. herodias* and *Casmerodius* within the *Ardea* clade.

Regardless of the consensus-tree finding, the reliability of these two remaining short branches is in question. The branch separating *Bubulcus* from *Casmerodius* and *A. herodias* is only 0.07 in length, which is less than one average delta-value standard deviation. These three taxa, therefore, should probably be depicted as derived from a trifucation. The branch distinguishing the egret clade depends, in part, on the data of *E. caerulea* and *Syrigma,* which, as mentioned, are less extensive than those of other labeled taxa. When *E. caerulea* and *Syrigma* distances are omitted from the clustering process and negative branches are allowed, the short branch that distinguishes the egret clade disappears. Thus, the most conservative estimate of heron phylogeny, and the one accepted in this study, not only has a trifurcation in the *Ardea* clade, but a quintifurcation at the node joining the typical herons (see Fig. 2).

Placement of unlabeled taxa.—To illustrate the relationships among all herons compared in this study, unlabeled taxa have been added to the fundamental tree described above to produce a summarizing tree (Fig. 2). The location where most unlabeled taxa belong is obvious, because unlabeled species lie less than ΔTm 2.0 from other members of their assigned clade or more

TABLE 9. Delta Tm values for taxa compared with labeled *Nycticorax violaceus*.

Species	$\bar{x}$	*n*	SD	Range
Nycticorax violaceus (homo)[a]	0.0	4		
Nycticorax violaceus (het)	0.5	3	0.19	0.4
Nycticorax caledonicus	3.1	5	0.15	0.4
Nycticorax nycticorax	3.1	3	0.12	0.2
Ardea herodias	3.2	5	0.10	0.3
Casmerodius albus egretta	3.2	3	0.04	0.1
Bubulcus ibis	3.5	5	0.27	0.3
Butorides striatus virescens	3.5	6	0.13	0.4
Butorides s. striatus	3.6	2		0.4
Egretta thula	3.9	5	0.10	0.2
Egretta caerulea	3.9	2		0.1
Syrigma sibilatrix	4.0	5	0.17	0.4
Ixobrychus minutus	5.3	2		0.1
Botaurus lentiginosus	5.7	5	0.14	0.4
Ixobrychus cinnamomeus	5.8	1		
Ixobrychus exilis	5.8	5	0.12	0.3
Tigrisoma lineatum	5.9	5	0.18	0.4

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

TABLE 10. Delta Tm values for taxa compared with labeled *Nycticorax nycticorax*.

Species	$\bar{x}$	*n*	SD	Range
Nycticorax nycticorax (homo)[a]	0.0	8		
Nycticorax nycticorax (het)	0.6	5	0.10	0.3
Nycticorax caledonicus	1.1	6	0.15	0.4
Ardea melanocephala	3.2	1		
Ardea cocoi	3.3	2		0.1
Casmerodius albus egretta	3.3	7	0.30	0.9
Ardea herodias	3.4	8	0.21	0.6
Nycticorax violaceus	3.4	12	0.21	0.7
Bubulcus ibis	3.5	6	0.25	0.6
Butorides striatus virescens	3.6	10	0.37	1.0
Egretta intermedia	3.6	1		
Butorides s. striatus	3.7	2		0.2
Syrigma sibilatrix	3.8	12	0.24	0.7
Egretta vinaceigula	3.9	1		
Egretta thula	4.0	11	0.26	0.9
Egretta tricolor	4.0	3	0.06	0.1
Egretta caerulea	4.1	5	0.40	1.0
Ixobrychus minutus	5.3	3	0.28	0.6
Ixobrychus exilis	5.6	4	0.09	0.2
Botaurus lentiginosus	5.7	7	0.20	0.6
Cochlearius cochlearius	5.8	6	0.26	0.7
Tigrisoma lineatum	6.0	7	0.28	0.9
Plegadis falcinellus	10.1	2		0.4

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

than 3.0 from members of other clades, or both. To be misplaced, they would have had to undergo large rate changes, and such rate differences would have been discovered through out-group comparisons.

Three species whose distances are summarized in Tables 2–15 were excluded from Fig. 2. *Botaurus stellaris* (Table 14) appears to be farther from *B. lentiginosus* than from *I. exilis*. The DNA of *stellaris* was short stranded and in such small quantities that only a few comparisons were possible, and none was made with labeled *exilis*. Thus, little weight can be given to the marked distance between *stellaris* and *lentiginosus*, even though this finding agrees with the view of Parkes (1955) that the two taxa are more distinct than generally recognized. *Ardeola grayii* DNA was compared only with that of *E. thula* and *N. nycticorax*. These comparisons show that *grayii* is a typical heron, but are too few to pinpoint the position of *grayii* in the tree. *Ardea pacifica* was found to be a member of the *Ardea* clade, but because of abnormally low NPH values (all <87%), it was omitted from the tree for the reasons discussed in the section on reproducibility. The cause of the low NPH values is unknown. It is likely to have been protein or glycogen contamination.

Summary of the phylogeny.—In the discussion that follows, suggested taxonomic category changes are based on the distance criteria of Sibley and Ahlquist (1985).

Although the tree depicted in Fig. 2 has multifurcations and, thus, is not entirely resolved, it nevertheless contains a great deal of information. Insight into the relationships of problematical taxa is provided, and perhaps as importantly, relative distances between branching events are apparent. In fact, the most powerful feature of DNA hybridization may be its ability to detect periods of rapid lineage origination, i.e. radiations that lack an obvious adaptive or biogeographic component (Bledsoe 1984, Sibley and Ahlquist 1985). These periods are signaled in DNA data by clusters of genetic distances, which are translated into multifurcations or a quick succession of nodes. If simply the branching pattern were provided, the time required for the development of adaptive changes would be unknown, and little could be said about causal forces, especially in a group as constrained morphologically as the herons.

TABLE 11. Delta Tm values for taxa compared with labeled *Cochlearius cochlearius*.

Species	$\bar{x}$	*n*	SD	Range
Cochlearius cochlearius (homo)[a]	0.0	4		
Cochlearius cochlearius (het)	0.4	4	0.67	1.5
Tigrisoma lineatum	5.1	6	0.25	0.6
Casmerodius albus egretta	5.3	1		
Nycticorax violaceus	5.5	6	0.42	0.9
Nycticorax nycticorax	5.5	7	0.40	0.9
Bubulcus ibis	5.6	5	0.25	0.7
Ardea herodias	5.8	6	0.44	1.1
Butorides striatus virescens	5.8	5	0.24	0.6
Syrigma sibilatrix	5.8	6	0.27	0.7
Egretta thula	6.3	7	0.47	1.3
Egretta caerulea	6.4	1		
Botaurus lentiginosus	6.9	6	0.46	1.3
Ixobrychus cinnamomeus	6.9	1		
Ixobrychus exilis	7.0	5	0.54	1.3
Plegadis falcinellus	9.1	1		

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

TABLE 12. Delta Tm values for taxa compared with labeled *Tigrisoma lineatum*.

Species	$\bar{x}$	*n*	SD	Range
Tigrisoma lineatum (homo)[a]	0.0	7		
Tigrisoma lineatum (het)	0.2	5	0.03	0.1
Cochlearius cochlearius	4.8	12	0.29	0.9
Ardea herodias	5.6	8	0.28	0.9
Bubulcus ibis	5.6	7	0.20	0.6
Casmerodius albus egretta	5.7	7	0.17	0.5
Nycticorax nycticorax	5.7	8	0.12	0.3
Butorides striatus virescens	5.8	7	0.18	0.5
Egretta intermedia	5.8	1		
Egretta caerulea	5.8	5	0.16	0.4
Nycticorax violaceus	5.8	7	0.12	0.3
Egretta novaehollandiae	5.9	1		
Egretta thula	6.1	6	0.11	0.3
Nycticorax caledonicus	6.1	1		
Syrigma sibilatrix	6.1	8	0.13	0.3
Egretta tricolor	6.2	1		
Ardeola grayii	6.4	4	0.28	0.6
Ixobrychus minutus	6.4	6	0.18	0.5
Botaurus lentiginosus	6.6	13	0.20	0.7
Ixobrychus exilis	6.7	10	0.22	0.6
Plegadis falcinellus	9.3	2		0.3

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

Cochlearius and *T. lineatum*, although distant enough to be placed in separate tribes, are closer to one another than to any other herons examined. They form a subfamily that is the sister group of the rest of the herons and bitterns. The relationship of *Cochlearius* and *T. lineatum* is indicated not only by their genetic similarity (ΔTm 4.9), but also by the similarity of their rates of evolution. Other researchers have noted similarities between these two taxa (e.g. Verheyen 1959, Vanden Berge 1970, Dickerman 1971). Payne and Risley (1976) found by a cladistic compatibility test of character states that one of two equally likely relationships placed *Tigrisoma* and *Tigriornis* in the same clade with *Cochlearius*. Regardless of these similarities, most recent investigators have placed *Cochlearius* with the night herons, either as a member of the same tribe, Nycticoracini (e.g. Bock 1956, Hancock and Elliott 1978), or as the sole member of an adjacent tribe, the Cochleariini (e.g. Verheyen 1959, Cracraft 1967, Dickerman 1971, Mock 1976, Payne 1979). If the DNA distances are correct, the similarity of *Cochlearius* to night herons is simply the result of adaptation to night feeding.

The DNA hybridization data do not resolve the issue of how recently the Boat-bill's bill originated. If *Cochlearius* were a night heron with a strange bill, then that bill, having a presumed special function, would have evolved rapidly. But the bill appears to serve a general function, stabbing (Biderman and Dickerman 1978), as well as scooping (Willard 1979). Thus, in light of the genealogical distance between Boat-bills and night herons, the bill may or may not have differentiated with unusual rapidity.

Bitterns are the sister group of the day and night herons at the level of tribe. This arrangement has never been proposed, the bitterns usually being considered the sister group of all herons, including tiger herons. Within the bitterns, the relative distances between *Ixobrychus* species is perhaps the most unexpected finding of this study. The higher rate of bittern evolution has been demonstrated (Sheldon in press) and could explain, in part, why *I. cinnamomeus* and *I. minutus* are so different from *I. exilis*. But, the greater similarity of *exilis* to *B. lentiginosus* than to its congeners (particularly *minutus*) is not easily explained by adaptation. Comparisons using radio-labeled, multiple preparations of *minutus* DNA are needed before its position in the tree of Fig. 2 can be accepted with confidence.

TABLE 13. Delta Tm values for taxa compared with labeled *Ixobrychus exilis*.

Species	$\bar{x}$	*n*	SD	Range
Ixobrychus exilis	0.0	6		
Botaurus lentiginosus	2.6	7	0.46	1.2
Ixobrychus minutus	3.3	7	0.09	0.3
Ixobrychus cinnamomeus	3.9	5	0.07	0.1
Casmerodius albus egretta	5.5	5	0.14	0.3
Ardea melanocephala	5.5	1		
Ardea herodias	5.6	5	0.06	0.2
Butorides striatus virescens	5.6	5	0.08	0.2
Bubulcus ibis	5.6	5	0.17	0.4
Nycticorax nycticorax	5.6	6	0.14	0.4
Nycticorax violaceus	5.7	5	0.09	0.2
Egretta thula	6.0	5	0.20	0.5
Syrigma sibilatrix	6.1	5	0.16	0.4
Tigrisoma lineatum	6.9	6	0.15	0.5

The day and night herons comprise a tribe, the typical herons, and no genealogical distinction appears to exist between these two adaptive forms (grades).

TABLE 14. Delta Tm values for taxa compared with labeled *Botaurus lentiginosus*.

Species	$\bar{x}$	*n*	SD	Range
Botaurus lentiginosus (homo)[a]	0.0	7		
Botaurus lentiginosus (het)	0.5	5	0.23	0.5
Ixobrychus exilis	2.4	8	0.14	0.4
Botaurus stellaris	2.9	3	0.18	0.3
Ixobrychus minutus	3.5	7	0.17	0.4
Casmerodius albus egretta	5.5	3	0.48	0.9
Nycticorax violaceus	5.6	10	0.24	0.5
Nycticorax nycticorax	5.6	10	0.21	0.6
Ardea cocoi	5.7	1		
Ardea herodias	5.7	9	0.28	0.9
Bubulcus ibis	5.7	10	0.31	1.1
Ardeola grayii	5.8	2		0.2
Butorides striatus virescens	5.9	8	0.18	0.4
Ardea melanocephala	6.0	1		
Egretta thula	6.0	10	0.25	0.9
Egretta caerulea	6.1	8	0.31	1.0
Syrigma sibilatrix	6.2	9	0.12	0.4
Egretta tricolor	6.3	1		
Cochlearius cochlearius	6.8	9	0.45	1.2
Tigrisoma lineatum	6.9	12	0.20	0.5
Plegadis falcinellus	10.9	6	0.19	0.5

[a] Homo = homoduplex; het = intrasubspecific heteroduplex.

TABLE 15. Delta Tm values for taxa compared with labeled *Plegadis falcinellus*.

Species	$\bar{x}$	*n*	SD	Range
Plegadis falcinellus	0.0	3		
Tigrisoma lineatum	9.5	9	0.21	0.7
Cochlearius cochlearius	9.7	13	0.21	0.6
Ardea herodias	10.0	5	0.20	0.6
Egretta caerulea	10.0	4	0.05	0.1
Bubulcus ibis	10.1	4	0.15	0.4
Butorides striatus virescens	10.1	2		0.3
Casmerodius albus egretta	10.1	4	0.23	0.5
Nycticorax nycticorax	10.2	5	0.11	0.3
Egretta thula	10.4	7	0.27	0.8
Nycticorax violaceus	10.4	1		
Syrigma sibilatrix	10.4	3	0.12	0.2
Botaurus lentiginosus	10.8	7	0.37	1.0
Ixobrychus exilis	10.9	6	0.23	0.6

The night herons, *N. nycticorax* and *N. violaceus*, though outwardly similar, are as divergent genetically from one another as either is from most other day herons. Several morphologists have recognized the significant difference between *nycticorax* and *violaceus* (e.g. Payne and Risley 1976), but none has appreciated how close these taxa are to day herons, even though the affinity of day and night herons is evidenced by the fact that some taxa (e.g. *Pilherodius pileatus* and *Syrigma*) have been placed alternately in one and then the other of these two groups (Bock 1956, Humphrey and Parkes 1963, Payne and Risley 1976) and by the fact that day and night herons share displays (Mock 1976, Payne and Risley 1976). *Nyctanassa* should be resurrected for *violaceus*, and night herons should not be segregated in a category of their own.

Adaptation to night living may have been a factor in the heron radiation that occurred at ca. ΔTm 3.5. Other taxonomically controversial "night herons," such as *Pilherodius* and the Old World *Gorsachius*, whose DNAs were not obtainable, probably also derived during this radiation, which would have occurred in the Early to Middle Miocene, judging from the heron fossil record (e.g. Becker 1985) and Sibley and Ahlquist's (1983) absolute dating calibration (ΔT 1.0 ≃ 4.5 million years).

Butorides is not especially close to any day- or night-heron taxa and should be kept as a separate genus. Its genetic relationship to pond herons, *Ardeola*, remains to be solved.

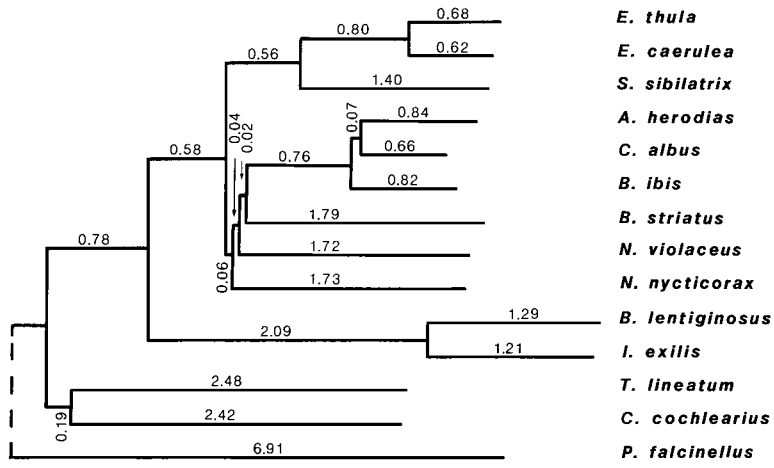

Fig. 1. The best tree found by PHYLIP when the computing options were set so that (1) the data were fit by least squares, (2) negative branches were not allowed, and (3) equality in sister-branch lengths was not assumed. Residual sum of squares equals 2.97.

The egret clade comprises *Syrigma* and most of Payne's (1979) *Egretta* species, including *novaehollandiae*, which was placed in *Ardea* by Bock (1956) and Curry-Lindahl (1971). *Syrigma*, a monotypic genus, has been difficult to place morphologically because of its adaptation to upland feeding (e.g. Humphrey and Parkes 1963, Kushlan et al. 1982).

Egretta and *Ardea* are not necessarily sister taxa. In the past, they have been associated because they share many similar characters and because the large egrets, *Casmerodius* and *E. intermedia*, appear (figuratively) to bridge the gap between them (e.g. Parkes 1955, Mayr and Short 1970, Payne and Risley 1976). The phenetic data of Payne and Risley (1976) also indicate a significant difference between *Egretta* and *Ardea*.

Bubulcus, *Casmerodius*, and *E. intermedia* are as close to *A. herodias* as any typical *Ardea* species and should be included in *Ardea*. *Bubulcus*, like *Syrigma*, has been a taxonomic problem because of its adaptation to field hunting. *Casmerodius*

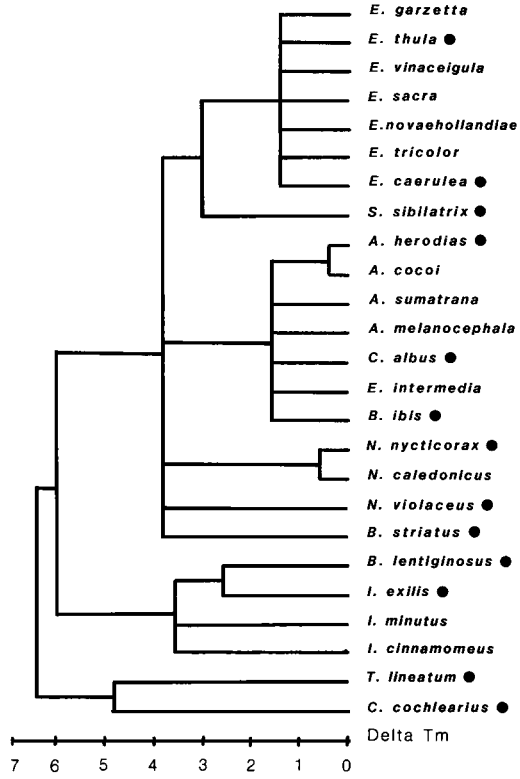

Fig. 2. A tree summarizing the relationships of all currently recognized heron species whose DNAs were compared in this study. This tree is based on the modified Jackknife Strict-Consensus Tree described in the *Evaluating estimated phylogenies* section. Branch lengths were computed by least squares from the ΔTm distances between labeled species (marked ●) under the assumption of constant rates of evolution. Unlabeled taxa have been added by hand according to their distances from labeled taxa.

and *E. intermedia* appear simply to be *Ardea* species that have retained or reacquired the presumably primitive character of white plumage, which commonly occurs in colonial, diurnal herons as well as in ibises, storks, pelicans, and other related groups. The southeast Asian subspecies of *Casmerodius albus, modestus,* is as distinct from the North American subspecies, *egretta,* as is *E. intermedia.* This genetic distance is consistent with Hancock's (1984) suggestion, based on the presence of an aerial stretch display in *modestus,* that these two races may be different species.

Linear arrangement of taxa.—Payne and Risley (1976) began their classification of the Ardeidae with day and night herons and ended it with tiger herons and bitterns. This arrangement was chosen because the day and night herons share plesiomorphic characters with the ardeids' presumed closest allies, the Ciconiiformes. These symplesiomorphic characters are primarily osteological, but also include white plumage and colonialism. On the basis of similarity in powder-down patch structure, Olson (1979) found that the nearest allies of the herons are the gruiform families, Mesoenatidae, Eurypygidae, and Rhynochetidae. In his view, solitary nesting and cryptic coloring would be symplesiomorphic characteristics, and therefore the linear arrangement should begin with bitterns and tiger herons, which exhibit these primitive characters.

DNA hybrid comparisons between herons and members of other families provide evidence that supports Payne and Risley's (1976) linear arrangement (Sheldon 1986, Sibley and Ahlquist pers. comm.). The sister group of the herons appears to comprise other ciconiiform birds, and perhaps some Pelecaniformes as well. Distances between herons and *Plegadis falcinellus,* for example, average ΔTm 10.2 (Tables 2–15). Those between herons and *Eurypyga helias* (the only "primitive" gruiform for which DNA was available) average ΔTm 13.2.

Acknowledgments

I thank Charles Sibley and Jon Ahlquist, who provided the inspiration and wherewithal for this research. R. Payne and two anonymous reviewers commented on the manuscript. Others to whom I am particularly indebted for ideas and help are: A. Bledsoe, E. Brownell, F. Catzeflis, J. Kennard, J. Kirsch, E. Nowicki, F. Sibley, P. Schochet, A. Schulman, E. Stickney, and L. Wallace. The following persons provided material for DNA extraction: H. Abdulali, J. Colebrook-Robjent, J. Eckert, C. Francis, M. Marin, T. Osborne, R. Schodde, J. Spendelow, R. Stuebing, G. Stiles, E. and N. Wheelwright, and G. Wilson. The Malaysian Prime Minister's and the Sabah Chief Minister's departments kindly permitted the collection of Bornean specimens. Financial support was provided by the Yale University Graduate School, Yale Peabody Museum, the Western Foundation of Vertebrate Zoology, and NSF grants DEB 77-02594, DEB 79-26746, and BSR 83-15155 to C. G. Sibley.

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