A Mitogenomic Timescale for Birds Detects Variable Phylogenetic Rates of Molecular Evolution and Refutes the Standard Molecular Clock

Sergio L. Pereira and Allan J. Baker*†*

*Department of Natural History, Royal Ontario Museum, Toronto, Ontario, Canada; and †Department of Zoology, University of Toronto, Toronto, Ontario, Canada

Current understanding of the diversification of birds is hindered by their incomplete fossil record and uncertainty in phylogenetic relationships and phylogenetic rates of molecular evolution. Here we performed the first comprehensive analysis of mitogenomic data of 48 vertebrates, including 35 birds, to derive a Bayesian timescale for avian evolution and to estimate rates of DNA evolution. Our approach used multiple fossil time constraints scattered throughout the phylogenetic tree and accounts for uncertainties in time constraints, branch lengths, and heterogeneity of rates of DNA evolution. We estimated that the major vertebrate lineages originated in the Permian; the 95% credible intervals of our estimated ages of the origin of archosaurs (258 MYA), the amniote–amphibian split (356 MYA), and the archosaur–lizard divergence (278 MYA) bracket estimates from the fossil record. The origin of modern orders of birds was estimated to have occurred throughout the Cretaceous beginning about 139 MYA, arguing against a cataclysmic extinction of lineages at the Cretaceous/Tertiary boundary. We identified fossils that are useful as time constraints within vertebrates. Our timescale reveals that rates of molecular evolution vary across genes and among taxa through time, thereby refuting the widely used mitogenomic or cytochrome *b* molecular clock in birds. Moreover, the 5-Myr divergence time assumed between 2 genera of geese (*Branta* and *Anser*) to originally calibrate the standard mitochondrial clock rate of 0.01 substitutions per site per lineage per Myr (s/s/l/Myr) in birds was shown to be underestimated by about 9.5 Myr. Phylogenetic rates in birds vary between 0.0009 and 0.012 s/s/l/Myr, indicating that many phylogenetic splits among avian taxa also have been underestimated and need to be revised. We found no support for the hypothesis that the molecular clock in birds "ticks" according to a constant rate of substitution per unit of mass-specific metabolic energy rather than per unit of time, as recently suggested. Our analysis advances knowledge of rates of DNA evolution across birds and other vertebrates and will, therefore, aid comparative biology studies that seek to infer the origin and timing of major adaptive shifts in vertebrates.

Introduction

The observation that nucleotide changes or amino acid replacements accumulate at a roughly constant rate among related species led to the proposition of the molecular clock hypothesis (Zuckerkandl and Pauling 1962, 1965). This became one of the most important and influential hypotheses in evolutionary biology because it was a fundamental expectation of the neutral theory of evolution (Kimura 1968) and could be used to estimate divergence times among taxa and construct timescales for the evolution of life. Many phylogenetic studies of homeotherms (Klicka and Zink 1997; Aleixo 2004) made use of this hypothesis and applied a "standard mitochondrial clock rate" of DNA divergence of 2%/Myr, equivalent to a rate of molecular evolution of 0.01 substitutions per site per lineage per Myr (s/s/l/Myr). Calibration of this clock was originally obtained from mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) data from chimpanzees and humans (Brown et al. 1979) and was supported by similar rates obtained for 2 genera of geese (Shields and Wilson 1987) and other vertebrates (Wilson et al. 1985).

However, a recent review pointed out the apparently heterogeneous nature of the mtDNA clock in birds and noted that studies supporting the standard rate could not be compared directly because they were derived from different types of data (i.e., RFLP vs. DNA sequence) or excluded third-codon positions or transitions, and some accounted for among-site rate variation in DNA sequences whereas others ignored it (Lovette 2004). This problem can be addressed only with more sophisticated methods (Sanderson 1997; Thorne et al. 1998; Sanderson 2002; Thorne and Kishino 2002) that account for rate variation among sites and lineages and that are based on multiple calibration points from the fossil record or geological events. Among these methods, the Bayesian approach (Thorne et al. 1998; Thorne and Kishino 2002) is very appealing as it accounts for uncertainty in fossil ages, branch lengths, divergence times, and rates of DNA substitution or amino acid replacement and allows for changes in the rate of molecular evolution through time.

Despite methodological advances in dating methods, many recent ornithological studies have still applied the standard 2%/Myr rate without critical assessment of its validity in the groups under investigation (Cheviron et al. 2005; Eberhard and Bermingham 2005; Gill et al. 2005; Bollmer et al. 2006). A new timescale is, therefore, a high priority to derive rates of molecular evolution across birds and to advance our knowledge of the tempo and mode of avian evolution generally. Two factors currently limit the construction of such a timescale. First, there is a need for a well-supported phylogenetic hypothesis among avian orders. There is a consensus between morphological and molecular hypotheses for the basal relationships among modern birds (Neornithes), but most relationships at the ordinal level are not fully resolved (fig. 1). Briefly, it is well accepted that the Palaeognathae (tinamous and ratite birds) is a sister group to the Neognathae (all other Neornithes), and within Neognathae, Galloanserae (Galliformes and Anseriformes) is a sister clade to Neoaves (all other birds) (Cracraft et al. 2004; Slack et al. 2006).

The second limiting factor is the mostly incomplete and fragmentary fossil record of Neornithes, which makes it very difficult to place fossils phylogenetically (Brodkorb

Key words: divergence times, Aves, vertebrates, fossil, rate of DNA evolution, molecular clock.

E-mail: sergio.pereira@utoronto.ca.

Mol. Biol. Evol. 23(9):1731–1740. 2006
doi:10.1093/molbev/msl038
Advance Access publication June 14, 2006

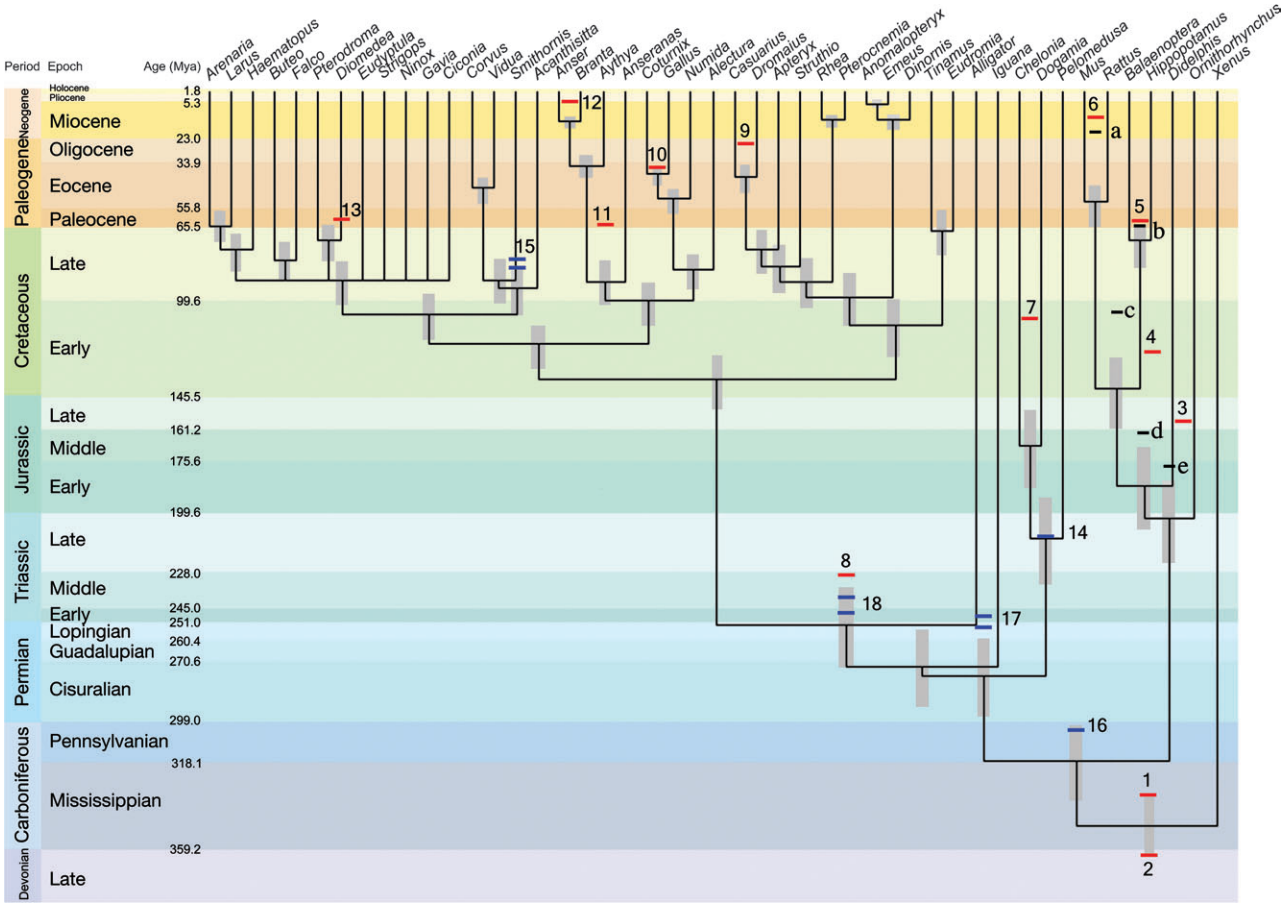

FIG. 1.—Timescale for avian and nonavian vertebrates. The topology is based on current knowledge of avian and vertebrate relationships. Gray vertical bars correspond to 95% CrI of the Bayesian posterior distribution of molecular time estimates. Red horizontal bars numbered 1–13 are time constraints used to derive the timescale. Blue horizontal bars numbered 14–18 are other suggested suitable time constraints and were not used in our analyses. Black horizontal bars labeled "a–e" are revised average age for mammals when the *Mus–Rattus* split is constrained to lie within 12 and 21 MYA; the 95% CrI for these nodes are—a: 20.3, 21.0; b: 63.0, 66.8; c: 97.4, 113.4; d: 146.4, 173.1, and e: 163.5, 192.2. Age estimates for mammals. The geologic timescale is based on Gradstein et al. (2004).

1964; Crowe and Short 1992; Dyke 2001). This limits the use of fossils as time constraints or calibration points and hampers tests of alternative hypotheses on whether a massive extinction occurred at the Cretaceous/Tertiary (K/T) boundary, with a subsequent quick radiation of Neornithes (Feduccia 1995), or whether many modern orders originated before the K/T boundary (Hedges et al. 1996; Cooper and Penny 1997). If the avian fossil record is too limited to set time boundaries, it may be necessary to look for other possible time constraints among nonavian vertebrates. For example, the diapsid–synapsid split at approximately 310 MYA is one of the most frequently used external calibration points in molecular dating studies (Hedges et al. 1996; Kumar and Hedges 1998; Nei et al. 2001; van Tuinen and Hedges 2004; Zhang et al. 2005). However, this calibration point has been criticized recently because of a revised age for the fossil beds on which this split is based and the use of a normal or uniform distribution for fossil age uncertainty (Graur and Martin 2004; Hedges and Kumar 2004; Reisz and Mueller 2004).

Here we present a new timescale for avian evolution derived from a Bayesian approach to molecular dating that accounts for uncertainties in fossil data and in branch lengths in the higher level phylogeny and allows for change in rates of evolution through time (Thorne et al. 1998; Thorne and Kishino 2002). Specifically, we applied this method to a data set of 48 mitochondrial genome sequences of vertebrates including 33 extant and 3 extinct birds and 12 other nonavian vertebrates and used several independent time constraints suggested by the fossil record. Our goals were to approximate the Bayesian posterior distribution of molecular time estimates and rates of DNA substitution in birds and other selected vertebrates, to assess the uncertainty and variability of molecular time estimates and rates of DNA substitution at the mitogenomic, single-gene, and among-taxon levels, and to evaluate whether the standard mitochondrial clock rate of 0.01 s/s/l/Myr is a good approximation of the rate of mtDNA evolution in birds as has been claimed. Although long-term phylogenetic rates of molecular evolution have been shown recently to decline monotonically from much faster rates estimated from intraspecific mutation studies, thus leading to overestimation of divergence times if the latter are extrapolated beyond about 1–2 Myr or vice versa (Ho et al. 2005), our study

involves deeper divergences for which the slower phylogenetic rate is appropriate.

Materials and Methods

Data and Phylogenetic Hypothesis

Complete mtDNA sequences of 35 avian species and 13 nonavian vertebrates were retrieved from GenBank under accession numbers NC_000891, NC_001610, NC_005089, NC_001665, NC_001601, NC_000889, NC_001947, NC_000886, NC_002780, NC_002793, NC_001922, NC_005933, NC_000877, NC_004539, NC_007011, NC_007227, NC_006382, NC_004575, NC_001323, NC_004538, NC_002196, NC_007007, NC_005932, NC_005931, NC_007174, NC_007172, NC_003713, NC_003712, NC_007006, NC_003128, NC_000878, AY325307, NC_000879, NC_002069, NC_000880, NC_002672, NC_002779, NC_002673, NC_000846, NC_002783, NC_002785, NC_002782, NC_002778, NC_002784, NC_002781, NC_002772, NC_001573, and NC_001708. Individual genes were visually aligned in MacClade 4.0 (Maddison DR and Maddison WP 2000) and are abbreviated as follows: ATP6 and ATP8, ATP synthase F0 subunits 6 and 8; CO1–3, cytochrome *c* oxidase subunits 1, 2, and 3; cyt *b*, cytochrome *b*; ND1–6, reduced form of nicotinamide adenine dinucleotide reductase subunits 1–6; 12S and 16S, small (12S) and large (16S) subunits of ribosomal genes; and tRNAs, transfer ribosomal genes. The 22 tRNAs were concatenated in one single data set and analyzed together because individual tRNA sequences are too short to provide reliable branch length estimates at this taxonomic depth. The mitochondrial control region was not included because the alignment of those sequences is virtually impossible due to a large number of highly variable sites, insertions, and deletions. The tree depicted in figure 1 summarizes the phylogenetic relationships among vertebrates (Cracraft and Donoghue 2004; Slack et al. 2006) and was used as our phylogenetic hypothesis.

Bayesian Approximation of Molecular Time Estimates and Phylogenetic Rates of Molecular Evolution

We used the MULTIDISTRIBUTE package (Thorne and Kishino 2002) (available from J. Thorne, North Carolina State University) to integrate uncertainty in branch length estimates from each gene and approximate the posterior distribution of molecular time estimates and rates of molecular evolution. The African lungfish *Protopterus dolloi* was used as an outgroup to root the tree as required by the program. Gamma priors were set as follows: expected time between the tip and the ingroup root (rttime) = 340 MYA (Paton et al. 1999; Ruta and Coates 2004), with standard deviation (SD) = 50 MYA; rate of the root node (rtrate) and its SD = 1.629 substitution per site per 100 Myr determined as the median of all the tip-to-root branch lengths divided by rttime; and rate of change between ancestral and descendant nodes (brownmean) = 0.294. Because a priori information for rtrate and brownmean are largely unknown, the SD was set as the same values to allow a gene to have a priori a large variation in rate at the node and rate change over time (Thorne and Kishino 2002). We also assumed a priori that genes have a different tendency to change rates, which will lead to posterior estimates of rates of evolution that are less biased toward an unrealistic prior distribution for autocorrelation of rate change among genes. We also applied the Bayesian method to each individual mitochondrial gene to evaluate variability in age estimates and uncertainty for each gene. In this case, rtrate and the SD (in substitutions per site per 100 Myr) were set as 2.634 (for ATP6), 4.865 (ATP8), 1.622 (CO1), 3.066 (CO2), 1.466 (CO3), 1.011 (cyt *b*), 1.692 (ND1), 1.377 (ND2), 2.153 (ND3), 1.405 (ND4), 2.015 (ND4L), 0.906 (ND5), 2.384 (ND6), 1.626 (12S), 1.099 (16S), and 0.568 (tRNAs).

To approximate an overall rate of molecular evolution for the complete mtDNA sequence excluding the control region, all gene alignments were concatenated, and the Bayesian method was applied. In this case, only the posterior distribution of rates of DNA substitution is of interest, and therefore, the ages of all nodes were fixed as the posterior mean estimate obtained from the mitogenomic analysis, in which branch length uncertainties were accounted separately for each gene. Unfortunately, this approach has a poorer model fit because the parameters of the available substitution model (Hasegawa-Kishino-Yano with within-site heterogeneity) and rate of evolution at the root of the tree are highly variable across genes. The rtrate and its SD for the concatenated data set were set to 1.038 substitutions per site per unit time. In all Bayesian analyses, the parameters for the Monte Carlo Markov Chain (MCMC) were set as follows: burn-in period = 2,000, sample frequency = 200, and number of samples = 10,000. We ran all analyses at least twice, each one starting with a different randomly selected initial state. Convergence of the MCMC runs was checked by comparing the posterior distribution of molecular time estimates and rates of molecular evolution between replicates of the same run, by comparing the proportion of successful changes of those parameters in each run. If the first 3 figures of these parameters were very similar or identical in different runs, convergence has been achieved.

Time Constraints

We used the fossil record to provide minimum time constraints at several points in our phylogenetic hypothesis (numbers as in fig. 1). 1) Origin of Amniotes at 340 Myr and 2) a maximum of 370 MYA (Paton et al. 1999; Benton 2000; Ruta and Coates 2004); 3) origin of Monotremata at 160 MYA (Benton 1993); 4) origin of Metatheria and Eutheria at 125 MYA (Luo et al. 2003); 5) *Balaenoptera–Hippopotamus* split at 63 MYA (Gingerich and Uhen 1998); 6) *Mus–Rattus* split at 12 MYA (Jacobs and Downs 1994); 7) crown Cryptodira at 110 MYA (Meylan et al. 2000); 8) Aves–Crocodylia split at 235 MYA (Benton 1993); 9) *Casuarius–Dromaius* split at 25 MYA (Boles 1992); 10) *Coturnix–Gallus* split at 38 MYA (Brodkorb 1964); 11) *Anseranas* divergence from other Anseriformes at 65 MYA (Clarke et al. 2005); 12) *Anser–Branta* split at 4.5 MYA (Bickart 1990); and 13) Neoaves radiation, excluding Passeriformes, at 62 MYA (Slack et al. 2006).

Additionally, the utility of the following commonly suggested constraints were tested by mapping them on

Table 1
Bayesian Posterior Estimates of Divergence Times

	13 Time Constraints			4 Time Constraints		
Divergence	Node Age	SD	95% CrI	Node Age	SD	95% CrI
Tinamus–Eudromia	68.2	8.2	57.9, 79.6	68.2	8.2	57.9, 79.6
Dromaius–Casuarius	41.5	6.7	34.4, 49.3	41.5	6.7	34.4, 49.3
Apteryx–Dromaius, *Casuarius*	76.8	8.7	66.0, 88.3	76.8	8.7	66.0, 88.3
Struthio–Apteryx, *Dromaius*, *Casuarius*	84.9	8.8	73.6, 97.0	84.9	8.8	73.6, 97.0
Pterocnemia–Rhea	13.9	4.5	11.0, 17.0	13.9	4.5	11.0, 17.0
Rheas–other ratites	92.2	9.0	80.5, 104.7	92.2	9.0	80.5, 104.7
Anomalopteryx–Emeus	6.5	4.1	4.8, 8.1	6.5	4.1	4.8, 8.1
Dinornis–other moas	14.2	5.7	11.0, 17.5	14.2	5.7	11.0, 17.5
Moas–other ratites	99.7	9.1	87.5, 112.7	99.7	9.1	87.5, 112.7
Tinamous–ratites	113.6	9.3	100.5, 127.4	113.6	9.3	100.5, 127.4
Corvus–Vidua	46.7	5.9	39.8, 54.2	46.7	5.9	39.8, 54.2
Oscines–Suboscines	91.8	8.0	81.4, 103.2	91.8	8.0	81.4, 103.2
Acanthisitta–other Passeriformes	95.3	8.0	84.8, 106.9	95.3	8.0	84.8, 106.9
Buteo–Falco	81.8	7.7	72.1, 92.4	81.8	7.7	72.1, 92.4
Arenaria–Larus	65.2	7.0	57.1, 74.1	65.2	7.0	57.1, 74.1
Haematopus–Arenaria, *Larus*	76.7	7.3	67.9, 86.3	76.7	7.3	67.9, 86.3
Pterodroma–Diomedea	72.6	7.2	63.7, 82.1	72.6	7.2	63.7, 82.1
Non-Passeriformes Neoaves radiation	91.9	7.8	82.1, 102.6	91.9	7.8	82.1, 102.6
Passeriformes–other Neoaves	108.0	8.3	97.0, 120.2	108.0	8.3	97.0, 120.2
Gallus–Coturnix	40.4	4.1	38.1, 44.9	40.4	4.1	38.1, 44.9
Numida–Gallus, *Coturnix*	52.4	5.1	47.5, 58.6	52.4	5.1	47.5, 58.6
Alectura–other Galliformes	86.6	7.4	77.9, 96.3	86.6	7.4	77.9, 96.3
Anser–Branta	14.5	2.7	11.7, 17.8	14.6	2.7	11.7, 17.9
Aythya–Anser, *Branta*	36.0	4.5	30.3, 42.4	36.0	4.5	30.3, 42.4
Anseranas–other Anseriformes	92.1	7.8	82.4, 102.8	92.1	7.8	82.4, 102.8
Galliformes–Anseriformes	101.7	8.0	91.7, 112.8	101.7	8.0	91.7, 112.8
Galloanserae–Neoaves	122.2	8.6	110.4, 135.2	122.2	8.6	110.4, 135.2
Palaeognathae–Neognathae	139.2	9.0	126.0, 153.6	139.2	9.0	126.0, 153.6
Aves–Crocodylia	257.9	10.6	238.0, 278.7	257.9	10.6	238.0, 278.7
Iguana–Archosauria	277.9	10.3	258.0, 298.2	277.9	10.3	258.0, 298.2
Crown Cryptodira	171.7	11.2	151.8, 192.3	171.7	11.2	151.8, 192.3
Pleurodira–Cryptodira	216.5	11.5	195.6, 238.9	216.5	11.5	195.6, 238.9
Testudines–other reptiles including Aves	282.4	10.3	262.8, 302.6	282.4	10.3	262.8, 302.6
Balaenoptera–Hippopotamus	72.2	7.9	63.5, 85.7	72.2	7.9	63.5, 85.7
Mus–Rattus	53.6	7.4	43.7, 65.4	53.6	7.4	43.7, 65.4
Rodentia–Cetartiodactila	144.1	10.1	127.6, 163.2	144.1	10.1	127.6, 163.2
Eutheria–Metatheria	190.9	10.8	171.6, 212.0	190.9	10.8	171.6, 212.0
Monotremata–other mammals	206.8	10.9	187.3, 228.1	206.8	10.9	187.3, 228.1
Diapsids–Synapsids	323.6	9.8	305.1, 342.5	323.6	9.8	305.1, 342.5
Amniotes–Amphibia	355.7	8.4	341.2, 369.2	355.7	8.4	341.2, 369.2

NOTE.—Node age, SD, and 95% CrI are given in million years for the analyses using 13 and the 4 best-fitting time constraints.

the timescale obtained from the corresponding nodes in our analyses: 14) Cryptodira–Pleurodira turtle split at around 210 MYA (Gaffney 1990); 15) the drift of New Zealand from Gondwanaland at 82–85 MYA (*Acanthisitta* vs. other Passeriformes) (Ericson et al. 2002; Barker et al. 2004); 16) the diapsid–synapsid split at 310 MYA (Benton 1993); 17) the bird–lizard split between 252 and 257 MYA (Reisz and Mueller 2004), and 18) the bird–crocodile split between 243 and 251 MYA (Muller and Reisz 2005).

Results and Discussion

A Mitogenomic Molecular Timescale and Key Divergence Times among Vertebrates

The times of divergence of major clades of reptiles (turtles, lizards, birds, and crocodilians) were estimated to have occurred in the Permian (Gradstein et al. 2004) according to the posterior distribution of molecular time estimates (table 1 and fig. 1). These divergence times are compatible with published estimates obtained from Bayesian analysis using different priors for substitution parameters and time constraints (Blair and Hedges 2005; Zhang et al. 2005). For example, our estimate of the divergence of Amniotes and Amphibia at around 355 MYA (95% credible interval [CrI] 341, 369 MYA) is very close to the 354-MYA (95% CrI 341, 367 MYA) estimate derived from mitogenomic data of amphibians and other vertebrates (Zhang et al. 2005). Additionally, the 323 MYA (95% CrI 305, 342 MYA) we estimated for the diapsid–synapsid split is very close to that of 326 MYA (95% CrI 311, 314 MYA) obtained from 325 nuclear-encoded protein sequences, assuming the same minimum and maximum time constraints for the Amniote–Amphibia split (Blair and Hedges 2005). These concordances in divergence times using mitogenomic and nuclear DNA show that saturation of DNA substitutions at this taxonomic depth does not seem to negatively impact the estimates of divergence times at deeper nodes and add to the evidence that the Bayesian method is normally very robust to variation in age priors (Aris-Brosou and Yang 2002; Thorne and Kishino 2005).

On the contrary, we estimated that the diapsid–synapsid split likely occurred in the Later Mississippian (fig. 1) of the Carboniferous period rather than in the Early Pennsylvanian, as suggested by the revised geological age of 313–316 MYA for the oldest fossils of both lineages (Menning et al. 2000). Moreover, the Later Mississippian split is in agreement with a recent paleontological analysis that suggested that even the 313–316 MYA age for the fossil beds in Joggins, Nova Scotia, is an underestimate of the divergence time between diapsids and synapsids, as at least 4 ghost lineages of amniotes are older than the age of Joggins (Reisz and Mueller 2004). The earlier timing of this split contrasts with a previous vertebrate molecular timescale (Kumar and Hedges 1998; Hedges and Kumar 2003) that was based on earlier interpretations of the fossil record (Benton 1993).

Our Bayesian estimate for the *Mus–Rattus* split at around 44 and 65 Myr is much older than the 12–14 Myr suggested by the fossil record and accepted by most mammalogists (e.g., Springer et al. 2003). We, therefore, performed two further rounds of Bayesian dating analyses, imposing a maximum divergence time between *Mus* and *Rattus* at 21 Myr, which is the upper limit of the 95% CrI of the Bayesian posterior distribution obtained in studies with more adequate taxon sampling (Springer et al. 2003; Delsuc et al. 2004). Our analyses returned estimates of divergence time that are on average 30 Myr younger for mammals compared with the analysis not using this constraint (see caption of fig. 1). However, the addition of this maximum constraint does not seem to have any appreciable effect on the ages of avian nodes (not shown) as age estimates were on average only 2.7 Myr younger than the analysis without it. Additionally, the 95% CrI of the avian age estimates from the two approaches largely overlapped. Estimation of the age of the *Mus–Rattus* split is problematical using molecular data because of the much higher rate of evolution in these 2 taxa relative to other mammals, and thus, a maximum constraint is required to reasonably model rate variation among mammals in our limited set of taxa (Bromham et al. 1999; Douzery et al. 2003; Blair et al. 2005). This problem, which is beyond the scope of our study, has been further explored elsewhere with adequate taxon sampling and time constraints (Springer et al. 2003).

Cretaceous Origin for Modern Birds

Our estimates of divergence times suggest that birds started to radiate into modern lineages in the Early Cretaceous at around 139 MYA (upper 95% CrI extending back to the Late Jurassic), well before the K/T boundary (table 1 and fig. 1). Cretaceous diversification has been suggested previously for birds and mammals based on DNA or amino acid sequences from the nuclear and mitochondrial genomes (Hedges et al. 1996; Cooper and Penny 1997; Kumar and Hedges 1998). These estimates are in agreement with the fossil record, which detects the presence of derived Anseriformes in the Cretaceous, and therefore suggest a Cretaceous age for Galliformes, ratites, tinamous (Clarke et al. 2005), and possibly other modern avian orders (Dyke 2001) and argue against a cataclysmic extinction of lineages at the K/T boundary.

Vicariance Biogeography of Palaeognathae

Analyses of complete mitochondrial genomes of Palaeognath birds suggested that their current distribution can be explained mainly by vicariance, with the breakup of Gondwanaland, with 2 exceptions: *Struthio* may have dispersed from Australia–Antarctica to Indo-Madagascar by a connection through the Kerguelen plateau and later reached Eurasia and Africa and *Apteryx* reached New Zealand through land connections along the Norfolk Ridge and Lord Howe Rise (Cooper et al. 2001; Haddrath and Baker 2001). Our molecular time estimates (table 1) do not reject a fully vicariant hypothesis of ratite evolution. The radiation of ratites began in the Late Cretaceous with the separation of New Zealand moa and extended throughout the Late Cretaceous and most of the Cenozoic (fig. 1). The molecular time estimates we obtained for *Struthio* (85 MYA) and *Apteryx* (77 MYA) are younger than the separation of Africa from South America around 130–90 MYA (Salgado-Labouriau 1994) and New Zealand from Antarctica around 82–85 MYA (Cooper and Millener 1993), respectively. However, the 95% CrI includes a time span from 74 to 97 MYA and 66 to 88 MYA for the origin of the *Struthio* and the *Apteryx* lineages, respectively (table 1). These time intervals support suggestions that land vertebrates may have moved between South America and Africa until as recently as 80–70 MYA (Sibley and Ahlquist 1990) and between New Zealand and Australia until 80–75 MYA (Cooper and Millener 1993).

Anseriformes–Galliformes Split

The split between Galliformes and Anseriformes was estimated at 89.9 $\pm$ 6.97 MYA, based on 12 nuclear genes that were evolving at a constant rate, and was mooted as a major anchor point for dating other divergences in birds (van Tuinen and Hedges 2001). However, this divergence time was based on an external calibration point for the diapsid–synapsid split at 310 MYA, which is now known to be an underestimate. We estimated that the ordinal split in the Galloanserae occurred earlier around 101 MYA (95% CrI 92, 112 MYA), consistent with a separate Bayesian analysis of partial sequences of 3 mitochondrial genes (Pereira and Baker 2006).

Basal Passeriformes Split

The endemic biota of New Zealand is thought to have evolved in isolation from the rest of the world when New Zealand separated from Antarctica about 82–85 MYA (Cooper and Millener 1993). This geological rifting event has been used to calibrate molecular clocks of Passeriformes because the New Zealand wrens are sister to all other Passeriformes (Ericson et al. 2002; Barker et al. 2004). We estimated that the endemic New Zealand *Acanthisitta* split from other Passeriformes in the Late Cretaceous (fig. 1) about 10 Myr before the maximum limit of 85 MYA for the separation of New Zealand from Antarctica. However, the lower 95% CrI includes the maximum geological age for this event (85, 107 Mya). We conclude that basal Passeriformes, like the endemic moa, may have

diverged from their respective sister groups during or prior to the separation of New Zealand from Antarctica.

Variation among Time Estimates Obtained from Single Mitochondrial Genes

Molecular time estimates above 250 MYA obtained with single genes were similar to those using mitogenomic sequences (fig. 2*A*), with ratios of single-gene to mitogenomic estimates in the range of 1.0–1.1. At the phylogenetic depth represented by these divergence times, single genes have accumulated enough DNA substitutions to be informative, and therefore, this concordance is not surprising. Conversely, for divergences less than 250 MYA, single genes provided older molecular time estimates than the mitogenomic data (fig. 2*A*). In general, CO1, ND1, ND2, and the concatenated set of tRNAs seem to provide the most similar molecular time estimates for most nodes compared with the mitogenomic estimates.

As expected (Thorne and Kishino 2005; Pereira and Baker 2006), the uncertainty in single-gene estimates, as measured by the ratios of the size of the 95% CrI of the single-gene to mitogenomic estimates, increased by up to 18.6 times (fig. 2*B*). This happens because shorter sequences and/or lower rates of molecular evolution introduce more stochastic errors in molecular time estimates and because the divergence times, which are the shared parameters across genes in a multigene analysis, are not internally constrained by the model when using single genes (Yang and Yoder 2003). In other words, integrating multiple gene sequences reduces the variance of molecular time estimates that is due to branch length uncertainties but does not reduce the uncertainty of molecular time estimates that are due to variable rates of molecular evolution (Thorne and Kishino 2005). Therefore, we strongly recommend the use of multiple genes to derive estimates of divergence time in birds and other organisms.

Fit of Fossil Age to the Bayesian Posterior Distribution of Molecular Time Estimates

The Bayesian dating approach uses fossil data as minimum or maximum time constraints, therefore allowing us to evaluate the fit of fossil ages to the posterior distribution of molecular time estimates. Mapping the time constraints on the timescale indicates a close approximation between the posterior distribution of molecular time estimates and the fossil age for Cetartiodactila (*Balaenoptera–Hippopotamus*) (Gingerich and Uhen 1998), the divergence of *Gallus* and *Coturnix* (Brodkorb 1964; Pereira and Baker 2006), and the Amphibia–Amniote split (Paton et al. 1999; Ruta and Coates 2004). However, the majority of the fossils used as constraints were younger than the estimated age of the corresponding node and not included within the respective lower 95% CrI (fig. 1). This result is not unexpected as the origin of a clade might not be recognized promptly in the fossil record (Benton and Ayala 2003), and major morphological changes do not necessarily co-occur with molecular changes.

We also evaluated the differences between age estimates obtained in the analysis using 13 time constraints and one that used only those time constraints that best fitted within our estimates of divergence time (time constrains 1, 2, 5, and 10 in fig. 1). The later analysis was performed in the MULTIDISTRIBUTE package with the exact conditions described for the analysis with 13 time constraints. The posterior mean age obtained from the analysis using only the 4 "best-fitting" constraints differed no more than 0.2 Myr compared with the posterior mean age of the analysis using 13 time constraints; similarly, the CrI from both analyses differed on average by about 0.4 Myr (range −1.9 to 0.8 Myr) (table 1). These results point to the robustness of Bayesian dating methods with this data set and show that time constraints falling outside the 95% CrI of the posterior mean age produce no bias in age estimates and that most of the information on the posterior mean node age is extracted from the DNA sequence data.

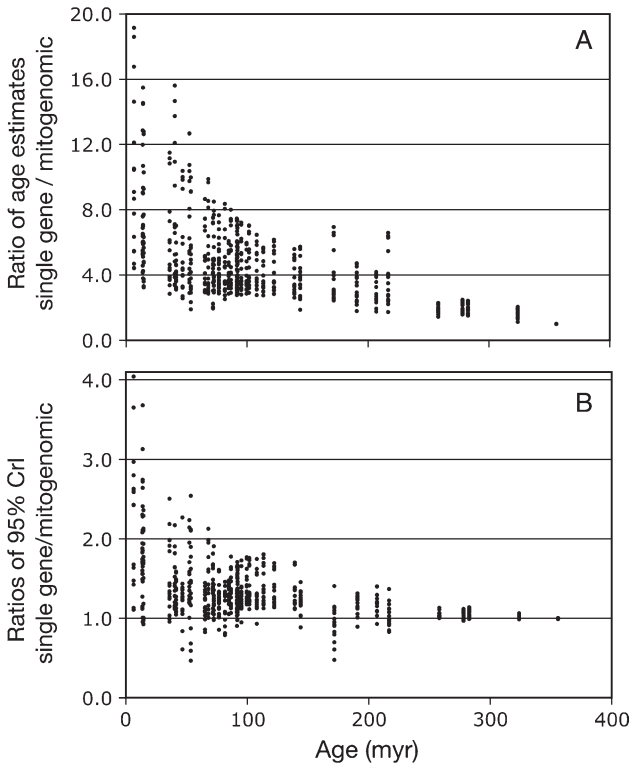

FIG. 2.—Variability in molecular time estimates for all nodes across genes. (*A*) Ratio of single-gene to mitogenomic age estimates. (*B*) Ratio of the size of the 95% CrI of the single-gene to mitogenomic estimates.

The most remarkable concordance between a time constraint not used in our analyses and the estimate of the mean of the posterior distribution in our timescale (fig. 1) was the radiation of crown Testudines (Gaffney 1990; Near 2005). Additionally, the maximum geological constraints for the separation of New Zealand from Antarctica at 85 MYA (Cooper and Millener 1993), the bird–crocodile split between 251 and 243 MYA (Muller and Reisz 2005), and the diapsid–synapsid split at 310 MYA, which were not used as time constraints in our study, fell within or close to the lower limit of the 95% CrI of the posterior distribution of molecular time estimates (fig. 1). Our estimate of the origin of archosaurs (birds, dinosaurs, and crocodiles) at 258 MYA (95% CrI 240, 278) not only neatly brackets

their Triassic diversification as preserved in the fossil record but also accommodates the oldest known fossil of dinosaurs about 230 MYA.

We have identified some time constraints that can be used to derive estimates of divergence times across a range of vertebrates (fig. 1). However, we discourage the use of a fossil as a single, fixed calibration point because in doing so one aggravates the problem of estimating divergence times if the constraint does not lie close enough to the divergence time between taxa or the phylogenetic placement of the fossil is incorrect.

Phylogenetic Rates of Molecular Evolution and the Use of the Standard Mitochondrial Molecular Clock in Birds

On the assumption that *Anser* and *Branta* diverged about 5 MYA (Bickart 1990), RFLP sequence divergences of 7.8% and 9.9% were translated into phylogenetic rates of molecular evolution between 0.0078 and 0.0099 s/s/l/Myr (Shields and Wilson 1987), similar to the rate of 0.01 s/s/l/Myr estimated in mammals (Brown et al. 1979). Using this same fossil calibration, mitochondrial cyt *b* sequences were subsequently estimated to have a phylogenetic rate of molecular evolution of 0.0105 s/s/l/Myr (Paxinos et al. 2002). This rate became known as the standard mitochondrial clock rate and has been used extensively to estimate divergence times among birds (Lovette 2004). Our estimates, however, do not support the generality of this rate. There is considerable variation and uncertainty in phylogenetic rates of molecular evolution in the mitochondrial genome through time in the vertebrates generally and in birds in particular (fig. 3; Supplementary Material online). The phylogenetic rates of molecular evolution for the mitochondrial genome at terminal and internal nodes vary between 0.0009 and 0.012 s/s/l/Myr; in several cases, the standard rate was not included in the 95% CrI of the estimates (fig. 3). The same pattern was observed for all mitochondrial genes (see Supplementary Material online). Moreover, the estimated uncertainties in rates of some genes at many nodes do not overlap (e.g., CO1 and tRNAs in fig. 3). Our estimates agree with phylogenetic rates we reported earlier for mitochondrial protein-coding genes of ratites (Cooper et al. 2001), the control region, cyt *b* and ND2 of cracid birds (Pereira et al. 2004), and the control region of extinct moa (Baker et al. 2005). This implies that many published studies of avian evolution may have substantially underestimated divergence times by applying the standard mitochondrial clock.

Furthermore, there are several shortcomings with the use of the *Anser–Branta* fossil calibration, which are usually overlooked in studies of molecular dating in birds. First, the resulting rate of molecular evolution was estimated without correction for among-site rate variation (Shields and Wilson 1987). Even among closely related species, in which DNA substitutions have not reached saturation, rate variation among sites can be extensive for mitochondrial sequences, which can severely underestimate the number of substitutions (Golding 1983). Second, the claimed similarity of phylogenetic rates of molecular evolution found in vertebrates (Wilson et al. 1985) is inconsistent with the suggestions that avian mtDNA evolves slower

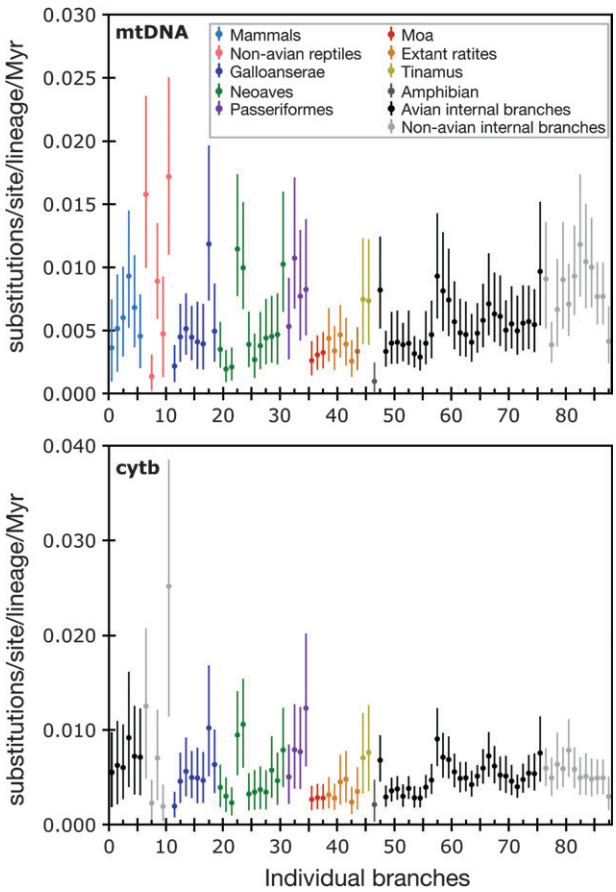

FIG. 3.—Variability of phylogenetic rates of mitochondrial molecular evolution. Dots and bars are the mean phylogenetic rate of evolution in substitutions per site per lineage per Myr and its 95% CrI, respectively, for the mitochondrial genome and cyt *b*. Branches are plotted on the *x* axis and colored by taxonomic groups indicated in the inset.

than in mammals and faster than nonavian reptile and fish mtDNA (Kessler and Avise 1985; Mindell et al. 1996). Finally, the suitability of the *Anser–Branta* calibration point (Bickart 1990) used to estimate the avian mitochondrial clock rate (Shields and Wilson 1987) has never been questioned or independently confirmed. Moreover, a recent study has suggested that the molecular clock "ticks" at a constant rate of substitution per unit of mass-specific metabolic energy rather than per unit of time, and therefore, body size and temperature should be taken into account (Gillooly et al. 2005). Avian taxon sampling was limited in that study, and when we applied the mass-specific correction to the substitution rates derived from our mitogenomic data, there was no significant regression between body mass and the rate of DNA evolution ($r^2 = 0.0335$).

In our study, we were able to revisit the *Anser–Branta* fossil calibration, and our Bayesian inference for the split of these genera (table 1) was approximately 9.5 Myr older (14.5 MYA; 95% CrI 11.7, 17.8 MYA) than that suggested by the fossil record (Shields and Wilson 1987; Bickart 1990). Consequently, our estimated mean age for *Anser* and *Branta* translates into a revised phylogenetic rate of molecular evolution for *Anser* and *Branta* between 0.0027 and 0.0034 s/s/l/Myr for the RLFP data (Shields

and Wilson 1987) and 0.0032 s/s/l/Myr for cyt *b* sequences (Paxinos et al. 2002). These revised rates are in agreement with the Bayesian posterior distribution we obtained from mitogenomic sequences using a range of different time constraints. We estimated that the mitochondrial genomes of *Anser* and *Branta* (excluding the control region) are evolving at phylogenetic rates of 0.0051 s/s/l/Myr (95% CrI 0.0031, 0.078) and 0.0044 s/s/l/Myr (95% CrI 0.0027, 0.0068), respectively. For cyt *b* sequences, our estimates were 0.0056 s/s/l/Myr (0.0031, 0.0091) for *Anser* and 0.0049 s/s/l/Myr (0.0028, 0.0076) for *Branta*. We conclude that the use of the standard molecular clock of 0.01 s/s/l/Myr for mtDNA is untenable in birds and that future studies need to account for the variation in the rate of molecular evolution among lineages and among sites in DNA sequences as well as uncertainty in fossil ages. This clearly can be achieved in a Bayesian framework where these sources of uncertainty can be integrated in the posterior distributions of these evolutionary parameters.

Our analysis advances knowledge of rates of DNA evolution across birds and other vertebrates and will, therefore, have a significant impact on inferences about the tempo and mode of evolution in these organisms. However, we do not recommend the extrapolation of divergence times estimated here as calibration point or time constraints in intraspecific studies. The Bayesian method was devised to estimate divergence times from interspecific sequence data for which it is biologically plausible to assume that 2 lineages have independent rates of evolution after splitting from a common ancestor (Kishino et al. 2001). The rate of evolution within populations is more likely to be influenced by factors such as population size, genetic drift, and natural selection (Kishino et al. 2001; Ho et al. 2005), and it is still unclear how these factors would affect the method. We anticipate that in the future a method will be developed to account for differences in the evolutionary dynamics of interspecific and intraspecific sequence data, allowing a combined analysis of both types of data in a Bayesian framework to approximate the posterior distribution of divergence times and rates of evolution above, at, and below the species level.

Supplementary Material

Supplementary materials are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We are very grateful to Dr Jeff Thorne for the helpful discussion on the Bayesian dating method and 2 anonymous reviewers for their helpful comments. This work was supported by grants to A.J.B. from the National Science and Engineering Research Council of Canada and the Royal Ontario Museum Foundation.

Literature Cited

Aleixo A. 2004. Historical diversification of a terra-firme forest bird superspecies: a phylogeographic perspective on the role of different hypotheses of Amazonian diversification. Evolution 58:1303–17.

Aris-Brosou S, Yang Z. 2002. Effects of models of rate evolution on estimation of divergence dates with special reference to the metazoan 18S ribosomal RNA phylogeny. Syst Biol 51: 703–14.

Baker AJ, Huynen LJ, Haddrath O, Millar CD, Lambert DM. 2005. Reconstructing the tempo and mode of evolution in an extinct clade of birds with ancient DNA: the giant moas of New Zealand. Proc Natl Acad Sci USA 102:8257–62.

Barker FK, Cibois A, Schikler P, Feinstein J, Cracraft J. 2004. Phylogeny and diversification of the largest avian radiation. Proc Natl Acad Sci USA 101:11040–5.

Benton MJ. 1993. The fossil record 2. London: Chapman and Hall.

Benton MJ. 2000. Vertebrate paleontology. New York: Chapman and Hall.

Benton MJ, Ayala FJ. 2003. Dating the tree of life. Science 300:1698–700.

Bickart KJ. 1990. The birds of the Late Miocene-Early Pliocene big sandy formation, Mohave County, Arizona. Ornithol Monogr 44:1–72.

Blair JE, Hedges SB. 2005. Molecular phylogeny and divergence times of deuterostome animals. Mol Biol Evol 22:2275–84.

Blair JE, Shah P, Hedges SB. 2005. Evolutionary sequence analysis of complete eukaryote genomes. BMC Bioinformatics 6:53.

Boles WE. 1992. Revision of *Dromaius gidju* Patterson and Rich 1987 from Rivesleigh, northwestern Queensland, Australia, with a reassessment of its generic position. Nat Hist Mus Los Angel Cty Sci Ser 36:195–208.

Bollmer JL, Kimball RT, Whiteman NK, Sarasola JH, Parker PG. 2006. Phylogeography of the Galapagos hawk (*Buteo galapagoensis*): a recent arrival to the Galapagos Islands. Mol Phylogenet Evol 39:237–47.

Brodkorb P. 1964. Catalogue of fossil birds, part 2 (Anseriformes through Galliformes). Bull Fla State Mus Biol Sci 8:195–335.

Bromham L, Phyllips MJ, Penny D. 1999. Growing up with dinosaurs: molecular dates and the mammalian radiation. Trends Ecol Evol 14:113–8.

Brown WM, George M, Wilson AC. 1979. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76:1967–71.

Cheviron ZA, Hackett SJ, Capparella AP. 2005. Complex evolutionary history of a Neotropical lowland forest bird (*Lepidothrix coronata*) and its implications for historical hypotheses of the origin of Neotropical avian diversity. Mol Phylogenet Evol 36:338–57.

Clarke JA, Tambussi CP, Noriega JI, Erickson GM, Ketcham RA. 2005. Definitive fossil evidence for the extant avian radiation in the Cretaceous. Nature 433:305–8.

Cooper A, Lalueza-Fox C, Anderson S, Rambaut A, Austin J, Ward R. 2001. Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. Nature 409:704–7.

Cooper A, Penny D. 1997. Mass survival of birds across the Cretaceous-Tertiary boundary: molecular evidence. Science 275:1109–13.

Cooper RA, Millener P. 1993. The New Zealand biota: historical background and new research. Trends Ecol Evol 8:429–33.

Cracraft J, Barker FK, Braun M, et al. (14 co-authors). 2004. Phylogenetic relationships among modern birds (Neornithes): towards an avian tree of life. In: Cracraft J, Donoghue M, editors. Assembling the tree of life. New York: Oxford University Press. p 468–89.

Cracraft J, Donoghue M. 2004. Assembling the tree of life. New York: Oxford University Press.

Crowe TM, Short LL. 1992. A new gallinaceous bird from the Oligocene of Nebraska, with comments on the phylogenetic

position of the Gallinuloididae. Nat Hist Mus Los Angel Cty Sci Ser 36:179–85.
Delsuc F, Vizcaíno SF, Douzery EJP. 2004. Influence of tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within xenarthrans. BMC Evol Biol 4:11.
Douzery EJP, Delsuc F, Stanhope MJ, Huchon D. 2003. Local molecular clocks in three nuclear genes: divergence times for rodents and other mammals and incompatibility among fossil calibrations. J Mol Evol 57:S201–13.
Dyke GJ. 2001. The evolutionary radiation of modern birds: systematics and patterns of diversification. Geol J 36:305–15.
Eberhard JR, Bermingham E. 2005. Phylogeny and comparative biogeography of *Pionopsitta* parrots and *Pteroglossus* toucans. Mol Phylogenet Evol 36:288–304.
Ericson PG, Christidis L, Cooper A, Irestedt M, Jackson J, Johansson US, Norman JA. 2002. A Gondwanan origin of passerine birds supported by DNA sequences of the endemic New Zealand wrens. Proc Biol Sci 269:235–41.
Feduccia A. 1995. Explosive evolution in tertiary birds and mammals. Science 267:637–8.
Gaffney ES. 1990. The comparative osteology of the Triassic turtle *Proganochelys*. Bull Am Mus Nat Hist 194:1–263.
Gill FB, Slikas B, Sheldon FH. 2005. Phylogeny of titmice Paridae: II. Species relationships based on sequences of the mitochondrial cytochrome-b gene. Auk 122:121–43.
Gillooly JF, Allen AP, West GB, Brown JH. 2005. The rate of DNA evolution: effects of body size and temperature on the molecular clock. Proc Natl Acad Sci USA 102:140–5.
Gingerich PD, Uhen MD. 1998. Likelihood estimation of the time of origin of Cetacea and the time of origin of Cetacea and Artiodactyla. Paleontol Electron [serial online] 1:1–47.
Golding GB. 1983. Estimates of DNA and protein sequence divergence: an examination of some assumptions. Mol Biol Evol 1:125–42.
Gradstein FM, Ogg JG, Smith AG, et al. (39 co-authors). 2004. A geologic time scale 2004. Cambridge: Cambridge University Press.
Graur D, Martin W. 2004. Reading the entrails of chickens: molecular timescales of molecular evolution and the illusion of precision. Trends Genet 20:80–6.
Haddrath O, Baker AJ. 2001. Complete mitochondrial DNA genome sequences of extinct birds: ratite phylogenetics and the vicariance biogeography hypothesis. Proc Biol Sci 268:939–45.
Hedges SB, Kumar S. 2003. Genomic clocks and evolutionary timescales. Trends Genet 19:200–6.
Hedges SB, Kumar S. 2004. Precision of molecular time estimates. Trends Genet 20:242–7.
Hedges SB, Parke PH, Sibley CG, Kumar S. 1996. Continental breakup and the ordinal diversification of birds and mammals. Nature 381:226–9.
Ho SY, Phillips MJ, Cooper A, Drummond AJ. 2005. Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. Mol Biol Evol 22:1561–8.
Jacobs LL, Downs WR. 1994. The evolution of murine rodents in Asia. In: Tomida Y, Li C-K, Setoguchi T, editors. Rodent and lagomorph families of Asian origins and diversification. Tokyo: National Science Museum Monographs. p 149–56.
Kessler LG, Avise JC. 1985. A comparative description of mitochondrial DNA differentiation in selected avian and other vertebrate genera. Mol Biol Evol 2:109–25.
Kimura M. 1968. Evolutionary rate at the molecular level. Nature 217:624–6.
Kishino H, Thorne JL, Bruno WJ. 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol Biol Evol 18:352–61.
Klicka J, Zink RM. 1997. The importance of recent ice ages in speciation: a failed paradigm. Science 277:1666–9.
Kumar S, Hedges SB. 1998. A molecular timescale for vertebrate evolution. Nature 392:917–20.
Lovette IJ. 2004. Mitochondrial dating and mixed support for the "2% rule" in birds. Auk 121:1–6.
Luo ZX, Ji Q, Wible JR, Yuan CX. 2003. An Early Cretaceous tribosphenic mammal and metatherian evolution. Science 302:1934–40.
Maddison DR, Maddison WP. 2000. MacClade 4.0. Sunderland: Sinauer Associates, Inc.
Menning W, Weyer D, Drozdzewski G, van Ameron HWJ, Wendt I. 2000. A carboniferous timescale 2000: discussion and use of geological parameters as time indicators from Central and Western Europe. Geol Jahrb A 156:3–44.
Meylan PA, Moody RTJ, Walker CA, Chapman SD. 2000. *Sandownia harrisi*, a highly derived trionychoid turtle (Testudines: Cryptodira) from the Early Cretaceous of the Isle of Wight, England. J Vertebr Paleontol 20:522–32.
Mindell DP, Knight A, Baer C, Huddleston CJ. 1996. Slow rates of molecular evolution in birds and the metabolic rate and body temperature hypothesis. Mol Biol Evol 13:422–6.
Muller J, Reisz RR. 2005. Four well-constrained calibration points from the vertebrate fossil record for molecular clock estimates. Bioessays 27:1069–75.
Near TJ. 2005. Assessing concordance of fossil calibration points in molecular clock studies: an example using turtles. Am Nat 165:137–46.
Nei M, Xu P, Glazko G. 2001. Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organism. Proc Natl Acad Sci USA 98:2497–502.
Paton RL, Smithson TR, Clack JA. 1999. An amniote-like skeleton from the Early Carboniferous of Scotland. Nature 398:508–13.
Paxinos EE, James HF, Olson SL, Sorenson MD, Jackson J, Fleischer RC. 2002. mtDNA from fossils reveals a radiation of Hawaiian geese recently derived from the Canada goose (*Branta canadensis*). Proc Natl Acad Sci USA 99:1399–404.
Pereira SL, Baker AJ. 2006. A molecular timescale for galliform birds accounting for uncertainty in time estimates and heterogeneity of rates of DNA substitutions across lineages and sites. Mol Phylogenet Evol 38:499–509.
Pereira SL, Grau ET, Wajntal A. 2004. Molecular architecture and rates of DNA substitutions of the mitochondrial control region of cracid birds. Genome 47:535–45.
Reisz RR, Mueller J. 2004. Molecular timescales and the fossil record: a paleontological perspective. Trends Genet 20: 237–41.
Ruta M, Coates MI. 2004. Bones, molecules and crown-tetrapod origins. In: Donoghue PCJ, Smith MP, editors. Telling the evolutionary time: molecular clocks and the fossil record. New York: CRC Press. p 224–62.
Salgado-Labouriau M. 1994. Historia Ecologica da Terra. Sao Paulo, Brazil: Editora Edgard Blücher Ltda.
Sanderson MJ. 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol Biol Evol 19:1218–31.
Sanderson MJ. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol Biol Evol 19:101–9.
Shields GF, Wilson AC. 1987. Calibration of mitochondrial DNA evolution in geese. J Mol Evol 24:212–7.
Sibley CG, Ahlquist JE. 1990. Phylogeny and classification of birds: a study in molecular evolution. New Haven: Yale University Press.

Slack KE, Jones CM, Ando T, Harrison GL, Fordyce RE, Arnason U, Penny D. 2006. Early penguin fossils, plus mitochondrial genomes, calibrate avian evolution. Mol Biol Evol 23: 1144–55.

Springer MS, Murphy WJ, Eizirik E, O'Brien SJ. 2003. Placental mammal diversification and the Cretaceous-Tertiary boundary. Proc Natl Acad Sci USA 100:1056–61.

Thorne JL, Kishino H. 2002. Divergence time and evolutionary rate estimation with multilocus data. Syst Biol 51:689–702.

Thorne JL, Kishino H. 2005. Estimation of divergence times from molecular sequence data. In: Nielsen R, editor. Statistical methods in molecular evolution. New York: Springer. p 233–56.

Thorne JL, Kishino H, Painter IS. 1998. Estimating the rate of evolution of the rate of molecular evolution. Mol Biol Evol 15:1647–57.

van Tuinen M, Hedges SB. 2001. Calibration of avian molecular clocks. Mol Biol Evol 18:206–13.

van Tuinen M, Hedges SB. 2004. The effect of external and internal fossil calibrations on the avian evolutionary timescale. J Paleontol 78:45–50.

Wilson AC, Cann RL, Carr S, et al. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol J Linn Soc 26:375–400.

Yang Z, Yoder AD. 2003. Comparison of likelihood and Bayesian methods for estimating divergence times using multiple gene loci and calibration points, with application to a radiation of cute-looking mouse lemur species. Syst Biol 52:705–16.

Zhang P, Zhou H, Chen YQ, Liu YF, Qu LH. 2005. Mitogenomic perspectives on the origin and phylogeny of living amphibians. Syst Biol 54:391–400.

Zuckerkandl E, Pauling L. 1962. Molecular disease, evolution, and genetic heterogeneity. In: Marsha M, Pullman B, editors. Horizons in biochemistry. New York: Academic Press. p 189–225.

Zuckerkandl E, Pauling L. 1965. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, editors. Evolving genes and proteins. New York: Academic Press. p 97–166.

Peter Lockhart, Associate Editor

Accepted June 1, 2006