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

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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 mitochondrial 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.

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

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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, Galliformes (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...
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 the Mus–Rattus split is based (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_007006, NC_003713, NC_003712, NC_000879, NC_002069, NC_003712, NC_002784, NC_003713, NC_003712, NC_007172, NC_000886, NC_000887, NC_002782, NC_002784, NC_002778, NC_002772, NC_001922, NC_005933, NC_000877, NC_004539, NC_001947, NC_000886, NC_002784, NC_003713, NC_003712, NC_007172, NC_000886, NC_000887, NC_002782, NC_002784, NC_002778, NC_002772, NC_001970, and NC_001708. Individual genes were visualized in MacClade 4.0 (Maddison DR and Maddison NC_001573, and NC_001708. Individual genes were visualized in MacClade 4.0 (Maddison DR and Maddison WP 2000) and are abbreviated as follows: ATP6 and ATP8, CO1–3, cytochrome WP 2000) and are abbreviated as follows: ATP6 and ATP8, CO1–3, cytochrome WP 2000) and are abbreviated as follows: ATP6 and ATP8, 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; 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 rate of the root node (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 Anniotes 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
the timescale obtained from the corresponding nodes in our analyses: 14) Cryptodira–Placodonta turtle split at around 210 MYA (Gaffney 1990); 15) the drift of New Zealand from Gondwanaland at 82–85 MYA (Acantholiths 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 Müller 2004), and 18) the bird–crocodile split between 243 and 251 MYA (Muller and Reisz 2005).

### Results and Discussion

**A Mitogenic 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 354–352 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 320, 342 MYA) estimated for the diapsid–synapsid split is very close to that of 326 MYA (95% CrI 305, 341 MYA) obtained from 325 nuclear-encoded protein sequences, assuming the same minimum and maximum time constraints for the Amnioite–Amphibia split (Blair and Hedges 2005). These concordances in divergence times using metagenomic 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).

| Divergence | 13 Time Constraints | | | 4 Time Constraints | | |
|---|---|---|---|---|---|
| **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 |
| **Rhea–other ratites** | 92.2 | 9.0 | 80.5, 104.7 | 92.2 | 9.0 | 80.5, 104.7 |
| **Anomolopexy–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 |

Note:—Node age, SD, and 95% CrI are given in million years for the analyses using 13 and the 4 best-fitting time constraints.
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; Delsee 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 ± 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 diversifications 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. 2A), 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. 2A). 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. 2B). 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 Cetartiodactyla (Balaenoptera–Hippopotamus) (Gingerich and Uhen 1998), the divergence of Gallus and Coturnix (Brodkorb 1964; Pereira and Baker 2006), and the Amphibia–Anniote 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.

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 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 (Gilloyl 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 mitogenetic 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 RFLP 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, 0.0078) 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 points 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/).

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Brodkorb P. 1964. Catalogue of fossil birds, part 2 (Anseriformes excluding the control region) are evolving at phylogenetic rates of 0.0051 s/s/l/Myr (95% CrI 0.0031, 0.0078) 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 points 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.

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