Calibration of Avian Molecular Clocks

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Molecular clocks can be calibrated using fossils within the group under study (internal calibration) or outside of the group (external calibration). Both types of calibration have their advantages and disadvantages. An internal calibration may reduce extrapolation error but may not be from the best fossil record, raising the issue of nonindependence. An external calibration may be more independent but also may have a greater extrapolation error. Here, we used the advantages of both methods by applying a sequential calibration to avian molecular clocks. We estimated a basal divergence within birds, the split between fowl (Galliformes) and ducks (Anseriformes), to be 89.8 ± 6.97 MYA using an external calibration and 12 rate-constant nuclear genes. In turn, this time estimate was used as an internal calibration for three species-rich avian molecular data sets: mtDNA, DNA-DNA hybridization, and transferrin immunological distances. The resulting time estimates indicate that many major clades of modern birds had their origins within the Cretaceous. This supports earlier studies that identified large gaps in the avian fossil record and suggests that modern birds may have coexisted with other avian lineages for an extended period during the Cretaceous. The new time estimates are concordant with a continental breakup model for the origin of ratites.

Introduction

Many questions concerning the evolution, ecology, and behavior of birds require knowledge of an evolutionary timescale. As with most groups of organisms, the fossil record and first occurrences of lineages provide a temporal framework. Unfortunately, the fossil record of birds is often fragmentary and difficult to interpret. In part, this is because modern birds lack teeth, and most possess hollow bones that preserve poorly. Molecular clocks are especially helpful for groups with such problematic fossil records, and several recent studies have estimated divergence times among major clades of birds (Hedges et al. 1996; Cooper and Penny 1997; Härlid, Janke, and Arnason 1997, 1998; Härlid and Arnason 1999; Waddell et al. 1999). Although these studies used different data sets and methods, each reached the same general conclusion that some or most major clades of modern birds arose in the Cretaceous. In one case, continental breakup was suggested as the mechanism for the diversification of avian orders (Hedges et al. 1996). On the other hand, the fossil record (Benton 1993, 1997) has long indicated that modern avian orders arose more recently, in the early Tertiary, possibly from a shorebird ancestor (Wyles, Kunkel, and Wilson 1983; Feduccia 1995, 1996). Reconciling these two competing hypotheses will require greater scrutiny of the avian fossil record and of molecular clocks.

Avian molecular clocks have used internal (Cooper and Penny 1997; Waddell et al. 1999) and external (Hedges et al. 1996; Härlid, Janke, and Arnason 1997, 1998; Kumar and Hedges 1998; Härlid and Arnason 1999) calibrations. An advantage to the use of an internal calibration is that the time estimated is closer to the calibration time and therefore requires less extrapolation and associated error. One disadvantage is that the fossil record of the group in question (e.g., birds) may be poorer than that of another group (e.g., mammals) and therefore more likely to yield a significant underestimate of divergence time unless the fossil calibrations are chosen carefully. Another disadvantage is that an internal calibration could be considered nonindependent or paralogical. For example, if the question being posed involves the origin of avian orders, and the fossil record of avian orders is used to calibrate the clock, then the two are not independent. External calibrations have the advantage that they are independent and do not rely on the fossil record of the group in question. On the other hand, the larger extrapolation involved may lead to greater statistical error in the time estimate. An additional problem with external calibrations is that genes appropriate for resolving relationships within a group may be too fast-evolving for comparisons outside of the group.

A possible solution is to use the advantages of both internal and external calibrations without the disadvantages. This can be accomplished with a sequential calibration method. First, the divergence time is estimated for a basal divergence (anchor point) within the ingroup using an external calibration. Because it is a basal (early) divergence, the extrapolation error is reduced. Afterward, other genes and molecular data sets more appropriate for the ingroup can be calibrated with this anchor point. Here, we used this sequential calibration method to refine the avian molecular timescale. In this case, we chose the divergence between the order comprising the ducks (Anseriformes) and the order comprising the game fowl (Galliformes) as an anchor point because of its basal position and because those two groups are best represented in the sequence databases. Most avian orders are represented by only one or two protein sequences, whereas hundreds to thousands of sequences, representing dozens of genes, are available for galliforms and anseriforms. Several studies have supported a close relationship between those two orders and a close relationship between that clade (Galloanserae)

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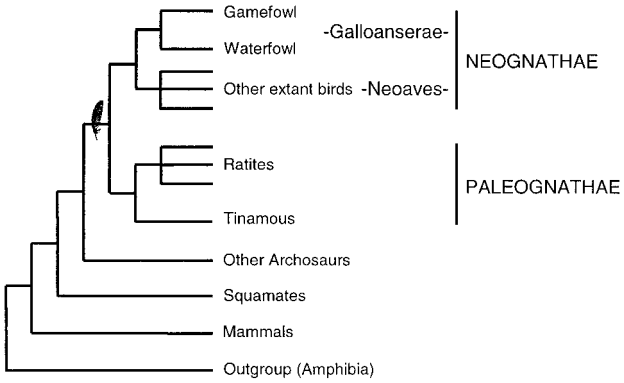

FIG. 1.—Amniote phylogeny showing the relative position of modern birds (Neornithes) and the established groupings within birds. Paleognathae includes the tinamous and ratites; Neognathae includes Galloanserae (galliform and anseriform birds) and Neoaves (all other birds).

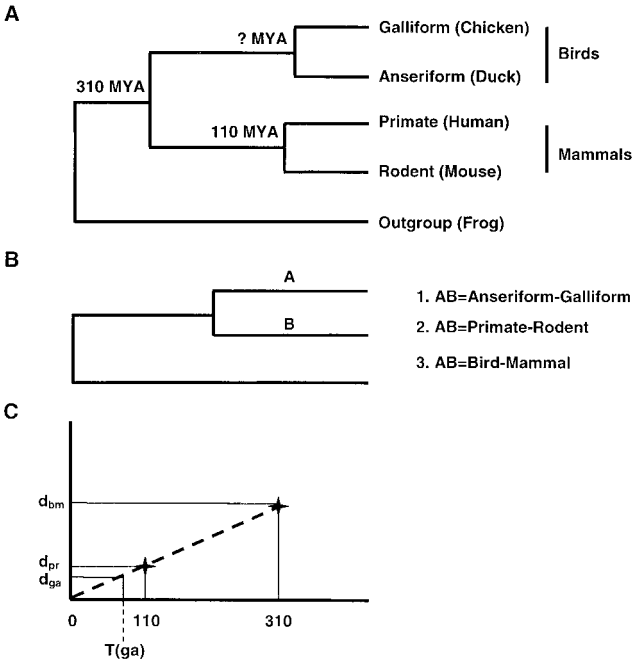

FIG. 2.—Types of calibrations and rate tests employed to determine the Galliformes-Anseriformes divergence time. *A,* Use of a fossil-based reptile-mammal calibration (310 MYA) and a molecular-based primate-rodent calibration (110 MYA). Nuclear genes included at least one galliform, one anseriform, two mammals (primate and rodent), and an outgroup in order to perform sufficient rate testing. The most frequently used representatives are shown in parentheses. *B,* Relative-rate tests were performed on galliform-anseriform, bird-mammal, and primate-rodent taxa. *C,* Use of a third combined (reptile-mammal + primate-rodent) calibration with a slope fixed through the origin.

and other neognathous birds (Neoaves; Cracraft 1988; Sibley and Ahlquist 1990; Caspers et al. 1997; Groth and Barrowclough 1999; van Tuinen, Sibley, and Hedges 2000; fig. 1). Alternative arrangements obtained with some mitochondrial sequences (Mindell et al. 1997, 1999; Härlid and Arnason 1999) may be the result of taxon-sampling biases (van Tuinen, Sibley, and Hedges 2000).

With a time estimate for this anchor point from all available nuclear protein-coding gene sequences, we calibrated the avian timescale for several species-rich molecular data sets.

Materials and Methods

Nuclear protein sequences were obtained from the public databases (GenBank release 113.0) to establish a galliform-anseriform time estimate. Two external calibration points were used (fig. 2*A*): a reptile-mammal (RM = 310 MYA; Benton 1997) primary calibration and a primate-rodent (PR = 110 MYA) secondary calibration. The first calibration was one of the best in the fossil record, and the second was a molecular time estimate derived from the first calibration (Kumar and Hedges 1998). Another advantage of both calibrations was that primates and rodents represent the most abundant nonavian sequences for possible calibration. Therefore, nuclear genes were selected if they included protein sequences from at least one galliform, one anseriform, two mammals (including primate and rodent), and an outgroup (fig. 1; most frequently within amphibia: Xenopus, Rana, or Bufo). Short (<100 residues) protein sequences were omitted. To investigate orthology, BLAST searches were performed on all sequences, and gene-specific topologies were constructed. Orthologous sequences were tested for rate homogeneity (Takezaki, Rzhetsky, and Nei 1995; Kumar 1996) between the galliform and the anseriform, between birds (Galloanserae) and the mammal(s), and between the primate and the rodent (fig. 2*B*). Most often, the galliform-anseriform divergence was based on pairwise distances between *Gallus gallus* and *Anas platyrhynchos,* but multiple galliform and anseriform taxa were included in the analyses when more than one taxon was available. Multiple mammalian taxa also were included, resulting in a mean number of nine total taxa (per gene) in the rate tests. Finally, a third and more general calibration was derived from the slope of the two point estimates (110 and 310 MYA) fixed through the origin (fig. 2*C*). In all cases, the amino acid distances were corrected for multiple hits (Poisson distance), tree topologies were constructed, and rate heterogeneity across sites was calculated (gamma-parameter) for each gene using GZ-GAMMA (Gu and Zhang 1997). Intergroup distances were estimated by four-cluster analysis in PHYLTEST (Kumar 1996) using a gamma distance.

Using these criteria, protein sequence alignments of 21 orthologous nuclear genes were available for timing analysis. Gene alignments and accession numbers are available at http:carib.bio.psu.edu/avianclock/. Of those 21 genes, 6 did not pass one or more relative-rate tests, depending on the calibration used (table 1). The divergence time between galliform and anseriform birds was calculated for each gene by applying the rate determined from the calibration to the divergence estimate (average distance method; Kumar and Hedges 1998). A mean time estimate and standard error were obtained by averaging time estimates of the rate-constant genes, excluding the highest and lowest values (outliers). The reason for excluding outliers was to reduce the effect of

Table 1
Genes Used in the Calculation of the Galliformes-Anseriformes Divergence Time

Gene	Taxa	Amino Acids (aa)	Amino Acid Variation (% GA)	Gamma Value	Branch Length GA (SE)	Branch Length RM (SE)	Branch Length PR (SE)	Regression Slope ($\times 10^3$)	GA Divergence Time RM Calibration	GA Divergence Time PR Calibration	GA Divergence Time Regression Slope
Alcohol dehydrogenase	7	185	11.4	0.74	0.066 0.075 (0.032)	0.162 0.185 (0.047)	0.088 0.065 (0.032)	1.2	126.9	101.8	123.5
Alpha-A globin	12	141	20.6	0.84	0.104 0.103 (0.036)	0.218 0.214 (0.058)	0.092 0.130 (0.045)	1.5	148.8*	102.5	141.6*
Alpha-B crystallin	7	173	4.0	0.87	0.016 0.027 (0.016)	0.186 0.126 (0.049)	0.004 0.023 (0.012)	0.9	42.0	173.6	45.9
Apolipoprotein A1 precursor	11	250	8.8	4.18	**0.075** 0.036 (0.020)	0.323 0.473 (0.058)	0.169 **0.288** (0.045)	2.8	—	—	—
Cytochrome C	11	104	2.9	0.20	0.029 0.030 (0.020)	0.037 0.082 (0.037)	**0.115** 0 (0.045)	0.3	93.0	—	93.0
Delta2 Crystallin	6	461	9.1	1.02	0.059 0.049 (0.016)	0.222 0.213 (0.038)	0.048 0.065 (0.017)	1.4	77.0	104.9	79.4
Epsilon Crystallin	8	332	3.6	0.36	0.012 0.027 (0.012)	0.085 0.063 (0.024)	0.007 0.013 (0.008)	0.5	81.3	210.3*	87.3
Epsilon globin	12	139	13.7	1.53	0.028 0.126 (0.037)	0.252 0.160 (0.054)	0.124 0.087 (0.039)	1.4	116.3	80.6	110.8
Fatty acid synthase	5	352	8.8	1.14	0.027 0.069 (0.018)	0.127 0.161 (0.032)	0.063 0.043 (0.019)	0.9	103.3	99.4	102.8
Gamma interferon	14	156	34.6	3.30	0.202 0.239 (0.062)	0.374 0.799 (0.099)	0.273 0.262 (0.075)	3.9	116.3	90.5	112.7
Growth hormone	13	183	1.6	1.60	0.021 0.013 (0.012)	0.091 **0.199** (0.034)	**0.399** 0.049 (0.066)	1.1	—	—	—
Histone 1	5	201	5.5	1.45	0.020 0.026 (0.016)	0.106 **0.248** (0.044)	0.212 0.172 (0.057)	1.4	—	—	—
Histone 2b	6	125	2.4	0.30	0.005 0.020 (0.015)	0.027 0.025 (0.020)	0 0.010 (0.008)	0.2	151.1[a]	343.6[a]	161.2[a]
Lysozyme	15	121	19.0	1.06	0.146 0.106 (0.053)	0.380 0.287 (0.081)	0.158 0.172 (0.064)	2.3	117.0	84.0	112.1
MX2a protein	8	613	36.2	1.29	**0.306** 0.149 (0.038)	**0.492** 0.393 (0.053)	0.272 0.275 (0.043)	3.1	—	—	—
Prion protein precursor	9	185	9.2	3.72	0.046 0.022 (0.020)	0.581 0.314 (0.087)	0.056 0.036 (0.023)	2.7	23.5*	81.3	25.5*
RAG-1	9	945	6.1	0.43	0.036 0.025 (0.007)	0.166 0.178 (0.024)	0.038 0.073 (0.013)	1.1	55.4	60.7	56.0
Rhodopsin	11	322	3.4	0.42	0.028 0.008 (0.011)	0.052 0.077 (0.020)	0.022 0.034 (0.012)	0.4	86.8	71.0	84.7
Tau Crystallin	5	434	2.8	0.36	0.008 0.021 (0.009)	0.024 **0.076** (0.016)	0.022 0.046 (0.014)	0.4	—	47.7*	—
TCRbeta	8	160	22.5	4.17	0.114 0.090 (0.039)	0.442 0.521 (0.095)	0.108 0.102 (0.036)	3.0	65.9	107.7	68.9
WNT5A	8	116	2.6	0.34	**0.047** 0 (0.017)	0.054 0.015 (0.024)	0 0 (0)	1.2	—	—[b]	—[b]
Average	9.1	271.3	10.9	1.40	0.066 0.059 (0.024)	0.210 0.229 (0.047)	0.108 0.093 (0.034)	1.5	90.1	96.5	89.8

Table 2
Average Galliformes-Anseriformes Divergence Times

	Type of Calibration		
	Reptile-Mammal	Primate-Rodent	Regression Slope
Average for rate-constant genes (N = 12)	90.1 ± 7.8	96.5 ± 8.2	**89.8 ± 7.0**
Average including outliers (N = 14)	89.5 ± 10.1	101.1 ± 11.5	88.9 ± 8.5
Average for all genes (N = 20–21)	92.6 ± 8.8	87.8 ± 11.2	86.2 ± 8.4

Note.—The boldface divergence time was chosen for further calibration.

possible paralogy problems (Kumar and Hedges 1998). The galliform-anseriform divergence time estimate derived from the combined (RM+PR) calibration (table 2) was used as an internal calibration point for three large molecular data sets: (1) an mtDNA data set containing the complete sequences of the 12S rRNA, tRNA-Valine, and 16S rRNA genes (a ~3-kb region) in 54 taxa representing all avian orders (van Tuinen, Sibley, and Hedges 2000); (2) a DNA-DNA hybridization distance data set with approximately 1,700 taxa (Sibley and Ahlquist 1990), also representing all avian orders; and (3) a transferrin immunological distance data set with 21 avian taxa representing 13 avian orders (Prager et al. 1974; Ho et al. 1976; Prager et al. 1976).

The mtDNA alignment was taken from a recent study of avian phylogeny (van Tuinen, Sibley, and Hedges 2000). To this alignment, five taxa were added from GenBank in order to include all available turtle and crocodilian mtDNA sequences and to increase the number of galloanserine taxa useful for calibration. The additional taxa include *Chelonia mydas* AB012104, *Pelomedusa subrufa* AF039066, *Chrysemys picta* AF069423, *Aythya americana* AF090337 (Mindell et al. 1999), and *Coturnix coturnix* X57245 (Desjardins and Morais 1991). The *C. coturnix* sequence (AF302070) was a partial sequence that was completed through DNA sequencing following a standard protocol (van Tuinen, Sibley, and Hedges 2000).

To investigate the extent of rate constancy among the mtDNA sequences, a branch length test was performed, after which the taxa showing significantly different branch lengths at the 1% level were excluded (Takezaki, Rzhetsky, and Nei 1995). A second branch length test was performed on the pruned data set to determine if other taxa now exhibited heterogeneous branch lengths because of a possible change in the mean. Additional branch length tests were performed until all remaining sequences (n = 26) exhibited rate constancy (mean $BL_{root\text{-}tip}$ = 0.013). Of the pruned sequences, several showed branch lengths smaller than the final mean. Rate constancy among the rate-constant taxa was confirmed by performing a two-cluster test (Takezaki, Rzhetsky, and Nei 1995). Branch lengths and tree topologies in both tests were produced from a Kimura two-parameter distance including transversions only and the neighbor-joining tree building method. A linearized tree (Takezaki, Rzhetsky, and Nei 1995) was produced from this pruned data set, and nodes were timed using the galliform-anseriform calibration. This molecular rate was then applied to the linearized tree of all taxa, and additional nodes were timed with a lineage-specific method ($t = l_1/r_A$; Takezaki, Rzhetsky, and Nei 1995; Kumar and Hedges 1998; Schubart, Diesel, and Hedges 1998). To account for the difference in branch lengths between the calibration taxon and these slower- or faster-evolving taxa, branch length ratios were estimated for each of these taxa to the calibration taxon, and time estimates were multiplied by this ratio to obtain final weighted estimates.

Divergence time estimates for the DNA hybridization data set were based on the mean ΔT_{50} values reported in figs. 353–369 in Sibley and Ahlquist (1990). For the immunological transferrin protein data, distance values were obtained from neighbor-joining branch lengths calculated from a complete data matrix of published reciprocal values (Ho et al. 1976; Prager and Wilson 1976; Prager et al. 1976) under the assumption of rate homogeneity. Divergence times within neornithine birds were estimated from the three molecular data sets only for well-established groupings (see Olson 1985; Cracraft 1988; Sibley and Ahlquist 1990; Feduccia 1996) or for groupings consistent between all three molecular data sets (table 3).

Results

Galliform-Anseriform Calibration

To assess the extent of branch length homogeneity in each gene, we compared branch lengths of the gal-

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Note.—Data shown are general information about the data sets used, the branch lengths leading from the most recent node to Galliformes and Anseriformes (GA), to birds and mammals (RM), and to primates and rodents (PR), the standard errors of the average branch lengths leading from the GA, RM, and PR nodes, the regression slopes fitted through zero, and the gene-specific divergence dates calculated from the three calibration types. Rejections by relative-rate tests are indicated with bold type, and the divergence time outliers are indicated with asterisks. Of the 21 available genes, 9 were excluded for time estimation because they did not pass relative-rate tests (6 genes), because they were outliers (2 genes), or because of paralogy suspicions (1 gene, Histone 2B).

[a] Histone 2b–based time estimations were excluded from final analyses because of large relative standard errors, suspected paralogy, and every calibration yielding times older than the oldest known nonmodern bird (*Archaeopteryx*).

[b] Identical primate and rodent sequences.

Table 3
Avian Divergence Times, Standard Errors, and Fossil Gaps (in MYA) Estimated by Applying the Galliform-Anseriform Calibration to Three Molecular Data Sets

	mtDNA[a]	DNA-DNA Hybridization[b]	Transferrin[c]	Mean ± SE	Absolute Fossil Gap[d]	% Fossil Time/ Molecular Time
Superordinal						
Palaeognathae-Neognathae	118.1 ± 17.2	109.7	128.0	118.6 ± 5.3	48.6	59.0
Galloanserae-Neoaves	101.8 ± 14.4	109.7	101.1	104.2 ± 2.8	34.2	67.2
Basal Neoaves	89.6 ± 13.4	105.8	72.5[c]	89.3 ± 9.6	19.3	78.4
Ordinal						
Within Palaeognathae[f]						
Tinamiformes-ratites	88.9 ± 14.8	85.4	74.8	83.0 ± 4.2	23.0	72.3
Ostrich-other ratites	79.5 ± 14.4	70.7	73.2	74.5 ± 2.6	14.5	80.5
Rhea-kiwi/emu	79.5 ± 14.4	64.9	50.4	64.9 ± 8.4	4.9	92.4
Kiwi-emu	55.6 ± 12.4	51.3	42.6	49.8 ± 3.8	44.8	10.0
Within Galloanserae						
Galliformes-Craciformes	77.7 ± 14.8	84.7	77.4	79.9 ± 2.4	26.9	66.3
Within Neoaves						
Apodiformes-Trochiliformes	67.9 ± 11.1	83.5	NA	75.7 ± 7.8	22.7	70.1
Intraordinal						
Ciconiiformes (Shorebird/Gull-other)	74.3 ± 10.6	73.3	NA	73.8 ± 0.5	3.8	94.9
Coraciformes (Kingfisher-Hornbill)	67.6 ± 10.5	91.7	NA	79.7 ± 12.1	26.7	66.5
Cuculiformes (Ani-Cuckoo)	52.0 ± 9.2	69.0	NA	60.5 ± 8.5	7.5	87.6
Galliformes (Chicken-Quail)	36.1 ± 7.5	40.4	39.8	38.8 ± 1.3	5.8	85.1
Passeriformes (Oscine-Suboscine)	77.1 ± 11.6	77.0	NA	77.1 ± 0.1	22.1	71.3

NOTE.—NA = data not available.

[a] 12S rRNA, tRNA-Valine, and 16S rRNA genes; from this study and van Tuinen, Sibley, and Hedges (2000). Standard errors shown are propagated errors of the three standard errors involved: of the calibration time estimate, of the mean calibration distance in the linearized tree, and of the mean distance between the terminal taxa on a node in the linearized tree. The distances and standard errors were obtained from the two-cluster analysis (Takezaki, Rzhetsky, and Nei 1995). Propagated error = $\sqrt{rSE_1^2 + rSE_2^2 + rSE_3^2} \times$ (mean nodal time estimate). rSE (relative standard error) = the proportion of the standard error of the mean to the mean estimate.

[b] ΔT_{50}; Sibley and Ahlquist (1990).

[c] Ho et al. (1976), Prager and Wilson (1976), and Prager et al. (1976).

[d] Oldest fossil occurrences from the literature: duck, loon, and shorebird, 70 MYA (Padian and Chiappe 1998); rhea, 60 MYA (Alvarenga 1983); emu, 5 MYA; cracid, 50 MYA (Olson 1985); galliform, swift, coraciform, and cuculiform, 53 MYA (Feduccia 1996); quail, 33 MYA (Olson 1985); and passeriform, 55 MYA (Boles 1995).

[e] Although no pairwise distances are available between 13 additional avian taxa, distances have been reported for each of these taxa to the other published taxa for which reciprocal values are available (Ho et al. 1976). To investigate if addition of any of these 13 taxa would alter the basal node within Neoaves, each taxon was added separately to the complete data matrix, and neighbor-joining trees were compared. The timing of the basal Neoaves is based on the addition of a woodpecker to the complete data matrix.

[f] van Tuinen, Sibley, and Hedges (1998); see figure 2 for hybridization and immunological distance data.

[g] The lineage-specific method was used to time the divergences among palaeognaths, as well as the Galliformes-Craciformes, Apodiformes-Trochiliformes, and the Suboscine-Oscine divergences. Branch length ratios were estimated for each of these divergences with the calibration taxa and applied to obtain weighted branch lengths: Galloanserae/ratites = 1.44; Galloanserae/Craciformes = 1.14; Galloanserae/Apodiformes-Trochiliformes = 1.04; Galloanserae/Passeriformes = 0.95.

liform and anseriform taxa to the node that joined them, and we similarly compared the branches leading to birds and mammals and to primates and rodents (table 1). For the genes that indicated significant departure from rate homogeneity, galliformes evolved more than twice as fast as anseriforms (n = 3, ratio of branch lengths = 2.32), birds evolved slower than mammals (n = 4, ratio = 0.78), and primates evolved twice as fast as rodents (n = 3, ratio = 2.03). After excluding these genes, the ratios were all close to 1 (galliform-anseriform = 0.92, birds-mammals = 0.95, primate-rodent = 0.99), and no obvious directional trends were observed.

The galliform-anseriform divergence time obtained from the mean of the rate-constant genes ranged between 90 and 100 MYA, with two of the three calibrations yielding times close to 90 MYA (table 2). Similar times were obtained from a concatenation of the rate-constant genes (92.0 MYA using RM calibration), in combination with a mean gamma parameter of 1.4 (among genes), when including the outliers or when averaging over all genes regardless of rate constancy. A slightly older time estimate (112 ± 11.7 MYA, n = 5 genes) for the galliform-anseriform divergence was published earlier (Kumar and Hedges 1998), but it was based on fewer genes and a larger gamma parameter (a = 2). For subsequent timing within modern birds, a galliform-anseriform divergence time of 90 MYA (89.8 ± 6.97 MYA) was used as the internal calibration point because it was based on more than one external calibration and involved the most conservative (recent) estimate of the rate-constant genes.

Divergence Times Among Birds

Multiple standard errors were involved in the calculation of the time estimates based on the mtDNA data,

and these are shown as single propagated errors for each node in table 3. As a result, the calibration errors alone yielded a propagated standard error of about 12% of the final time estimate. For the DNA-DNA hybridization data set, the galliform-anseriform calibration yielded a rate of 0.255 Δ °C/MYR (= 3.92 Myr/Δ °C). A slightly slower rate of 0.213 Δ °C/MYR (= 4.69 Myr/Δ °C) was proposed by the authors of a study (Sibley and Ahlquist 1990) based on an ostrich-rhea vicariance calibration now considered to be both phylogenetically and temporally incorrect (van Tuinen, Sibley, and Hedges 1998). For the transferrin data set, the galliform-anseriform calibration yielded a rate of 0.72 units of immunological distance per Myr; a slower rate than the 1.0–1.2 units/Myr estimated by the original authors (Prager et al. 1974). The original transferrin rate was estimated from the fossil record under the assumption of 100 MYA for the mean divergence time between bird orders and using 70 MYA for the divergence between phasianoid galliform and megapodioid galliform birds (Prager et al. 1974). However, the bird fossil record is too sparse to be useful for accurate internal calibration. Among the different molecular data sets, general agreement exists between divergence times for each avian group, as shown by small standard errors (table 3). As described previously (van Tuinen, Sibley, and Hedges 2000), we found a high correlation between mtDNA versus hybridization pairwise distances ($r = 0.90$), but also high correlations ($r = 0.93$) between the other data sets (transferrin vs. mtDNA and vs. hybridization distances). These results suggest clocklike behavior in all three data sets (see also Wilson, Carlson, and White 1977; Sibley and Ahlquist 1990).

The earliest divergences within modern birds, between Palaeognathae and Neognathae and between Galloanserae and Neoaves, are estimated to have occurred in the mid-Cretaceous (100–120 MYA; table 3). Considering the three molecular data sets simultaneously, the deepest splits within Palaeognathae, Galloanserae, and Neoaves, as well as some intraordinal divergences, also are estimated to have occurred in the Cretaceous (75–90 MYA, fig. 3).

Fossil gaps were calculated from the molecular time estimates and the oldest fossil occurrence of a clade. As expected, a strong correlation ($r = 0.91$) existed between the fossil and molecular divergence times. However, substantial fossil gaps were present for the older, Mesozoic, divergences; the fossil record agrees better with Cenozoic than with Mesozoic divergences. A more modest correlation ($r = 0.52$) also existed between the fossil-based divergence times and the absolute sizes of fossil gaps. However, this correlation disappeared ($r = 0.11$) when the relative, rather than absolute, fossil gap was used (table 2). The large fossil gap for the divergence of kiwis and emus (Olson 1985) and the small gap for the basal divergence within Ciconiiformes (table 3) probably are explained by the bias for aquatic species in the fossil record.

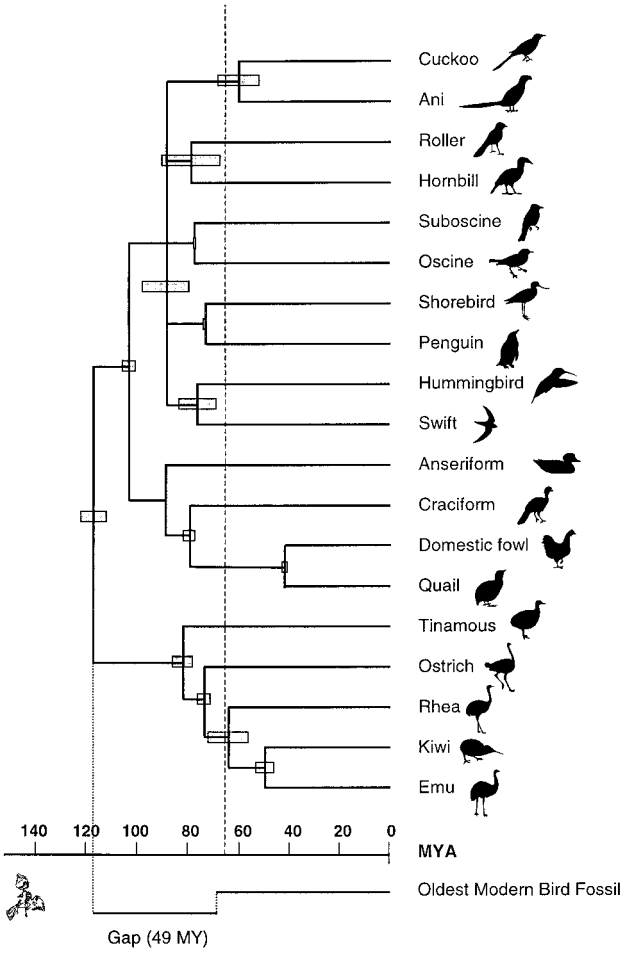

FIG. 3.—Linearized avian tree showing mean estimates and standard error bars from three molecular data sets (mtRNA, DNA-DNA hybridization, and transferrin immunological distances) based on the internal galliform-anseriform calibration. Also indicated are the estimated absolute fossil gap for the earliest neornithine divergence (below timescale), the earliest avian fossil occurrence (Archaeopteryx), and the Cretaceous-Tertiary boundary (dotted line). Data set–specific time estimates are provided in table 3.

Discussion

The divergence time estimates among major clades of birds (table 3) are in broad agreement with previous molecular-clock studies using fewer genes, different data sets, and different methods (Hedges et al. 1996; Cooper and Penny 1997; Cooper and Fortey 1998; Kumar and Hedges 1998; Rambaut and Bromham 1998; Härlid and Arnason 1999; Waddell et al. 1999). The primary point of agreement of all of these studies is that the major clades of modern birds began diverging from one another much earlier (Cretaceous) than indicated by the fossil record. A similar pattern is seen in placental mammals, where large Cretaceous gaps in the fossil record are implied by molecular time estimates (Hedges et al. 1996; Kumar and Hedges 1998; Easteal 1999). In both cases (birds and mammals), fossils of modern clades are known from the Cretaceous (Archibald 1996; Padian and Chiappe 1998; Stidham 1998; S. Hope, personal communication), but the taxonomic identity of those fossils remains in dispute (Feduccia 1996; Hope 1998; Dyke and Mayr 1999). In the future, molecular-clock studies of birds will have many additional nuclear

genes available for time estimation, and therefore divergence time estimates will be refined.

An alternative interpretation of the discordance between molecular and fossil divergence times is that molecular clocks have accelerated during rapid adaptive radiations (Benton 1999). However, it is often overlooked that most molecular-clock studies have tested for such deviations from rate constancy (e.g., in comparisons of lineages inside and outside of groups undergoing radiation). Moreover, clock studies using large numbers of genes and diverse taxa thus far have not encountered biases in species-rich versus species-poor lineages (Kumar and Hedges 1998; Hedges and Kumar 1999). In addition, the dichotomy between molecular estimates and the fossil record does not appear to be a general one, but is most pronounced for vertebrate divergences in the late Cretaceous (Kumar and Hedges 1998). An acceleration in molecular clocks during the radiation of avian and mammalian orders is not consistent with this restricted (Cretaceous) nature of the gap or with mechanisms of molecular evolution (Easteal 1999; Hedges and Kumar 1999). Finally, most knowledge on the origin and early evolution of birds has been gained only in the last 15 years, and therefore more discoveries should be expected (Chiappe 1995; Padian and Chiappe 1998). Nonetheless, the importance of this evolutionary question requires continued scrutiny of both molecular time estimates and the fossil record.

Times of divergence are of particular interest for one group of birds, the ratites. These are large, flightless birds currently distributed on the southern continents of South America (rheas), Africa (ostrich), Australia and New Guinea (emu and cassowary), and New Zealand (kiwi). Their closest relatives are the smaller, fowl-like tinamous of Central and South America. There is disagreement as to whether the current distribution and phylogeny of the ratites is the result of dispersal (Houde 1986; Feduccia 1996) or involves some continental breakup (Cracraft 1974; van Tuinen, Sibley, and Hedges 1998). Moreover, the specific timing of divergences will have a bearing on alternative scenarios within the context of continental breakup (van Tuinen, Sibley, and Hedges 1998).

Two recently proposed scenarios for the origin of ratites assume a close relationship between the South American (rhea) and Australasian (emu, cassowary, and kiwi) taxa based on molecular evidence (van Tuinen, Sibley, and Hedges 1998). Also, both scenarios suggest that the breakup of Antarctica and Australia (~65 MYA; Smith, Smith, and Funnell 1994) was the mechanism for the divergence of those two clades. The two scenarios differ in the location of the earliest ratite: Africa versus South America. One suggests that a proto-palaeognath stock was distributed on the Africa-South America supercontinent in the early to middle Cretaceous, leading to ratites (Africa) and tinamous (South America) when the two continents split 100–105 MYA. Under this scenario, there was subsequent dispersal of one African lineage back to South America via a late Cretaceous proto-Antillean land connection. Alternatively, the proto-palaeognaths may have been isolated in South America after that continent separated from Africa. Under this second scenario, the tinamou-ratite divergence took place within South America, and a subsequent dispersal of a proto-ratite northward to Laurasia, and then to Africa, led to the African ratites. The presence of fossil ratites in the Cenozoic of Laurasia (Houde 1986; Martin 1992) is compatible with both hypotheses (van Tuinen, Sibley, and Hedges 1998).

The mean divergence time estimates for palaeognaths (table 2 and fig. 1) support the second scenario. Specifically, the divergences between tinamous and ratites (83 MYA) and between ostrich and other ratites (75 MYA) are younger than predicted by the first scenario and therefore suggest that ratites arose in South America. The divergence time estimate for the rhea versus emu/kiwi (65 MYA; table 3) is also in agreement with tectonic and biogeographic models for the New World origin of Australasian taxa such as marsupials and hylid frogs (Maxson, Sarich, and Wilson 1975; Woodburne and Case 1996). The early Cenozoic divergence time (50 MYA; table 3) between kiwis and emus suggests that the ancestor of the kiwi lineage may have dispersed over water from Australia to New Zealand. However, in some of these cases, the 95% confidence intervals for the divergence times do not permit rejection of the alternative scenario (fig. 3). In the future, additional nuclear genes, particularly from nongalloanserine taxa, should help to further refine these time estimates and yield smaller confidence intervals.

Acknowledgments

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