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SYSTEMATIC RELATIONSHIPS AMONG WATERFOWL (ANATIDAE) INFERRED FROM RESTRICTION ENDONUCLEASE ANALYSIS OF MITOCHONDRIAL DNA

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Abstract.—To evaluate the potential of mitochondrial DNA (mtDNA) analysis for avian systematics, we have assayed mtDNA differences among 13 species of waterfowl in the genera *Anas* and *Aythya* (Anseriformes: Anatidae). Purified mtDNA was digested with each of 15 different type II restriction endonucleases which cleave at five- or six-base recognition sequences. Side-by-side comparisons of digestion profiles permitted the estimation of levels of fragment homology and nucleotide sequence divergence (p). Among nine *Anas* species, mean sequence divergence ($\bar{p}$) was 0.062 (range 0.004–0.088); among four *Aythya* species, 0.034 (0.025–0.043); between selected species belonging to separate genera, 0.109. Phylogenetic trees and dendrograms were constructed from qualitative and quantitative data bases by a variety of procedures including undirected parsimony (Penny and Wagner algorithms), undirected compatibility (Estabrook algorithms), and phenetic clustering. These trees were highly concordant with one another, and with traditional phylogenies derived from independent sources of information.

Previously published evidence from mammals has suggested a "saturation effect" on level of mtDNA differentiation: for p less than 0.15–0.20, mtDNA distances are reportedly linearly related to time since common ancestry, but for larger p values the relationship becomes curvilinear as differentiation approaches an observed plateau at a p of approximately 0.30. Our estimates of mtDNA sequence divergence among congeneric waterfowl fall in a broad range well within the expected linear portion of the curve. This observation, coupled with the general concordance of mtDNA-generated trees with those derived from independent information, demonstrates that the restriction fragment approach to mtDNA analysis should provide an important new molecular technique for studying evolutionary relationships among lower taxonomic levels in Aves. [Molecular evolution; phylogeny reconstruction; birds; waterfowl; restriction fragments.]

Only recently have relationships among birds been studied at the molecular level (Mayr, 1983). Protein electrophoresis (Avise, 1983; Barrowclough, 1983), immunological techniques (Wilson et al., 1977; Prager and Wilson, 1978), and DNA-DNA hybridization methods (Sibley and Ahlquist, 1982a, b) have provided especially important molecular results with which to evaluate earlier conclusions of systematists. As pointed out by Shields (1983), birds should provide excellent subjects for such molecular studies because much is known about this class of vertebrates at the organismal level. Comparisons of anatomical, behavioral, and molecular data, for example, may provide insights into the processes governing evolutionary change (Wyles et al., 1983). Furthermore, avian systematists have found that organismal level information alone has sometimes been inadequate to resolve phylogenetic issues. As stated by Diamond (1983), "No group of animals has been more intensively studied than birds. Yet taxonomic problems arising from convergent evolution and adaptive radiation are extreme for birds, so that taxonomists disagree widely about the relations between the groups . . . ." Of course, molecular characters themselves are not necessarily free from problems of convergence or heterogeneity in rates of change.

Here we utilize restriction fragment analysis of mitochondrial DNA (mtDNA) to estimate sequence divergence and evolutionary relationships among several species of waterfowl. In vertebrates, mtDNA is a double-stranded closed circular molecule inherited cytoplasmically through the maternal parent (Avise and Lansman, 1983). Available data indicate that mtDNA is conservative in size (15,700–19,500 base pairs) and gene orga-

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nization across all higher animals (Brown, 1983), but is strikingly nonconservative in primary nucleotide sequence evolution. Brown et al. (1979, 1982) reported that mammalian mtDNA is evolving at a rate at least 5 to 10 times faster than typical single copy nuclear DNA. The rapid evolution of mtDNA, and its extensive sequence heterogeneity, have permitted reconstructions of matriarchal phylogeny among conspecific and very closely related species of mammals (Avise et al., 1979, 1983; Ferris et al., 1983b; Lansman et al., 1983). Fewer data on mtDNA divergence are available for nonmammalian vertebrates, and we know of only a single published study on birds (Glaus et al., 1980).

The purpose of this report is to examine the possible utility of mtDNA restriction fragment analysis for avian systematics. Our focus is on relationships among species within the waterfowl (Anatidae), a group that has been extensively studied by systematists (Delacour and Mayr, 1945; Johnsgard, 1968; Sibley and Ahlquist, 1972; Bellrose, 1976; Patton and Avise, 1983). Phylogenies for this group have been ascertained on the basis of nonmolecular data (comparative morphology, development, physiology, behavior, and the fossil record), as well as biochemical characters (protein electromorphs). We examine mtDNA differentiation among 13 species belonging to the genera *Anas* and *Aythya*. As with the assessment of any new source of systematic information, we will be concerned specifically with: (1) calibration (determination of the levels of taxonomic divergence at which potentially informative mtDNA differentiation exists); and (2) evaluation (comparison of mtDNA-based phylogenies against those previously developed from independent sources of information).

MATERIALS AND METHODS

Laboratory procedures.—Mitochondrial DNA was purified from 35 individuals representing the following species: *Anas platyrhynchos* (mallard, $n = 2$); *A. crecca* (*carolinensis*, green-winged teal, $n = 3$); *A. fulvigula* (mottled duck, $n = 2$); *A. acuta* (northern pintail, $n = 4$); *A. strepera* (gadwall, $n = 2$); *A. americana* (American wigeon, $n = 3$); *A. discors* (blue-winged teal, $n = 4$); *A. clypeata* (northern shoveler, $n = 3$); *A. cyanoptera* (cinnamon teal, $n = 1$); *Aythya americana* (redhead, $n = 1$); *A. valisineria* (canvasback, $n = 2$); *A. affinis* (lesser scaup, $n = 3$); and *A. collaris* (ring-necked duck, $n = 5$). All specimens were collected from Aransas County or San Petricio County, Texas, except for the *A. platyrhynchos* which were obtained from a private dealer in Richmond County, Georgia and are of unknown native source. *Fresh* heart tissue proved to yield the cleanest mtDNA preparations.

Following gentle homogenization of tissues, nuclei and debris were removed by centrifugation at 700 × *g* for 5 min. Mitochondria were pelleted by recentrifugation at 20,000 × *g* for 20 min. Mitochondria were then lysed, and mtDNA purified by CsCl-ethidium bromide gradient centrifugation. The mtDNA-containing fraction from the gradient was dialyzed first against sodium acetate and then against Tris-HCl/EDTA. Details of all procedures can be found in Lansman et al. (1981).

MtDNAs were digested with each of 15 different type II restriction endonucleases (recognition sequences in parentheses): *Ava*I (CPyCGPuG); *Bam*HI (GGATTC); *Bcl*I (TGATCA); *Bgl*I ($GCCN_5GGC$); *Bgl*III (AGATCT); *Bst*EII (GGTNACC); *Cla*I (ATCGAT); *Hinc*II (GTPyPuAC); *Hind*III (AAGCTT); *Nde*I (CATATG); *Pst*I (CTGCAG); *Pvu*II (CAGCTG); *Sac*I (GAGCTC); *Stu*I (AGGCCT); and *Xba*I (TCTAGA). Fragments were radioactively end-labelled using the large fragment of *E. coli* DNA polymerase I and ^{32}P-αdCTP (Brown, 1980), and then separated on agarose gels. MtDNA digestion profiles were revealed by autoradiography of vacuum-dried gels (Maniatis et al., 1982). Lambda DNA digested by *Hind*III and pBR322 double-digested with *Pvu*II and *Hinc*II were utilized as size standards.

Because correct determination of fragment homologies is especially critical in a study such as this, several precautions were taken to minimize the probability of judg-

ing two nonhomologous fragments as identical due to chance comigration. Assays were confined to five- and six-base (as opposed to four-base) recognition enzymes, in order to produce digestion profiles with readily scorable numbers of fragments. Whenever necessary, electrophoresis was repeated with reordering of samples so that digestion profiles containing bands of questionable size identity could be compared side-by-side. Finally, for each restriction enzyme, samples were typically electrophoresed at a range of gel concentrations (0.6–2.2% agarose) depending on the size of fragments for which optimal resolution was required in comparisons of patterns (Maniatis et al., 1982). Although our focus was on examining all pairwise comparisons of individuals within each genus, the same series of 15 enzymes was also used to directly compare selected *Anas* versus *Aythya*.

Data analysis.—Raw data for *Anas* and *Aythya* were summarized in matrices of presence-absence information for each restriction fragment in each individual (see Appendix). Phylogenetic networks were computer-constructed from these matrices by two conceptually distinct approaches: (1) undirected compatibility (Estabrook et al., 1977); and (2) undirected parsimony employing both Penny (Hendy et al., 1978) and Wagner (Kluge and Farris, 1969; Farris et al., 1970) algorithms. The Penny algorithm, when run to completion, is reportedly guaranteed to find the minimum-length Wagner network. Felsenstein (1982) discussed the rationales and assumptions underlying parsimony and compatibility approaches.

We recognize that our method of use of fragment data technically violates one of the assumptions underlying most phylogenetic analyses. Namely, fragment gains or losses are not entirely independent events. Thus, loss of one fragment due to gain of a restriction site is accompanied by appearance of two new fragments, and vice versa. However, such redundancy of information should apply only to fragments produced by single enzymes, one at a time, and even here the redundancy is far from complete. For example, a given character-state loss can be associated with the formation of any of a large number of new pairs of character states, depending on the particular position of a site gain. We know of no specific phylogenetic algorithms designed to deal with this kind of partial non-independence; we suspect that the problem is likely to be rather unimportant for large data matrices, though this warrants further study.

The matrices also facilitated computations of total proportions of shared fragments between individuals:

$$F = 2N_{XY}/(N_X + N_Y),$$

where N_X and N_Y are the numbers of fragments in individuals X and Y and N_{XY} is the number of fragments shared by X and Y. Values of F were converted to estimates of nucleotide sequence divergence, p, by the approach of Nei and Li (1979). Final p values represent weighted means of calculations conducted separately for the five- and six-base enzymes. Phenograms were generated from matrices of p values by the unweighted pair-group method using arithmetic averages (UPGMA; Sneath and Sokal, 1973).

RESULTS

Grand totals of 193 and 109 mtDNA restriction fragments (character states) were scored in *Anas* and *Aythya*, respectively. Estimates of fragment homology between species and individuals differing in mtDNA genotype are presented in Tables 1 and 2. As might be expected given the small sample size and single collection locale for most species, observed intraspecific mtDNA differentiation was small. For example, among four specimens of *Anas discors*, F-values ranged from 0.927 to 0.982; between two *A. fulvigula*, F was 0.950; and between *A. americana* collected 80 kilometers apart in Aransas and San Petricio Counties, Texas, F was 0.974. Within *Aythya*, F-values among five specimens of *A. collaris* ranged from 0.970 to 1.000, and between two *A. valisineria*, F was 0.963. No intraspecific variation was observed in samples from our other species.

TABLE 1. Estimates of mtDNA genetic differentiation for nine species of *Anas* based on fragment patterns generated by 15 restriction endonucleases. Above diagonal: estimates of mtDNA nucleotide sequence divergence (p) from approach of Nei and Li (1979). Below diagonal: fraction of shared fragments (F) over all digests.

	Anas	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
I	*discors* 1	—	0.004	0.002	0.001	0.031	0.004	0.070	0.079	0.079	0.079	0.083	0.080	0.080	0.068
II	*discors* 2	0.955	—	0.005	0.005	0.024	0.000	0.071	0.080	0.080	0.080	0.084	0.081	0.081	0.072
III	*discors* 3	0.972	0.927	—	0.003	0.033	0.005	0.070	0.079	0.079	0.079	0.083	0.080	0.079	0.067
IV	*discors* 4	0.982	0.937	0.954	—	0.029	0.005	0.070	0.079	0.079	0.079	0.083	0.080	0.080	0.068
V	*clypeata* 1–3	0.627	0.672	0.598	0.644	—	0.024	0.065	0.077	0.077	0.084	0.088	0.078	0.077	0.083
VI	*cyanoptera* 1	0.955	1.000	0.927	0.937	0.672	—	0.071	0.080	0.080	0.080	0.084	0.081	0.081	0.072
VII	*crecca* 1–3	0.342	0.339	0.345	0.342	0.353	0.339	—	0.050	0.048	0.045	0.041	0.062	0.061	0.069
VIII	*fulvigula* 1	0.296	0.293	0.298	0.296	0.309	0.293	0.448	—	0.003	0.007	0.056	0.065	0.065	0.058
IX	*fulvigula* 2	0.296	0.293	0.298	0.296	0.309	0.293	0.466	0.950	—	0.007	0.056	0.060	0.059	0.057
X	*platyrhynchos* 1, 2	0.296	0.293	0.298	0.296	0.276	0.293	0.483	0.883	0.883	—	0.050	0.064	0.063	0.053
XI	*acuta* 1–4	0.276	0.274	0.278	0.276	0.258	0.274	0.513	0.413	0.413	0.446	—	0.067	0.067	0.050
XII	*americana* 1, 2	0.281	0.278	0.283	0.281	0.295	0.278	0.452	0.353	0.387	0.370	0.367	—	0.001	0.025
XIII	*americana* 3	0.283	0.281	0.286	0.283	0.297	0.281	0.456	0.356	0.390	0.373	0.370	0.974	—	0.027
XIV	*strepera* 1, 2	0.347	0.316	0.351	0.347	0.272	0.316	0.356	0.395	0.400	0.421	0.444	0.658	0.640	—

Perhaps less expected were the high fragment homologies also observed *between* many congeneric species. For example, between *Anas discors* and *A. cyanoptera*, F was 0.955, and in fact our single *cyanoptera* specimen could not be distinguished from one of the *discors* samples by any of the 15 restriction-digest profiles. Some other pairs of species exhibiting especially high fragment homologies included *A. fulvigula–A. platyrhynchos* (F = 0.883), *A. americana–A. strepera* ($\bar{F}$ = 0.649), and *Aythya americana–A. valisineria* (F = 0.654). Much lower fragment homologies were observed between some congeners such as *Anas platyrhynchos–A. clypeata* (F = 0.276). Between all assayed *Anas* species pairs, the average F was 0.408 (range 0.258–0.955); and among *Aythya*, $\bar{F}$ was 0.580 (0.509–0.654).

If certain assumptions are met (i.e., that all fragment changes arise from nucleotide substitution, that frequencies and distributions of cleavage sequences are similar to those expected in random sequences of same-base composition, and that nonhomologous fragments of similar weight are not scored as identical), then F-values can be converted to and interpreted as estimates of nucleotide sequence divergence, p. For mtDNA in other animals, available data suggest that these assumptions may often be valid, to a first approximation (Upholt, 1977; Nei and Li, 1979; Avise and Lansman, 1983). Matrices of p values for *Anas* and *Aythya* are presented in Tables 1 and 2. Between all assayed *Anas* species, $\bar{p}$ was 0.062 (range 0.004–0.088); and among *Aythya*, $\bar{p}$ was 0.034 (0.025–0.043). Nucleotide sequence divergence values for comparisons between genera were as follows: between *Aythya affinis* and *Anas discors*, p was 0.111, and between *Aythya affinis* and *Anas americana*, p was 0.107. Both of these distances are greater than any observed values among congeners in either *Anas* or *Aythya*.

For the waterfowl, we can only partially address the validity of assumptions underlying the conversion of F to p. There seem to be no large-scale mtDNA additions or deletions in Anatidae because ge-

TABLE 2. Estimates of mtDNA genetic differentiation for four species of *Aythya* based on fragment patterns generated by 15 restriction endonucleases. For explanation, see heading to Table 1.

	Aythya	I	II	III	IV	V	VI
I	*americana* 1	—	0.025	0.025	0.025	0.029	0.043
II	*valisineria* 1	0.654	—	0.002	0.032	0.034	0.041
III	*valisineria* 2	0.654	0.963	—	0.032	0.034	0.041
IV	*collaris* 1, 3	0.654	0.590	0.590	—	0.002	0.032
V	*collaris* 2, 4, 5	0.621	0.577	0.577	0.970	—	0.036
VI	*affinis* 1–3	0.513	0.509	0.509	0.595	0.564	—

nome size for all species appeared identical (within limits of resolution of our approach) at 16,500 ± 200 base pairs. Furthermore, our confidence in the correct appraisal of similarity in genome size, and in correct determination of fragment homologies, is strengthened in those many situations in which particular species were observed to share entire multiband mtDNA digestion profiles for particular enzymes (Fig. 1). For example, in the comparison between *Anas platyrhynchos* and *A. fulvigula*, 10 of 15 restriction enzymes produced identical multiband digestion profiles (and an additional three enzymes produced digestion profiles which could readily be interpreted as differing by a single restriction site change). Similarly, in the comparison between *A. discors* and *A. clypeata*, identical multiband profiles were produced by 5 of 15 enzymes (and the patterns produced by seven other enzymes appeared to be interconvertible by single restriction site changes).

Phylogenetic networks and dendrograms were constructed from the mtDNA data using several procedures, and results are summarized in Figures 2 and 3. We will describe results for the two genera separately.

Anas.—Although the Penny algorithm did not run to completion (due to the large size of the data matrix [193 character states of which 7 were invariant]), it nonetheless identified two equally-parsimonious networks that were of shorter total length (253 steps) than the observed Wagner estimate (258 steps). According to one Penny network, *A. acuta* shares a more recent hypothetical ancestor with the *A. platyrhynchos–A. fulvigula* assemblage than it does with *A. crecca*; in the other Penny network, *A. acuta* and *A. crecca* are more closely related than either is to the *A. platyrhynchos–A. fulvigula* cluster. In this network, 68% of the variable characters were nonhomoplasious (exhibited a single state change). The character compatibility network (134 consistent character-state changes in the largest clique) was identical in structure to the latter Penny network, and is thus presented in Figure 2. Branch lengths were drawn proportional to the number of character-state changes as determined by the undirected compatibility analysis. The network was *arbitrarily* rooted at a position which makes it most similar to the structure of the UPGMA phenogram (see below).

Results of the phenetic clustering procedure (UPGMA) for *Anas* are shown in the upper portion of Figure 3. The branching structure of the phenogram is identical to that of the compatibility and Penny parsimony networks (Fig. 2).

Aythya.—Due to the smaller size of the data matrix for *Aythya* (109 character states of which 22 were invariant), the Penny algorithm did run to completion and produced a guaranteed shortest network of length 99 steps (Fig. 2). Of the 87 variable characters, 75 (86%) were nonhomoplasious. The character compatibility network (Fig. 2), with 97 consistent character-state changes in the largest clique, was identical in structure to the Penny network, as was the UPGMA phenogram (bottom portion of Fig. 3). As before, the compatibility and parsimony networks were *arbitrarily* rooted to be similar to the UPGMA output.

In the UPGMA phenogram of Figure 3, *Anas* and *Aythya* were joined at a level of genetic distance ($\bar{p} = 0.109$) determined by

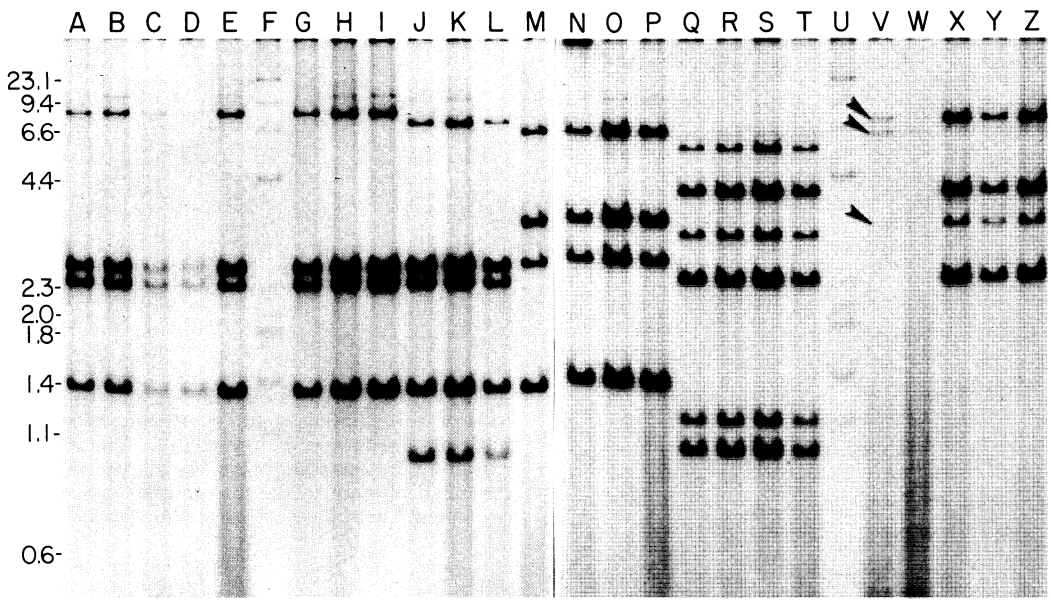

FIG. 1. *Hind*III digests (from two original gels) of mtDNA for individuals within *Anas*. Lanes A–D, *A. discors*; E, G, H, *A. clypeata*; I, *A. cyanoptera*; J–L, *A. crecca*; M, N, *A. fulvigula*; O, P, *A. platyrhynchos*; Q–T, *A. acuta*; V, W, *A. strepera*; X–Z, *A. americana*. Lanes F and U are lambda DNA digested by *Hind*III plus pBR322 double-digested with *Pvu*II and *Hinc*II. The arrows indicate the positions of the weakly visible mtDNA fragments in *A. strepera*. The sizes (in kilobases) of the molecular weight markers are indicated on the left.

the mean of the assayed comparisons between *Aythya affinis–Anas discors* and *Aythya affinis–Anas americana*.

DISCUSSION

From our data, it appears that restriction fragment analysis of mtDNA may provide a powerful new molecular approach for assessing evolutionary relationships among lower-level taxa of birds. This conclusion stems from the estimated magnitudes of mtDNA nucleotide sequence divergence among congeneric waterfowl and from the conformity of mtDNA-generated phylogenies with those derived from independent information.

Calibration.—Restriction enzyme assays of mtDNA have proven useful for estimations of matriarchal phylogenies within and among conspecific populations of several non-avian vertebrates (Avise and Lansman, 1983). Similar studies of geographic variation in mtDNA among bird populations will also be most desirable. However, for our preliminary calibration of mtDNA divergence in birds, we chose to focus on interspecific comparisons for the following reason. Studies of genetic divergence based on multilocus protein electrophoresis have revealed unexpectedly small distance ($\bar{D}$) values between avian congeners (Barrowclough and Corbin, 1978; Avise and Aquadro, 1982). For most assayed avian genera, $\bar{D}$s between closely related species are commonly within the range observed for conspecific populations of many fishes, amphibians, and other non-avian vertebrates. The conservative pattern of protein differentiation has also been observed among waterfowl (Patton and Avise, 1983). Is the magnitude of mtDNA divergence among avian congeners therefore within a range potentially useful for assessing systematic relationships?

Brown et al. (1979), Brown (1983) and Aquadro et al. (1984) summarized data suggesting a complex relationship between magnitude of mtDNA divergence between species and time since they last

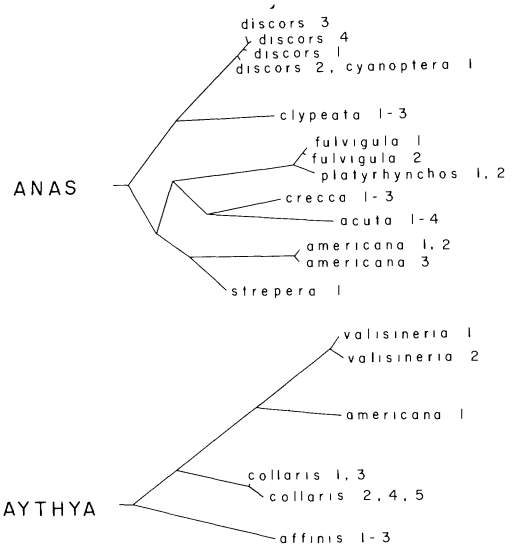

FIG. 2. MtDNA-based phylogenies for *Anas* and *Aythya* generated by parsimony and compatibility methods. Branch lengths are proportional to the number of character-state changes as determined by the compatibility method. Networks were arbitrarily rooted at a position most similar to the UPGMA dendrogram (Fig. 3).

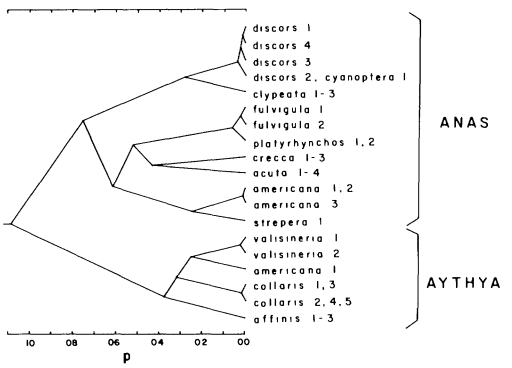

FIG. 3. UPGMA dendrogram for assayed waterfowl generated from a matrix of estimates of mtDNA nucleotide sequence divergence (p). The level of clustering of *Anas* and *Aythya* was determined from the mean distance in the intergeneric comparisons attempted. The cophenetic correlation equals 0.99.

shared a common ancestor. For mtDNA distances less than a p of about 0.15, differentiation appears to increase linearly with time, reflecting the rapid accumulation of nucleotide substitutions, the majority of which are transitions in silent positions of codons. A much slower rate of evolution characterizes the remaining positions (including replacement sites in protein-coding genes). Aquadro et al. (1984) argued that a substantial fraction of mtDNA is under strong selective constraints and exhibits a negligible rate of substitution. Overall, the magnitude of mtDNA divergence increases much less rapidly beyond a p of approximately 0.15–0.20 (corresponding to absolute divergence times of perhaps 8–15 million years). For meaningful comparisons among taxa, therefore, mtDNA distances should be well below the plateau region of the curvilinear relationship of p with time.

Between genera and subfamilies of galliform birds, Glaus et al. (1980) reported mtDNA distances (p) between 0.097 and 0.175. In the present study, distance estimates between *Anas* and *Aythya* also fell within that range ($\bar{p} = 0.109$); among congeneric waterfowl species, observed distances ranged from a p of 0.004 to 0.088. Although our p values were estimated indirectly from fragment comparisons, they do occupy a range potentially ideal for inferring evolutionary relationships. Whether this potential has been realized must be decided by comparisons of mtDNA networks with phylogenies derived from independent data.

Evaluation.—The mtDNA networks (Figs. 2 and 3) have revealed most of the well-defined species assemblages previously recognized by classical systematic criteria (Delacour and Mayr, 1945; Johnsgard, 1968; Sibley and Ahlquist, 1972; Bellrose, 1976; Patton and Avise, 1983) and by protein electrophoretic information (Patton and Avise, 1983). Details of these independent analyses can be found in the references provided. The established species clusters (all of which were also recognized in the mtDNA analyses) include the following:

(a) *Anas platyrhynchos–A. fulvigula.* By all information, these species are very similar, and some ornithologists consider *fulvigula* to be a sexually nondimorphic form of *platyrhynchos* (see Graham, 1979).

(b) *Anas americana–A. strepera.* Delacour

and Mayr (1945) placed these two species in adjacent groups in a taxonomic list, and noted that *americana* forms sterile hybrids with most species of *Anas* except *strepera*. These species are phenetically clustered in a protein-electrophoretic analysis, although no synapomorphic alleles were found to define an *americana-strepera* clade (Patton and Avise, 1983).

(c) *Anas discors–A. cyanoptera–A. clypeata*. The affinities of members of this well-defined "blue-winged" duck group are evident and have been noted by many authors (Delacour and Mayr, 1945). The species share a unique wing coloration, peculiar courtship methods, and feeding habits. They are also phenetically and cladistically allied in protein electromorph composition (Patton and Avise, 1983). *Anas discors* and *A. cyanoptera* may be especially closely related, since when "associated artificially they interbreed freely, producing fertile hybrids; and the stock soon becomes hopelessly mixed" (Delacour and Mayr, 1945). Among our samples, *discors* and *cyanoptera* could not be consistently distinguished in mtDNA genotype.

(d) *Aythya americana–A. valisineria*. These species belong to one of the four species-groups within *Aythya* recognized by Delacour and Mayr (1945) and are obviously closely related in traditional systematic characters and in mtDNA genotype.

(e) *Anas* versus *Aythya*. The supposed phylogenetic split between these genera is evidenced by a variety of morphological and behavioral traits (Delacour and Mayr, 1945) and by protein composition (Patton and Avise, 1983). The observed intergeneric distances in mtDNA were also larger than those between any *Anas* or *Aythya* congeners.

Most of the unresolved issues in *Anas* and *Aythya* phylogeny concern the relationships of different species groups to one another. Generally, the areas of uncertainty or ambiguity in the mtDNA phylogenies are the same as those remaining after analysis of morphological and other characters. For example, the mtDNA affinities of *Anas crecca* to the *A. fulvigula–A. platyrhynchos* assemblage, to *A. acuta*, and to the *A. americana–A. strepera* assemblage, are uncertain for two related reasons: (1) clustering distances between these various groups all fall within a narrow range ($p \approx 0.04$ to 0.06; Fig. 3); and (2) alternative but equally attractive parsimony networks display slightly different summaries of relationships among these organisms. Similar problems are encountered in attempts to relate unambiguously these groups by traditional systematic characters. For example, *A. crecca* exhibits the same courtship display as *A. platyrhynchos* but shares a similar drake call with *A. acuta*, while *A. platyrhynchos* and *A. acuta* exhibit occasional natural hybridization producing fertile hybrids (see Delacour and Mayr [1945] for a discussion of many other characters). We suspect that the remaining areas of uncertainty in the exact phylogenetic placement of some *Anas* and *Aythya* groups are attributable to biological considerations (such as similarities in evolutionary times of separation) rather than to unreliabilities in the various kinds of organismal or molecular traits assayed, or in particular methods of phylogenetic analysis. Overall then, given as evaluation criteria the extensive prior systematic experience with waterfowl, the mtDNA data appear to delineate quite reliably the evolutionary affinities among *Anas* and *Aythya*.

It was not a foregone conclusion that mtDNA analyses would provide an important approach for phylogenetic reconstruction. Two recent empirical studies have reported instances in which distributions of mtDNA genotypes apparently lack concordance with species boundaries defined by morphological and reproductive criteria (Ferris et al., 1983a; Powell, 1983). In these cases, secondary hybridization and recurrent backcrossing were purportedly responsible for the introduction of foreign mtDNA into a species. Barton and Jones (1983) pointed out that such phenomena may "obscure the evolutionary history of diverging populations . . . ." Another possible explanation for discordances between species boundaries and mtDNA genotype distributions involves

consideration of demographically influenced patterns of matriarchal lineage sorting before, during, and after speciations (Avise et al., 1983, 1984). Nonetheless, such potential complicating evolutionary factors have apparently not obscured probable relationships among waterfowl as assessed by mtDNA. Of course, the hope now is that mtDNA analyses can also be used to examine relationships in some other groups of birds for which systematic issues are less well resolved.

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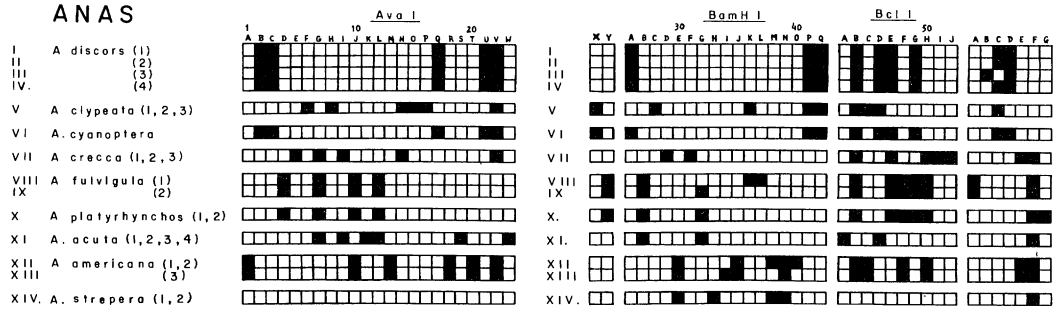
APPENDIX

Data Matrices. Presence Versus Absence of Restriction Fragments

For each restriction endonuclease, fragments observed within a genus are lettered sequentially in order of decreasing molecular weight, with presence of a fragment indicated by a solid square. The fragments are numbered cumulatively across enzymes so that, for example, a total of 193 character states was scored in *Anas*. The number and letter designations do not correspond between the two genera.



APPENDIX
(*continued*)