# A Comparative Description of Mitochondrial DNA Differentiation in Selected Avian and Other Vertebrate Genera[1]

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Levels of mitochondrial DNA (mtDNA) sequence divergence between species within each of several avian (*Anas, Aythya, Dendroica, Melospiza,* and *Zonotrichia*) and nonavian (*Lepomis* and *Hyla*) vertebrate genera were compared. An analysis of digestion profiles generated by 13–18 restriction endonucleases indicates little overlap in magnitude of mtDNA divergence for the avian versus nonavian taxa examined. In 55 interspecific comparisons among the avian congeners, the fraction of identical fragment lengths ($F$) ranged from 0.26 to 0.96 ($\bar{F}$ = 0.46), and, given certain assumptions, these translate into estimates of nucleotide sequence divergence ($p$) ranging from 0.007 to 0.088; in 46 comparisons among the fish and amphibian congeners, $F$ values ranged from 0.00 to 0.36 ($\bar{F}$ = 0.09), yielding estimates of $P$ >0.070. The small mtDNA distances among avian congeners are associated with protein-electrophoretic distances ($D$ values) less than ~0.2, while the mtDNA distances among assayed fish and amphibian congeners are associated with $D$ values usually >0.4. Since the conservative pattern of protein differentiation previously reported for many avian versus nonavian taxa now appears to be paralleled by a conservative pattern of mtDNA divergence, it seems increasingly likely that many avian species have shared more recent common ancestors than have their nonavian taxonomic counterparts. However, estimates of avian divergence times derived from mtDNA- and protein-calibrated clocks cannot readily be reconciled with some published dates based on limited fossil remains. If the earlier paleontological interpretations are valid, then protein and mtDNA evolution must be somewhat decelerated in birds. The empirical and conceptual issues raised by these findings are highly analogous to those in the long-standing debate about rates of molecular evolution and times of separation of ancestral hominids from African apes.

## Introduction

Species and genera of birds commonly exhibit smaller genetic distances ($D$ values) at protein-coding loci than do many nonavian vertebrates of same taxonomic rank (Martin and Selander 1975; Smith and Zimmerman 1976; Barrowclough and Corbin 1978; Avise et al. 1980*a*; Yang and Patton 1981; Zink 1982; Gutierrez et al. 1983). For example, from a multilocus electrophoretic survey of 10 species of waterfowl in the genus *Anas,* mean genetic distance was estimated to be 0.092

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(range, 0.00–0.19; Patton and Avise 1985), while among 10 species of fish in the genus *Lepomis,* mean distance was 0.62 (range, 0.16–1.01; Avise and Smith 1977). Although ranges of genetic distance for various vertebrate congeners do overlap considerably, the general trend toward relatively small $D$ values in birds remains (reviewed in Avise and Aquadro [1982]). At higher taxonomic levels also, protein distances in birds (as assayed by immunological techniques) have proved to be unexpectedly small (Prager et al. 1974; Prager and Wilson 1980).

To account for this "conservative" pattern of protein differentiation in Aves, the following two fundamental alternative hypotheses (which occupy the end points on a continuum of possibilities) have been suggested (Zink 1982; Avise 1983): (1) relative to many nonavian vertebrates, avian taxa on the average have a more recent shared common ancestry and (2) the rate of protein evolution is decelerated in birds. The former hypothesis subsumes the possibility, forcefully argued by Sibley (1982), that birds are taxonomically "oversplit" at all supraspecific levels. Various empirical approaches may help decide between these competing hypotheses (Avise 1983). One approach involves evaluation of observed protein distances against available biogeographic or fossil evidence on absolute times of avian speciations. In two such case-history studies—involving waterfowl (Patton and Avise 1985) and North American warblers (Avise et al. 1980*c*)—it was tentatively concluded that avian protein evolution may indeed have been decelerated; however, the validity of this conclusion hinges critically on the reliability of divergence times, which in these two studies were taken from rather meager fossil and biogeographic evidence, respectively.

A second approach to helping distinguish between recent ancestry and protein deceleration involves examination of divergence in other portions of the avian genome. This is the approach employed in this study. If low levels of protein differentiation reflect recent common ancestry, a conservative pattern of differentiation should also characterize other aspects of the avian genome. Alternatively, if avian congeners are not especially young, the conservative pattern of substitution in replacement positions of protein-coding genes may be atypical of the remainder of the avian genome, only a small fraction of which has such protein-coding function (Shields 1983). For example, one theoretical possibility that might account for protein deceleration involves body temperature (Avise and Aquadro 1982). Conceivably, the relatively high and stable internal temperatures of most birds might provide a physiologic environment conducive to selection against certain amino acid substitutions (see Hochachka and Somero 1973). Such stabilizing selection need not extend to silent-position nucleotide substitutions or to regions of DNA not coding for proteins. While many other selectionist scenarios with varying theoretical predictions about genome differentiation might be entertained, it remains of primary importance to assess genome divergence empirically.

Here we employ restriction endonuclease fragment analyses to assess levels of mitochondrial DNA (mtDNA) differentiation in selected avian and other vertebrate genera. We have assayed representatives of five taxonomic groups for which background data on allozyme distances are also available: sunfish (*Lepomis;* Centrarchidae); waterfowl (*Anas, Aythya;* Anatidae); warblers (*Dendroica;* Emberizidae); sparrows (*Melospiza, Zonotrichia;* Emberizidae); and tree frogs (*Hyla;* Hylidae). Is the conservative pattern of differentiation in avian proteins also characteristic of the avian mitochondrial genome?

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## Background

Mitochondrial DNA in higher animals is a closed circular duplex molecule, maternally transmitted across animal generations. It is conserved in size (~15.7–19.5 kb) and gene content yet rapidly evolving in primary nucleotide sequence (Brown 1983). Direct sequencing and fine-scale mapping studies with a few organisms have shown that the majority of mtDNA evolution arises from base substitutions (primarily transitions) plus some very small addition-deletions. Large-scale additions, deletions, or rearrangements are uncommon (Aquadro and Greenberg 1983; Cann and Wilson 1983; Greenberg et al. 1983). In protein-coding regions of the molecule, silent-position changes greatly outnumber amino acid–replacing substitutions (Anderson et al. 1982; Brown and Simpson 1982; Cann et al. 1984). The rate of base substitution in mammalian mtDNA is reportedly at least five to 10 times higher than that of single-copy nuclear DNA (Brown et al. 1979, 1982).

Nucleotide sequencing is not yet practical for large-scale population surveys. However, restriction endonuclease–site mapping or fragment analyses can be used to estimate sequence divergence indirectly. In this study we employ a restriction-fragment approach to analyze mtDNA. In interpreting results of such analyses, two previously established relationships are of special relevance. (1) The first involves the mathematical relationship between $F$ (the total proportion of shared fragments in the mtDNA digestion profiles of any compared samples) and $p$ (the estimated nucleotide sequence divergence). The relationship between $F$ and $p$ is markedly curvilinear (Upholt 1977; Nei and Li 1979), such that for small values of $F$ (i.e. $<0.25$), even small errors in estimating the fraction of identical fragment lengths (as might occur through chance electrophoretic comigration of nonhomologous fragments) will be reflected in large absolute differences in estimates of $p$. (2) The second involves the empirical relationship between $p$ and absolute divergence time ($t$), established on the basis of comparisons between 26 mammalian species pairs by Brown et al. (1979) and Brown (1983) (fig. 1). For $p < \sim 0.15$ (corresponding to $t = 8$ Myr), mtDNA sequence divergence appeared linearly related to time, but for long divergence times $p$ begins to plateau, until by 10 Myr "the readily-substituted positions in the mtDNA have become 'saturated' " (Brown et al. 1982). Much of the remaining mtDNA is presumably under strong selective constraints (Aquadro et al. 1984). If the dynamics of mtDNA sequence differentiation generally proceed as indicated in figure 1, meaningful estimates of divergence can be attempted only when values of $p$ are well within the linear portion of the curve, corresponding to $F$ values $> \sim 0.3$.

## Material and Methods

Between 13 and 18 informative restriction endonucleases were employed to assay the mtDNAs of various vertebrate species (table 1). Included in table 1 are only those enzymes found to produce multifragment digestion patterns in at least some species within each group. Enzymes that cleaved at no or one site in the mtDNAs of pairs of species being compared were excluded from the analyses. Primarily, five- and six-base-recognizing enzymes were used, because their mtDNA digestion profiles proved to be more readily interpretable than the more complex patterns produced by four-base enzymes.

Mitochondrial DNA was isolated from fresh tissue samples (heart, liver, or muscle) by the procedure of Lansman et al. (1981). In brief, the technique involves

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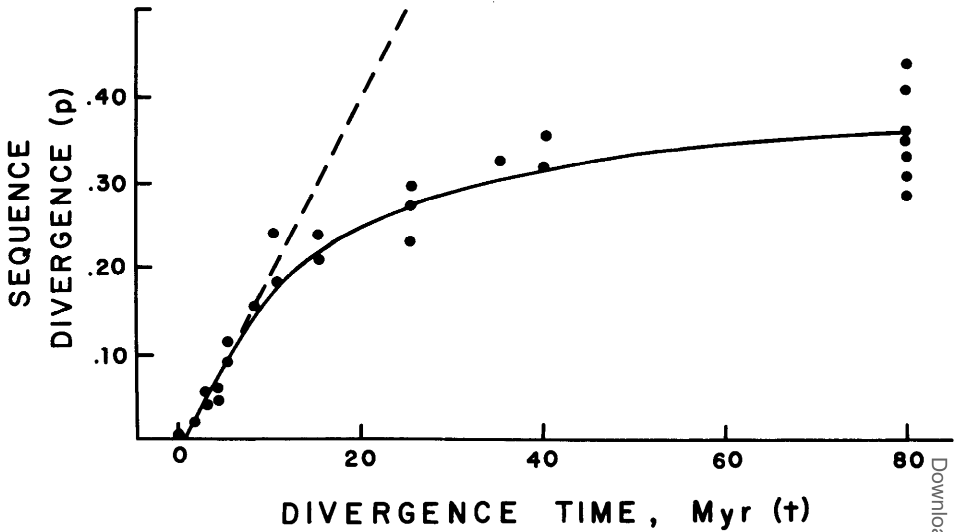


FIG. 1.—Empirical relationship between nucleotide sequence divergence ($p$) and time ($t$) described for mammalian mtDNA by Brown et al. (1979) and Brown (1983).

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homogenization of tissue, low-speed centrifugation to remove nuclei and debris, and subsequent lysis of mitochondria. MtDNA was purified by CsCl-ethidium bromide gradient centrifugation. Restriction endonuclease digestions of purified mtDNA were carried out under the vendor's (New England Biolabs) recommended conditions.

Digestion fragments were radioactively end-labeled using the large fragment of *Escherichia coli* DNA polymerase I and $^{32}$P-αdCTP (Brown 1980) and then electrophoresed through agarose gels ranging in concentration from 0.6% to 2.2%. Digestion profiles were revealed by autoradiography of vacuum-dried gels (Maniatis et al. 1982). Fragment (and genome) sizes were determined by comparisons against molecular weight markers provided by *Hin*dIII digests of λDNA and *Pvu*II-*Hin*cII double digests of pBR322.

For all waterfowl, warblers, sparrows, tree frogs, and some sunfish, digests were electrophoresed both at low and high gel concentrations to optimize resolution of large and small fragments, respectively. When necessary, electrophoresis was repeated to reorder samples for desired side-by-side comparisons. Fragment identity was assessed on the basis of comigration of fragments electrophoresed through the same gel. By including in our assays only those enzymes yielding digestion profiles with readily scorable numbers of fragments, by comparing fragments of questionable identity side-by-side on gels, and by employing a wide range of gel concentrations, we sought to minimize the possibility of judging as identical nonhomologous fragments that by chance comigrated.

The fraction of identical fragments was calculated for all pairwise comparisons of congeneric individuals by $F = 2N_{XY}/(N_X + N_Y)$, where $N_X$ and $N_Y$ are the numbers of fragments in genotypes $X$ and $Y$, and $N_{XY}$ is the number of fragments shared. Values of $F$ were converted to estimates of mtDNA nucleotide sequence divergence, $p$, by the method of Nei and Li (1979), which involves weighting

**Table 1**
**Species Used to Survey Vertebrate mtDNA**

| Common Name (Scientific Name)[a] (Sample Size) | Restriction Endonucleases[b] |
|---|---|
| Waterfowl: | |
| Mallard (*Anas platyrhynchos*) (2) | |
| Green-winged teal (*A. crecca*) (3) | |
| Mottled duck (*A. fulvigula*) (2) | |
| Northern pintail (*A. acuta*) (4) | |
| Gadwall (*A. strepera*) (2) | |
| American wigeon (*A. americana*) (3) | |
| Blue-winged teal (*A. discors*) (4) | 1–9, 11–14, 16, 18 |
| Northern shoveler (*A. clypeata*) (3) | |
| Cinnamon teal (*A. cyanoptera*) (1) | |
| Redhead (*Aythya americana*) (1) | |
| Canvasback (*A. valisineria*) (2) | |
| Lesser scaup (*A. affinis*) (3) | |
| Ring-necked duck (*A. collaris*) (5) | |
| Sparrows: | |
| Song sparrow (*Melospiza melodia*) (4) | |
| Swamp sparrow (*M. georgiana*) (3) | 1–5, 7–19 |
| Lincoln's sparrow (*M. lincolnii*) (2) | |
| White-throated sparrow (*Zonotrichia albicollis*) (4) | |
| Warblers: | |
| Chestnut-sided warbler (*Dendroica pensylvanica*) (2) | |
| Blackburnian warbler (*D. fusca*) (1) | |
| Magnolia warbler (*D. magnolia*) (2) | 1–5, 7–18 |
| Cape May warbler (*D. tigrina*) (1) | |
| Yellow-rumped warbler (*D. coronata*) (5) | |
| Sunfish: | |
| Redbreast sunfish (*Lepomis auritus*) (5) | |
| Green sunfish (*L. cyanellus*) (7) | |
| Pumpkinseed (*L. gibbosus*) (2) | |
| Warmouth (*L. gulosus*) (5) | |
| Dollar sunfish (*L. marginatus*) (2) | 1–6, 8–10, 12–14, 18 |
| Longear sunfish (*L. megalotis*) (2) | |
| Redear sunfish (*L. microlophus*) (4) | |
| Spotted sunfish (*L. punctatus*) (4) | |
| Bluegill (*L. macrochirus*) (10) | |
| Tree frogs: | |
| Bird-voiced tree frog (*Hyla avivoca*) (1) | |
| Spring peeper (*H. crucifer*) (3) | |
| Gray tree frog (*H. chrysoscelis*) (1) | 1–14, 16, 18 |
| Green tree frog (*H. cinerea*) (1) | |
| Barking tree frog (*H. gratiosa*) (5) | |

[a] According to the most recent American Ornithologists' Union checklist (1982).
[b] Listed according to the following numerical designations (recognition sequences in parentheses): 1 = *Ava*I(CPyCGPuG), 2 = *Bam*HI (GGATCC), 3 = *Bcl*I (TGATCA), 4 = *Bgl*I (GCCN$_5$GGC), 5 = *Bgl*II (AGATCT), 6 = *BstE*II (GGTNACC), 7 = *Cla*I (ATCGAT), 8 = *Hinc*II (GTPyPuAC), 9 = *Hind*III (AAGCTT), 10 = *Kpn*I (GGTACC), 11 = *Nde*I (CATATG), 12 = *Pst*I (CTGCAG), 13 = *Pvu*II (CAGCTG), 14 = *Sac*I (GAGCTC), 15 = *Sal*I (GTCGAC), 16 = *Stu*I (AGGCCT), 17 = *Tth*I (TCGA), 18 = *Xba*I (TCTAGA), 19 = *Xmn*I (GAAN$_4$TTC).

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**Table 2**
**Estimates of mtDNA Differentiation among Four Species of Sparrows**

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 (*Melospiza melodia*) ........ | | 0.026 | 0.030 | 0.068 |
| 2 (*M. georgiana*) ............ | 0.666 | | 0.030 | 0.073 |
| 3 (*M. lincolnii*) ............... | 0.630 | 0.636 | | 0.061 |
| 4 (*Zonotrichia albicollis*) ...... | 0.367 | 0.350 | 0.398 | |

NOTE.—Results are based on restriction profiles from 18 endonucleases; $F$ values among conspecifics were >0.93 (see text). Data above the upper-left-to-lower-right diagonal are nucleotide sequence divergence ($p$) values; those below the diagonal are total proportions of shared restriction fragments ($F$).

according to the numbers of fragments produced by four-, five-, and six-base enzymes. In addition, direct comparisons were made between the sparrow genera *Melospiza* and *Zonotrichia* and between selected representatives of the waterfowl genera *Anas* and *Aythya* (Kessler and Avise 1984).

Protein-electrophoretic distances (Nei's $D$ statistic [1972]) can be found in the following sources: waterfowl, Patton and Avise (1985); sparrows, Avise et al. (1980*b*); warblers, Avise et al. (1980*c*); sunfish, Avise and Smith (1977); treefrogs, Etges (1979). The mtDNA results have previously been presented (in the context of other evolutionary issues) for two groups: waterfowl (Kessler and Avise 1984) and sunfish (Avise and Saunders 1984).

## Results

Matrices of mtDNA genetic differentiation for sparrows, warblers, and tree frogs are presented in tables 2, 3, and 4, respectively. Mean $F$ values between species of *Melospiza* and between species of *Dendroica* were 0.65 (range, 0.63–0.67) and 0.52 (range, 0.44–0.61), respectively. These values are similar to previous estimates for the waterfowl genera *Anas* ($\bar{F}$ = 0.41; range, 0.26–0.96) and *Aythya* ($\bar{F}$ = 0.58; range, 0.51–0.65; Kessler and Avise [1984], tables 1 and 2) but are in marked contrast to the values found among *Lepomis* species of sunfish ($\bar{F}$ = 0.10; range, 0.00–0.36; Avise and Saunders [1984], table 3) and species of *Hyla* tree frogs ($\bar{F}$ = 0.07; range, 0.00–0.27; present study).

These differences are further reflected by the percentage of instances in which

**Table 3**
**Estimates of mtDNA Differentiation among Five Species of Warblers**

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 (*Dendroica pensylvanica*) ...... | | 0.043 | 0.035 | 0.031 | 0.048 |
| 2 (*D. fusca*) ................. | 0.529 | | 0.044 | 0.055 | 0.052 |
| 3 (*D. magnolia*) ............... | 0.581 | 0.515 | | 0.035 | 0.050 |
| 4 (*D. tigrina*) ................. | 0.612 | 0.436 | 0.585 | | 0.049 |
| 5 (*D. coronata*) ............... | 0.478 | 0.457 | 0.472 | 0.482 | |

NOTE.—Results are based on restriction profiles from 17 endonucleases; $F$ values among conspecifics were >0.92 (see text). Data above the upper-left-to-lower-right diagonal are nucleotide sequence divergence ($p$) values; those below the diagonal are total proportions of shared restriction fragments ($F$).

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**Table 4**
**Estimates of mtDNA Differentiation among Five Species of Tree Frogs**

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 *Hyla avivoca* ....... | | 0.160 | (0.156) | 0.189 | (0.314) |
| 2 *H. crucifer* ......... | 0.098 | | (0.253) | (0.600) | 0.237 |
| 3 *H. chrysoscelis* ...... | 0.268 | 0.056 | | 0.189 | (0.316) |
| 4. *H. cinerea* ......... | 0.063 | 0 | 0.063 | | 0.188 |
| 5 *H. gratiosa* ......... | 0.021 | 0.038 | 0.020 | 0.064 | |

NOTE.—Results are based on restriction profiles from 16 endonucleases; $F$ values among conspecifics were >0.83 (see text). In this table, some pairs of species exhibited $F = 0$ for six-base and/or five-base enzymes. For these species, estimates of sequence divergence are clearly large but unreliable in absolute magnitude, and the reported values (in parentheses) were generated under an arbitrary assumption that $p = 0.6$ for $F = 0$. Data above the upper-left-to-lower-right diagonal are nucleotide sequence divergence ($p$) values; those below the diagonal are total proportions of shared restriction fragments ($F$).

congeneric species shared identical, multifragment digest profiles for particular enzymes. As shown in table 5, within the four avian genera, species sharing identical patterns ranged from 18% to 28% of all comparisons of digests; however, within *Lepomis* and *Hyla,* frequencies of profile sharing were only 0.8% and 2.4%, respectively. (*Hyla crucifer* was excluded from the comparisons because it differed obviously in genome size from the other *Hyla* species, as noted below.) In addition to exact sharing of many multifragment patterns, the avian groups commonly exhibited digestion profiles that could readily be interpreted as differing by the gain or loss of a single restriction site. This is exemplified by the autoradiographs in figures 2 and 3, which present *Hin*dIII digests of mtDNA from warblers and sparrows (see also fig. 1 of Kessler and Avise [1984] for *Hin*dIII patterns in waterfowl). In contrast, congeners within *Lepomis* (see Avise and Saunders 1984) and *Hyla* (*Nde*I digest, fig. 4) typically exhibit few fragment identities.

Only a limited attempt was made to estimate intraspecific differentiation of mtDNA in the groups surveyed. In pairwise comparisons of conspecific sparrows (numbers of individuals given in table 1), $F$ values ranged from 0.93 to 1.0; among conspecific warblers, $F$ values ranged from 0.92 to 1.0. These ranges of $F$ values are similar to those observed among conspecific waterfowl ($F$ = 0.93–1.0; Kessler and Avise [1984]). Since most conspecifics were collected at a single locale, our data almost certainly underestimate levels of mtDNA genetic variability within species. However, in two cases for which we do have limited geographic samples, surprisingly little differentiation among individuals was observed. *Melospiza georgiana* collected from Clarke County, Georgia, and San Petricio County, Texas, were indistinguishable as to mtDNA genotype by 16 restriction enzymes ($F$ = 1.00). Two specimens of *Dendroica coronata* collected from these same two locales were also identical, while other individuals differed by a few mtDNA fragment changes ($F$ = 0.92–1.0).

Our data on intraspecific variability within *Hyla* or *Lepomis* are also limited. However, it is noteworthy that among five *H. gratiosa* collected from a single locale, $F$ values were as low as 0.83 and that no two individuals were identical in mtDNA genotype. Also, *L. macrochirus* is known to show extensive mtDNA sequence differentiation across the southern part of its geographic distribution, with $F$ values between two subspecies equal to 0.32 (Avise et al. 1984). In the future it will be

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**Table 5**
**Summary of Various Measures of mtDNA Divergence in Bird, Sunfish, and Tree Frog Species**

| Type of Comparison | Number of Pairwise Species Comparisons | Number of Multifragment Digestion Comparisons | Percentage Sharing of Multifragment Digestion Patterns | Mean Number of Fragments Scored ($N_x + N_y$) per Species Comparison | Mean (Range) of | |
|---|---|---|---|---|---|---|
| | | | | | $F$ | $p$ |
| Birds: | | | | | | |
| Among congeneric species: | | | | | | |
| *Anas* | 36 | 483 | 18.2 | 108.4 | 0.41 (0.26–0.96) | 0.062 (0.004–0.088) |
| *Aythya* | 6 | 79 | 21.5 | 108.7 | 0.58 (0.51–0.65) | 0.034 (0.025–0.043) |
| *Melospiza* | 3 | 43 | 27.9 | 117.6 | 0.65 (0.63–0.67) | 0.029 (0.026–0.030) |
| *Dendroica* | 10 | 167 | 18.0 | 142.6 | 0.52 (0.44–0.61) | 0.044 (0.031–0.055) |
| Between closely related genera: | | | | | | |
| *Anas/Aythya* | 2 | 27 | 3.6 | 115.0 | 0.19 (0.18–0.19) | 0.109 (0.107–0.111) |
| *Melospiza/Zonotrichia* | 3 | 45 | 2.2 | 129.0 | 0.37 (0.35–0.40) | 0.067 (0.061–0.073) |
| Other vertebrates (among congeneric species): | | | | | | |
| *Lepomis* | 36 | 378 | 0.8 | 70.4 | 0.10 (0.00–0.36) | 0.277 (0.070–0.6)[b] |
| *Hyla* | 10 | 83[a] | 2.4 | 102.6 | 0.07 (0.00–0.27) | 0.260 (0.156–0.6)[b] |

[a] Excluding comparisons with *H. crucifer*.
[b] Arbitrarily assumes $p = 0.6$ for $F = 0$ (see footnote to table 4).

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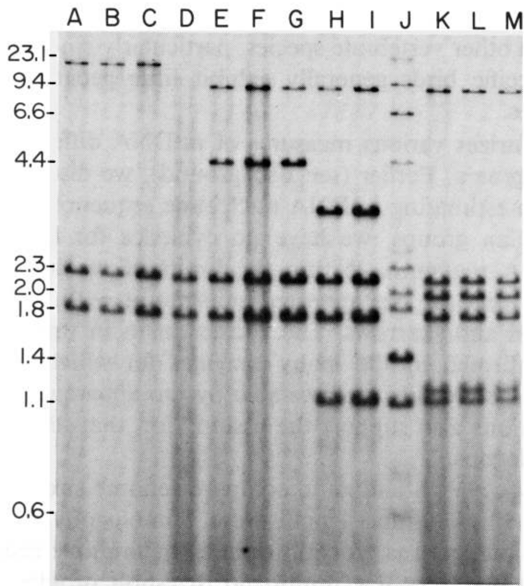


FIG. 2.—*Hin*dIII digests of mtDNA isolated from sparrows. Lanes *A–D* depict results for *Melospiza melodia;* lanes *E–G, M. georgiana;* lanes *H* and *I, M. lincolnii;* lane *J,* molecular-weight standards (sizes in kb indicated at left); and lanes *K–M, Zonotrichia albicollis.* Gel concentration is 1.5% agarose.

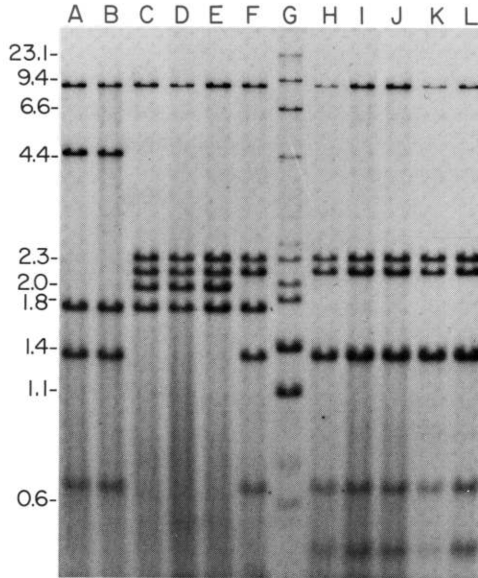


FIG. 3.—*Hin*dIII digests of mtDNA isolated from warblers. Lanes *A* and *B* depict results for *Dendroica pennsylvanica;* lane *C, D. fusca;* lanes *D* and *E, D. magnolia;* lane *F, D. tigrina;* lane *G,* molecular weight standards (sizes in kb indicated at left); and lanes *H–L, D. coronata.* Gel concentration is 1.5% agarose.



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desirable to study more thoroughly the geographic differentiation in mtDNA in a variety of avian and other vertebrate species, particularly since Barrowclough (1983) reports that conspecific birds generally exhibit little geographic heterogeneity in allozyme frequencies.

Table 5 summarizes various measures of mtDNA differentiation in the seven assayed vertebrate genera. Earlier (see Background), we discussed the assumptions that must be met in estimating mtDNA nucleotide sequence divergence, $p$, from $F$. For each of the avian groups, we have no evidence for large-scale additions or deletions of mtDNA sequence. Within the limits of resolution of our approach (±200 bp), mtDNA genome size appeared constant at ~16.5 kb for waterfowl and 17.3 kb for warblers and sparrows. The conservatism in mtDNA genome size for these groups is confirmed by the many enzymes for which the digestion profiles were either identical or readily interpretable by the apparent gain or loss of single sites. Such observations also support the assumption that identical fragments have usually been homologous.

In *Hyla* and *Lepomis,* attempts to compare genome sizes and accurately assess whether fragments of the same length were homologous were complicated (1) because most digestion patterns probably differed by multiple restriction site changes and (2) by the fact that very few multiband digestion profiles were shared across species. In one species we have unequivocal evidence for dramatic genome size variation. As judged by independent comparisons of many different digests (see, e.g., fig. 4), the mtDNA genome of *H. crucifer* is ~3 kb bases larger than those of

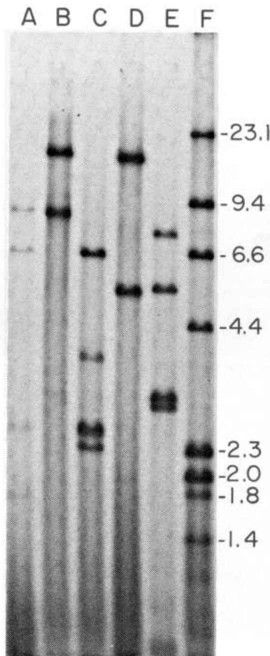


FIG. 4.—*Nde*I digests of mtDNA isolated from tree frogs. Lane *A* depicts results for *Hyla avivoca;* lane *B, H. crucifer;* lane *C, H. chrysoscelis;* lane *D, H. cinerea;* lane *E, H. gratiosa;* and lane *F*, molecular weight standards (sizes in kb indicated at right). Gel concentration is 0.7% agarose.

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the other assayed *Hyla*. We estimate its size to be ~23 kb, which would make it the largest mtDNA genome yet reported in a higher animal (Brown 1983).

Until direct sequence data become available, the estimates of $p$ derived from fragment or site analyses must remain provisional. Nonetheless, in terms of fragment identities in restriction-digest profiles, the assayed avian congeners clearly exhibit a conservative pattern of differentiation compared with the sunfish and tree frogs assayed. Even if it should prove true that the mtDNA digestion profiles of *Lepomis* or *Hyla* commonly differ because of large deletions, rearrangements, or other changes in addition to simple base substitution, this aspect of mtDNA differentiation itself would be of interest because of its contrast with the avian pattern.

## Discussion

In recent years, several studies have begun to exploit the potential of the mtDNA genome for evolutionary analysis (reviewed in Avise and Lansman 1983; Brown 1983). Most projects have dealt with mammals, and the need for data on other vertebrates has been apparent (Brown 1983). The purposes of this study have been to: (1) expand the available data base on mtDNA differentiation, particularly for birds; (2) examine the empirical relationship between protein electrophoretic distance and mtDNA nucleotide sequence divergence; and (3) use these data to readdress the issues of genome conservatism and rate of molecular evolution in birds.

### Relative Magnitudes of mtDNA Sequence Divergence

For the taxa considered in this study, mtDNA genetic distances among birds appeared smaller than those for the nonavian vertebrates (table 5 and fig. 5). For example, the *largest* mtDNA distance observed among congeneric birds in the genera *Anas, Aythya, Melospiza,* and *Dendroica* (a total of 55 pairwise species comparisons) was $p = 0.088$; only one distance value among *Lepomis* sunfish (36 interspecific comparisons) was less than this ($p = 0.070$). In 10 comparisons among species of *Hyla,* the smallest distance estimate was $p = 0.156$. Furthermore, even distances between the avian genera *Anas* and *Aythya* ($\bar{p} = 0.109$) and between *Melospiza* and *Zonotrichia* ($\bar{p} = 0.067$) are lower than most distances between assayed sunfish or tree frogs within a single genus. We realize that the *absolute* mtDNA distances reported in this study may be subject to question, since nucleotide sequences were not determined directly and because several assumptions underlie the conversion of $F$ to $p$. Nonetheless, the *relative* ordering of mtDNA distances appears clear. Overall, in terms of relative magnitudes of mtDNA differentiation reflected in restriction-digest profiles, the avian groups appear to be "shifted down" approximately one taxonomic level compared to the nonavian groups studied so far.

This conclusion is further substantiated by another published study of mtDNA differentiation in birds. From cleavage map comparisons, Glaus et al. (1980) report $p$ values ranging from 0.097 to 0.175 in comparisons *between genera* and *subfamilies* of galliform birds. These values are consistent with our estimate of $\bar{p} = 0.109$ between *Anas* and *Aythya* and fall within a range characteristic of many interspecific comparisons with *Lepomis.* They are also comparable to reported mtDNA distances (based on mapped sites) between two congeneric rat species, *Rattus rattus* and *R. norvegicus* ($p = 0.137$–$0.184$; Brown and Simpson [1981]), and between two

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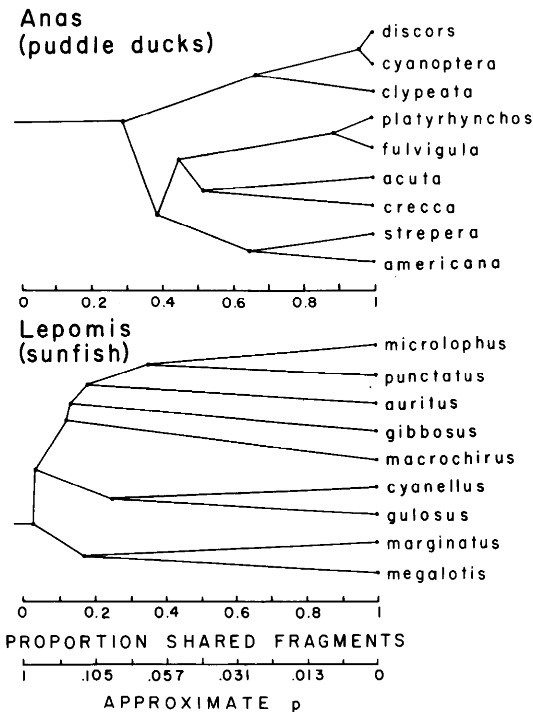


FIG. 5.—MtDNA-based cluster phenograms (UPGMA method; Sneath and Sokal [1973]) for *Anas* and *Lepomis*. These were plotted on common scales of the proportion of shared fragments and the associated nucleotide sequence divergence ($p$ value) to emphasize the contrast in magnitude of the mtDNA differentiation in these waterfowl versus that in sunfish. For *Lepomis*, the phenogram should not be considered an appraisal of phylogenetic relationships because the mtDNA distances between most species are too large (see text).

congeneric field mice, *Peromyscus maniculatus* and *P. leucopus* ($p$ = 0.120–0.167; Avise et al. [1983]).

## Empirical Relationship between $p$ and $D$

The availability of estimates of both nuclear gene divergence (as measured by conventional protein-electrophoretic procedures) and mitochondrial sequence divergence for particular taxa permits comparisons between these distance measures. In figure 6 we have plotted $p$ versus $D$ for species pairs within *Anas, Aythya,* and *Lepomis*. For the avian congeners, all $p$ values are $<0.09$, and these correspond empirically to $D$ values in the range of 0.00–0.20. For *Lepomis*, most $p$ values are $>0.10$, with associated $D$s ranging from 0.15 to 1.0 or more. Because of the nonlinear accumulation of sequence divergence in mtDNA with time beyond perhaps 8–10 Myr (see Background and fig. 1), Brown et al. (1982) suggest that study of relationships among organisms and estimates of absolute times of divergence be restricted to comparisons within the linear portion of the curve (where $p < \sim 0.15–0.20$). Data given in figure 6 suggest that such small $p$ values appear to be associated with protein-electrophoretic distances of $< \sim 0.20–0.40$. Elsewhere we

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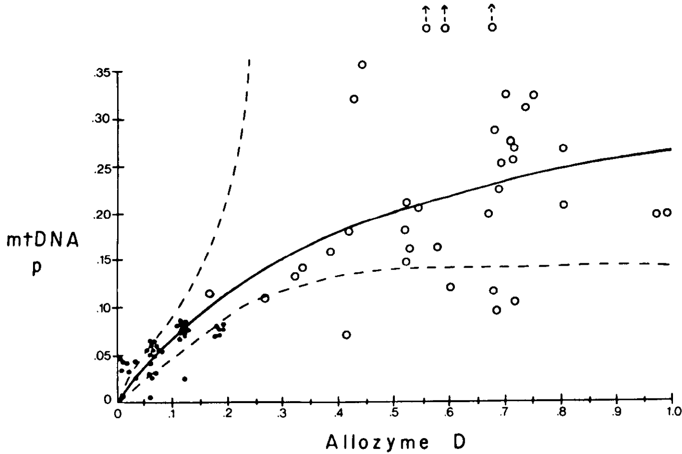


FIG. 6.—Empirical relationship between estimated nucleotide sequence divergence in mtDNA ($p$) and protein-electrophoretic distance ($D$; Nei [1972]). Closed circles represent comparisons among congeneric waterfowl in the *Anas* and *Aythya* genera; open circles represent comparisons among sunfish of the *Lepomis* genus. The solid line is a least-squares regression generated under the model $p = \alpha D/(1 + \beta D)$. The regression equation is $p = 0.79D/(1 + 2.13D)$, where the estimated SEs on the regression coefficients in the numerator and denominator are 0.14 and 0.68, respectively. (The three outlying points at the top of the graph were not included in the determination of the regression equation.) Dashed lines are boundaries about $p$ values along this regression assuming a $\pm 10\%$ error in estimation of fragment homology.

have exploited these small mtDNA distances in waterfowl to assess systematic relationships within the group (Kessler and Avise 1984).

Much of the large variance in $p$ values associated with $D$ values >0.4 may be attributable to the mathematical relationship between scored fragment identity, $F$, and $p$ (see Background). In figure 6 we plot one example of a least-squares regression line relating $p$ to $D$. The dashed lines represent boundaries about this empirical regression assuming a $\pm 10\%$ error in estimation of $F$ from which the $p$ values were derived.

## Divergence Times and Rates of Evolution in Birds

The conservative pattern of mtDNA differentiation in the avian versus nonavian taxa examined generally parallels the conservative pattern of protein differentiation reported previously. As argued in the Introduction, this result is compatible with the thesis that avian taxa may have a more recent shared common ancestry than do many comparable nonavian taxa. Can the magnitudes of mtDNA and protein divergence be reconciled with other information about absolute times of avian separation?

The fossil record for birds is notoriously poor, and it is usually not possible to accurately estimate separation times of particular pairs of extant species. However, previous interpretations of the somewhat better fossil remains for waterfowl indicated that both *Anas* and *Aythya* were already present by at least the Miocene epoch, that is, more than 15 Myr ago (Brodkorb 1964; Howard 1964; Romer 1966; Patton and Avise 1985). Available molecular data do not corroborate this interpretation even to a first approximation.

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Brown et al. (1979, 1982) have calibrated a rate of nucleotide sequence divergence in mtDNA of ~2%/Myr. If we accept this rate, our mtDNA-estimated split between *Anas* and *Aythya* occurred ~5.5 Myr ago ($\bar{p} = 0.109$). Based on the allozyme data of Patton and Avise (1985), we have calculated a genetic distance of $\bar{D} = 0.164$ (range, 0.113–0.313) between *Anas* and *Aythya* species. Using the *slowest* calibrated electrophoretic clock commonly employed in the literature (see Avise and Aquadro 1982), this genetic distance suggests a divergence time in the range of ~2.0–5.6 Myr ago. The protein- and mtDNA-based estimates are roughly comparable but are in sharp contrast to the fossil-based date of >15 Myr.

If we accept the paleontological estimates of divergence time, the molecular data would argue for a decelerated rate of *both* mtDNA and protein evolution in waterfowl. However, avian paleontologists themselves have emphasized the provisional nature of many fossil assignments. "When evaluating the avian fossil record, it must be borne in mind that, in most instances, fossil species are known from only a few disarticulated bones. As the subfamilies and tribes of the Family *Anatidae* are not always clearly defined even in life, the allocation of extinct species to these groups, on the basis of one or two fragments of the skeleton, may quite properly be subject to question. . . . Many missing links must yet be found, and much more must be known of the osteology of living anseriforms before the fossil record can offer a true picture of the evolution of the group" (Howard 1964, pp. 235, 237).

Alternatively, if we question the reliability of the fossil assignments for waterfowl, a scenario arises that is analogous to the current debate over the divergence time of ancestral humans from African apes. Early interpretations of paleontological evidence suggested that humans and chimps last shared a common ancestor ~30 Myr ago (Simons 1964, 1967; Pilbeam 1970). Subsequently, molecular data indicated that this split may have occurred as recently as 5 Myr ago (Sarich and Wilson 1967; Wilson et al. 1977; Andrews 1982; Andrews and Cronin 1982). While some evolutionists interpreted the molecular results to indicate a deceleration of sequence divergence in higher primates (Goodman 1976; Goodman et al. 1983), reevaluations of earlier paleontological studies and new fossil evidence have led many paleontologists to view more favorably the possibility of a recent human-chimp split (Johanson and White 1979; Greenfield 1980; Andrews 1982; Pilbeam 1984). Similarly, the notion that species of waterfowl (and of other avian genera) have speciated more recently than many nonavian vertebrate congeners should not be summarily dismissed. If the fossil evidence is indeed suspect, mtDNA- and protein-based estimates might be taken as more realistic indicators of avian divergence times.

Many possibilities remain. For example, it is conceivable that occasional introgression among hybridizing waterfowl or other avian species has inhibited genetic differentiation for some time after their "separation" (Prager and Wilson [1975] note that birds generally lose potential for interspecific hybridization slowly). Effects of such reticulate evolution in the early history of diverging species would be difficult to distinguish from more recent complete separation. It is also possible that the mtDNA rate calibrations, which were taken from mammalian data, will not apply to birds. As noted by Brown (1983), little is known about mtDNA replication repair in animals, and "rate of mtDNA evolution could vary considerably among various taxonomic groups." If avian mtDNA is evolving at the mammalian rate, then protein evolution alone may be somewhat decelerated in birds (reconcil-

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iation of mtDNA- and protein-based divergence times required use of a very *slow* protein clock). Overall, it is perhaps most likely that the final answer may include elements of *both* fundamental alternative hypotheses cited in the Introduction. Thus, rates of avian molecular evolution may be somewhat decelerated relative to other taxonomic groups, *and* many avian taxa are probably of more recent evolutionary age than their nonavian taxonomic counterparts. As with the human-chimp controversy, continued molecular and paleontological research will be required to shed additional light on these issues.

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