Four New Avian Mitochondrial Genomes Help Get to Basic Evolutionary Questions in the Late Cretaceous

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Good phylogenetic trees are required to test hypotheses about evolutionary processes. We report four new avian mitochondrial genomes, which together with an improved method of phylogenetic analysis for vertebrate mt genomes give results for three questions in avian evolution. The new mt genomes are: magpie goose (*Anseranas semipalmata*), an owl (morepork, *Ninox novaeseelandiae*); a basal passerine (rifleman, or New Zealand wren, *Acanthisitta chloris*); and a parrot (kakapo or owl-parrot, *Strigops habroptilus*). The magpie goose provides an important new calibration point for avian evolution because the well-studied *Presbyornis* fossils are on the lineage to ducks and geese, after the separation of the magpie goose. We find, as with other animal mitochondrial genomes, that RY-coding is helpful in adjusting for biases between pyrimidines and between purines. When RY-coding is used at third positions of the codon, the root occurs between paleognath and neognath birds (as expected from morphological and nuclear data). In addition, passerines form a relatively old group in Neoaves, and many modern avian lineages diverged during the Cretaceous. Although many aspects of the avian tree are stable, additional taxon sampling is required.

Good evolutionary trees are required to test hypotheses. For example, we wish to know how many lineages of birds survived from the Cretaceous to the present (Cooper and Penny 1997) in order to test models of apparent "mass extinctions" and "explosive radiations" (Feduccia 1995, 2003). A well-resolved avian tree is also required for testing biogeographic (Cracraft 2001; Ericson et al. 2002) and/or ecological hypotheses (Cooper and Penny 1997; see later).

It is almost an offense against birds that the deep mammalian evolutionary tree is virtually resolved (Waddell, Kishino, and Ota 2001; Lin et al. 2002; Springer et al. 2003) whilst there are still major uncertainties about many aspects of the avian evolutionary tree (see for example Cracraft 2001). A major uncertainty is the position of the root of the avian tree; mitochondrial (mt) data sets tend to place the root within the passerine birds (Mindell et al. 1999; Härlid, Janke, and Arnason 1999, although see Braun and Kimball 2002). In contrast, morphological and nuclear sequences tend to place the root between paleognath birds (ratites and tinamous) and all other birds (neognaths). There is also uncertainty over the time of origin of passerines (perching birds and/or song birds); Feduccia (1995, 2003) places them as a recent order of modern birds, other authors place their origin before the diversification of shore birds (Barker, Barrowclough, and Groth 2002; Ericson et al. 2002).

Part of our confidence that the higher-level mammalian tree is now quite accurate is that highly similar trees are being found using independent data sets—nuclear (for example, Springer et al. 2003) and mitochondrial (Lin et al. 2002). Agreement can be treated quantitatively; in the mammal example, a deep four-way split in the eutherian tree was defined with nuclear data sets. The probability of randomly selecting a tree with this same four-way split from mitochondrial data, given the number of taxa, was less than $\approx 10^{-14}$. The four-way split was found with mitochondrial data, confirming the high similarity of trees from the two data sets. We expect that a combination of mitochondrial and nuclear data should eventually give similar confidence in avian trees.

There is good progress toward resolving the avian tree using both nuclear and mitochondrial sequences (Sibley and Ahlquist 1990; Van Tuinen, Sibley, and Hedges 2000; Cracraft 2001; Cooper et al. 2001; Haddrath and Baker 2001; Ericson et al. 2002; Paton, Haddrath, and Baker 2002; Barker, Barrowclough, and Groth 2002; Garcia-Mareno, Sorenson, and Mindell 2003). In an unrooted avian tree, as expected from morphological data, ratites and tinamous unite to form paleognaths, and all remaining birds are neognaths (and separate into Gallianseres [chicken, geese, and relatives] and Neoaves (Cracraft 2001). The succession of divergences within Neoaves, which contains the vast majority of living birds, remains unclear. Cracraft (2001) has a six-way split between the following groups:

Passerines
Parrots
Cuckoos
Woodpeckers, rollers, bee-eaters, kingfishers, jacanas, and mousebirds (four orders)
Owls, nightjars, swifts, and turacos
Seabirds, shore birds, doves, cranes, raptors, rails, penguins, storks, loons, and grebes (a very diverse group, ~10 orders)

Despite this lack of resolution, we use the Cracraft (2001) tree as an informal prior for evaluating results. Of the six Neoaves groups, only two (passerines and the seabird/shorebird alliance) are currently represented in the complete mitochondrial set, showing the need for increased taxon sampling. The species sequenced here, together with

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some reasoning for the choices, is given below. All are Australasian taxa, which helps avoid duplication of effort (a list of taxa being sequenced by our group is available at http://awcmee.massey.ac.nz/mt_genomes.htm).

We have sequenced mt genomes of two of the four unrepresented groups namely an owl and a parrot. Parrots are a distinct and old group for which a Late Cretaceous fossil has been reported (Stidham 1998; Hope 2002); a kakapo (owl-parrot or night parrot, *Strigops habroptilus*: fam. Psittacidae) was selected for this study. Owls are another distinct avian group, and a New Zealand owl (morepork or ruru, *Ninox novaeseelandiae*: fam. Strigidae) was chosen.

The rifleman (a New Zealand wren, *Acanthisitta chloris*: fam Acanthisittidae) is a basal passerine. New Zealand wrens do not really fit the oscine/suboscine classification. Cracraft (2001) shows an unresolved three-way split between oscines (which form the large majority of passerine birds), suboscines, and New Zealand wrens. Ericson et al. (2002) reports nuclear sequences for the rifleman, analysis of which places it basal to all other passerines—oscine and suboscine. A rifleman mt genome should also help resolve the position of passerines within the avian tree, including the position of the root. In the earliest mitochondrial data sets (with only a small number of genomes) the root of the avian tree tended to fall within passerines (Mindell et al. 1997; Härlid, Janke, and Arnason 1999), rather than in the expected position between neognaths and paleognaths. Recently, and with more taxa in the data sets, it has not been possible to reject the classical (neognath/paleognath) rooting (Paton, Haddrath, and Baker 2002; Braun and Kimball 2002; Slack et al. 2003). In one case, with transversion likelihood, the results rejected the passerine rooting (Braun and Kimball 2002). In our work with eutherian mammals (Lin et al. 2002) we found that increased taxon sampling led to agreement between trees from nuclear and mitochondrial data.

A magpie goose (*Anseranas semipalmata*) was chosen because two major morphological studies (Ericson 1997; Livezey 1997) conclude that *Presbyornis* fossils are on the lineage to geese and ducks—after the divergence of the magpie goose lineage. Goose and duck mitochondrial genomes are available (see Slack et al. 2003), and the addition of a magpie goose mt genome therefore establishes an important calibration point for avian evolution. Some molecular results are available for the magpie goose (see Sibley and Ahlquist 1990; Sraml et al. 1996; Mindell et al. 1997) and support its placement outside geese and ducks, but still within Anseriformes. With respect to dates, Ericson (1997) places the *Anseranas*/*Presbyornis* divergence at least 60 MYA (Paleocene) but some older *Presbyornis* fossils are reported from about 66–67 MYA, in the very Late Cretaceous of Antarctica (Noriega and Tambussi 1995; Case and Tambussi 1999). These fossils are not yet fully published, and in the interim we use both the 60 MYA date as a lower bound on the time of divergence, and compare results using this to those with the older (66 MYA) calibration point.

As mentioned above, the rooting point of the avian tree is controversial. We take the view that, although the data are correct, inadequacies in analytical methods can lead to different results from nuclear and mitochondrial data. Rather than "blame the data," the onus is on theoreticians to improve techniques of analysis to reflect the unusual nucleotide composition of some vertebrate mitochondrial genomes. This includes differences between pyrimidines (C&T) and between purines (A&G) (see Schmitz et al. 2002; Phillips and Penny 2003). We also require criteria to evaluate which techniques are more powerful in capturing information in the data. One such measure is the *treeness* statistic, the sum of internal internode (branch) lengths divided by the sum of all internodes on the tree (see Lanyon 1988; Phillips and Penny 2003). Treeness increases when apparently saturated sites are omitted—such as third codon positions or, especially (for mitochondrial data), by reducing the nucleotides (A,G,C,T) to pyrimidines and purines (RY-coding). RY-coding reduces the effect of differences in nucleotide composition between species resulting from C-T differences (pyrimidine bias), or the lesser differences between A and G (purine bias) (Phillips and Penny 2003). The reduced bias is measured by the *relative compositional variability* (RCV, the average variability for character states between taxa). For nucleotides, RCV is defined as:

$$\mathrm{RCV} = \Big(\sum |\mathrm{A}_i, -\mathrm{A}^*| + |\mathrm{T}_i, -\mathrm{T}^*, | + |\mathrm{C}_i - \mathrm{C}^*| + |\mathrm{G}_i - \mathrm{G}^*|\Big)/n.t$$

(see Phillips and Penny 2003)

A_i, T_i, C_i, and G_i are the total numbers of each nucleotide for the *i*th taxon; A*, T*, C*, and G* are the averages for the n taxa, and t the number of sites. RCV allows direct comparison of the extent of composition bias for data sets and data treatments.

In summary, for data partitions or codings compared on the same tree, higher treeness and/or lower RCV values indicate a stronger phylogenetic signal and/or a lower composition bias that can mislead phylogenetic inference. Phylogeny estimates from data treatments (such as partitioning and/or coding) that have the highest treeness/RCV values are expected to be the least susceptible to composition bias (Phillips and Penny 2003). We find that treeness and RCV values are preferable to using chi-squared values of deviations in amino acid (aa) composition, because the chi-squared test loses sensitivity when coding sequences are expressed as amino acids. For the same original amount of data, the number of degrees of freedom is increased markedly, whereas the number of sites is reduced by two-thirds, making the analysis much less powerful. RY-coding is effective for mitochondrial sequences in that it results in more agreement between data sets. For example, monotremes (platypus and echidna) were placed just outside the therians (marsupial plus placental mammals; Phillips and Penny 2003), and the Hexapoda clade of insects plus Collembollans was recovered (Delsuc, Phillips, and Penny 2003; see also Nardi et al. 2003).

Materials and Methods

The owl was from Nga Manu Bird Sanctuary, Waikanae, New Zealand; Trevor Worthy provided a

rifleman sample from Northwest Nelson, N.Z.; David Lambert, Massey University, donated kakapo tissue; and Peter Whitehead and Julian Gorman, Northern Territory University, Darwin, Australia, provided magpie goose tissue. DNA was extracted from muscle, liver, or blood using standard kits. Mitochondrial DNA was amplified in fragments longer than 5 kb (to minimize the risk of amplifying nuclear copies) using the Expand Long template polymerase chain reaction (PCR) kit (Roche).

For the owl, parrot, and rifleman, long PCR DNA fragments were sequenced directly or used as template for a second round of short-range PCR of 1~2 kb. Primers were designed to match conserved regions of avian mtDNA genomes, allowing 0–3 degenerate sites to maximize their usefulness for other species. We used the Fasta search in the GCG program (Wisconsin Package, version 10.0) to search our primer database for appropriate targets for primer walking. Where possible, primers from Sorenson et al. (1999) and Cooper et al. (2001) were used. Any new primers required were designed using Oligo 4.03 (National Biosciences, Inc.). Sequencing reactions followed standard protocols for Applied BioSystems 377 and 3730 Sequencers. Sequences were assembled and checked using Sequencing Analysis and MT Navigator programs (ABI) and Sequencher 4.1 (Gene Codes Corp.).

For magpie goose, long-range PCR products were pooled and fragmented pneumatically with a nebulizer for 40 s at 40 psi into pieces about 2 kb in length, then cloned and sequenced using the TOPO Shotgun Subcloning Kit Version D (Invitrogen). This involved ligation into pCR 4Blunt-TOPO and transformation into TOPO10 *E. coli*. Plasmid DNA was extracted and purified using the GenElute Plasmid Miniprep Kit (Sigma), and insert size was determined by restriction digest. Plasmids containing inserts >800 bp were sequenced with the universal forward and reverse primers. The sequences were edited and assembled in Sequencher; any gaps were filled with short-range reamplifications from the appropriate long fragments.

In addition to the four new mt genomes, we used 20 other avian taxa: chicken (*Gallus gallus*; GenBank accession number X52392), quail (*Coturnix japonica*; AP003195), redhead duck (*Aythya americana*; AF090337), greater white-fronted goose (*Anser albifrons*; AF363031), rook (*Corvus frugilegus*; Y18522), gray-headed broadbill (*Smithornis sharpei*; AF090340), village indigobird (*Vidua chalybeata*; AF090341), peregrine falcon (*Falco peregrinus*; AF090338), common buzzard (*Buteo buteo*; AF380305), Oriental white stork (*Ciconia boyciana*; AB026193), ruddy turnstone (*Arenaria interpres*; AY074885), blackish oystercatcher (*Haematopus ater*; AY074886), little blue penguin (*Eudyptula minor*; AF362763), great spotted kiwi (*Apteryx haastii*; AF338708), emu (*Dromaius novaehollandiae*; AF338711), double-wattled cassowary (*Casuarius casuarius*; AF338713), ostrich (*Struthio camelus*; Y12025), greater rhea (*Rhea americana*; Y16884), great tinamou (*Tinamus major*; AF338707), and elegant crested-tinamou (*Eudromia elegans*; AF338710).

Six reptiles were used as outgroups: American alligator (*Alligator mississippiensis*; Y13113), eastern painted turtle (*Chrysemys picta*; AF069423), green turtle (*Chelonia mydas*; AB012104), blue-tailed mole skink (*Eumeces egregius*; AB016606), common iguana (*Iguana iguana*; AJ278511), and spectacled caiman (*Caiman crocodylus*; AJ404872). Data sets were prepared both with and without outgroups because in preliminary studies we found that the avian tree could change when the outgroup was added. A similar phenomenon has been reported with eutherian mammals (Lin et al. 2002).

Sequences were aligned manually in Se-Al version 1.0 a1 (http://evolve.zps.ox.ac.uk/Se-Al/Se-Al.html). rRNA sequences were aligned on the basis of secondary structure (www.rna.icmb.utexas.edu/RNA/) to maximize homologous positions. Data are available at http//imbs.massey.ac.nz/downloads.htm. Standard programs were used for all analysis, including PAUP* 4.0b8 (Swofford 1998), MOLPHY (Adachi and Hasegawa 1996), and MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Maximum parsimony, minimum evolution (with ML distances), maximum likelihood (ML), and Bayesian methods were employed on both the avian and avian plus outgroup data sets. Optimal parameters for the maximum likelihood models were determined using Modeltest (Posada and Crandall 1998). The hierarchical and AIC tests were in agreement for Modeltest.

Results

Description of mt Genomes

The GenBank numbers and lengths of the four new sequences are as follows:

Acanthisitta chloris, rifleman—AY325307 (missing tRNA-Phe and some control region)
Anseranas semipalmata, magpie goose—AY309455, 16,869 bp (complete)
Ninox novaeseelandiae, owl—AY309457, 17,122 bp (missing part of tRNA-Phe and control region)
Strigops habroptilus, parrot—AY309456, 16,311 bp (missing part of control region)

Each mitochondrial genome was sequenced from $tRNA^{Phe}$ or 12S RNA through to $tRNA^{Thr}$/$tRNA^{Pro}$/ ND6/ $tRNA^{Glu}$ and into the control region. These genomes have the same gene order as the chicken and not the alternative avian gene order ($tRNA^{Thr}$ /control region/ $tRNA^{Pro}$ / ND6/$tRNA^{Glu}$/noncoding; Mindell, Sorenson, and Dimcheff 1998). The sequences for ND6 and t-Glu in the kakapo appear to encode functional genes; this as well as the fact that $tRNA^{Pro}$ follows $tRNA^{Thr}$ indicates that the kakapo does not have the same rearrangement as found in the parrot genus *Amazona* (Eberhard, Wright, and Bermingham 2001). Unfortunately, for political reasons, we are unable to clone DNA fragments from native birds in New Zealand. Hence, parts of $tRNA^{Phe}$ and the control region are missing from all three native birds, as they have proved difficult to sequence without cloning, on account of the presence of repeats and heteroplasmy. Features such as start and stop positions for each gene (as in Slack et al. 2003) are reported in http://awcmee.massey.ac.nz/downloads.htm. The TψC loop of $tRNA^{Phe}$ has three variants that are potentially informative within birds. Paleognaths (ratites and tinamou), galliformes, anseriformes, and the owl have the same pattern (see fig. 1A). Other Neoaves (except penguin) have an

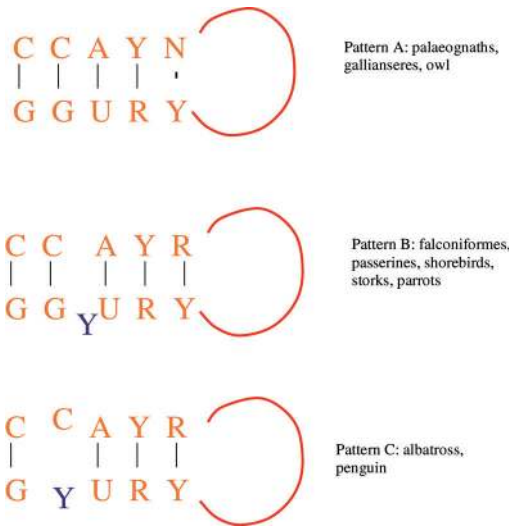

FIG. 1.—TψC stem patterns among birds for tRNA-Phe. Pattern A appears to be ancestral for modern birds, being shared by the four most basal groups (tinamous, ratites, anseriformes, and galliformes), as well as the owl. The inferred ancestral bases are in red. An additional unpaired base (indicated in blue) occurs in all other birds examined, for which pattern B appears primitive. A third pattern (C, in the penguin and petrel) can easily be derived from pattern B by the loss of a guanine.

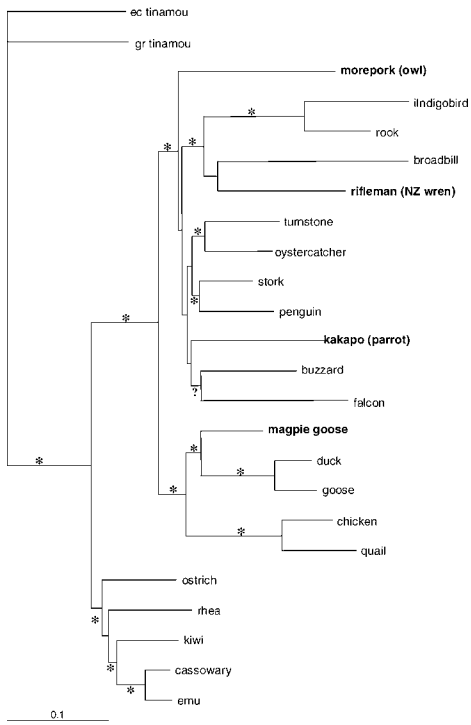

FIG. 2.—Unrooted MrBayes tree for 24 avian mt genomes. The data represent 13,439 base pairs of the combined protein and RNA data sets using $12_n3_rSL_n$ coding. Each of the resulting five partitions is optimized for its GTR + I + Γ_4 model. An asterisk indicates the groupings that have both high bootstrap support by several analyses on this data set, and also on prior information (nuclear and morphological). The falconiform (buzzard/falcon) group is marked by a question mark because it is only weakly supported on the present analyses, even though it is well-supported on other data. The four new taxa from this study are indicated in bold.

inserted pyrimidine, usually "C," which is unpaired. This is illustrated as pattern B in figure 1. The penguin/petrel pattern is similar to 1B but has lost a "G" and has one less set of paired bases (pattern C).

Two data sets were used for phylogenetic analysis: a 24 taxon bird-only data set; and a 30-taxon data set with the 24 birds plus the six outgroups (two turtles, two crocodilians, and two lizards) as used in Slack et al. (2003). To check the relative size of signal in different data partitions, we calculated RCV values on the data, and treeness values on the tree in figure 2 (see later). The results are given in table 1. The first, second, and third codon positions of the 12 proteins encoded on the heavy strand are indicated by 1, 2, and 3, and the values for coding as nucleotides (or as RY-coding) are shown by a subscript "n" (or "r"), respectively. RNA coding genes (ribosomal and transfer) were partitioned into stems (S) and loops (L). Protein-coding genes were also translated to amino acids. The number of nucleotides was 10,338 protein-coding sites (3,446 amino acids) and 3,101 RNA-coding sites, giving a combined total of 13,439 nucleotides (excluding gaps).

The most important conclusion from table 1 is that, compared to amino acid coding, or omitting the third codon position, RY-coding the third position improves the signal-to-noise ratio, thus retaining more phylogenetic information. This result is seen in several comparisons, for example, the values for third position coded as nucleotides (3_n), and as RY-coding (3_r). The 3_n value (0.70) is the lowest treeness/RCV value in the table; the 3_r value (2.76) is one of the highest. Similarly, it is informative to compare 12_n with 123_n and with 12_n3_r (that is, adding the third codon position to the first two, first as nucleotides, then as RY-coded). By itself, adding the third position as nucleotides reduces the treeness/RCV value from 1.74 to 1.15—consistent with the experience of many authors that the third codon position is "saturated." Adding the third position as RY-coded enhances the value from 1.74 to 2.32. Thus coding the third position as RY both increases the signal in the internal edges (branches) of the tree and reduces the variability of nucleotide composition between taxa. This can only happen if there is a large amount of information as purines and pyrimidines that is masked by within-purine and within-pyrimidine biases (Phillips and Penny 2003). Most of our results are therefore given under the $12_n3_rSL_n$ coding scheme, although others have been used (data not shown), such as reducing the first position to RY-coded (1_r), loops to RY-coding (L_r), etc. For analysis, each of the five partitions (codons one, two, and three, stems, and loops) has its own optimized model (including for gamma distribution and proportion of invariant sites).

Unrooted Trees

We find cases with both real (Lin et al. 2002; Slack et al. 2003) and simulated data (Holland, Penny, and Hendy 2003), where the unrooted tree changes when the outgroup is added. We therefore examine the unrooted tree first, and only include the outgroup taxa later. Figure 2 shows the unrooted (avian-only) tree for the combined protein and

Table 1
Treeness and RCV Values for Partitions of the 24 Taxon Avian-Only Data Set

Partition	treeness	RCV	treeness/RCV
$12_n3_rSL_n$	0.1376	0.0495	2.7798
12_n	0.0888	0.0511	1.7378
3_n	0.0652	0.0931	0.7003
3_r	0.1603	0.0580	2.7638
SL_n	0.1393	0.0530	2.6283
123_n	0.0829	0.0724	1.1450
12_n3_r	0.1384	0.0596	2.3221
PTN_{aa}	0.1584	0.0787	2.0127

NOTE.—Partitions of the data coding the third codon position as RY (3_r) rather than nucleotide (3_n) improves the signal-to-noise ratio (treeness/RCV). In addition, it retains all alignable sites. Values for other partitions are shown; PTN_{aa} are the protein-coding genes as amino acids. For the main analysis, other positions were retained as nucleotides (first and second codon positions and stems (S) and loops (L), that is, $12_n3_rSL_n$).

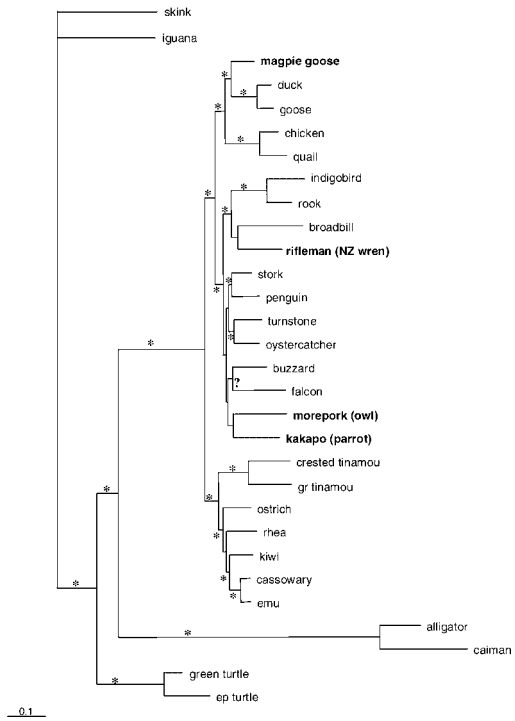

FIG. 3.—Avian MrBayes tree rooted by six outgroup taxa. The data is the combined protein and RNA genes using $12_n3_rSL_n$ coding, and with each partition optimized for its GTR + I + Γ_4 model. Posterior probabilities are >0.98 for all internal edges, except for the following groupings of the owl/parrot/falconiformes (0.82) and rhea/emu/kiwi/cassowary (0.97). These values do not include effects from model misspecification. Asterisks, question mark, and bold font are as in figure 2.

RNA data ($12_n3_rSL_n$), using MrBayes. Because we do not have any new paleognath taxa, we do not discuss them in detail. We simply note that they have the standard subdivision into tinamou and ratites, and that some details of the ratite subtree are not robust. Looking at the new taxa, the magpie goose (as expected on the basis of prior information) is always deep on the duck/goose lineage. These three anseriforms join with the two galliforms (chicken and quail), to form Gallianseres. This grouping has increasingly been supported in recent years by both molecular and morphological data (for example, Cracraft 2001; Livezey and Zusi 2001; Slack et al. 2003; Sorenson et al. 2003). Thus the unrooted avian tree has the predicted strong three-way subdivision into paleognaths (ratites and tinamou) and the two neognath subdivisions (Gallianseres and Neoaves).

With respect to the four passerines (rifleman, broadbill, indigobird, and rook), the first important point is that they are united on the unrooted tree. Slack et al. (2003) found that the passerines (then without the rifleman) grouped together on the unrooted mt tree; it was only on addition of the reptilian outgroup that the passerine grouping became (at best) paraphyletic. A second point is that, given the expected rooting point between paleognaths and neognaths, passerines appear to form an early division of Neoaves. As discussed in Boles (1997), passerines have traditionally been considered relatively recent within extant birds (see also Livezey and Zusi 2001; Feduccia 2003). Because there are still major Neoavian groups missing from this data set (cuckoos, woodpeckers, mouse birds, etc.; Cracraft 2001) it is possible these could form earlier divisions within Neoaves. Nevertheless, the deep placement of passerines is worth noting.

Turning to the new passerine, the rifleman (as expected) is deep within the passerines—either ancestral to all passerines (as in Ericson et al. 2002) or basal on the suboscine (broadbill) lineage (Sibley and Ahlquist 1990). In the present study, the highest bootstrap (PAUP*) and posterior support values (MrBayes) favor the broadbill/rifleman association. However, this latter grouping should be treated cautiously. Both the rifleman and broadbill are long branches in the tree and with, at most, a short edge between them. This pattern fits the classic long-branch attraction case, where misleading results are found even without differences in rates of evolution (Hendy and Penny 1989); this affects all tree selection criteria where the model is to some extent mis-specified. To check this possibility, we are now sequencing another suboscine (a New World tyrant flycatcher) to break up the long broadbill branch. However, in our terminology (Cooper and Penny 1997), the tree is locally stable; the alternatives are rearrangements around a single internal branch of the tree.

In the current data set, neither owl nor parrot has any strong associations. Both fall within the Neoaves (which is supported by 100% bootstrap, or posterior probabilities of 1.0, in all our analyses). In general, the owl is either deeper in the tree than the parrot (as in figure 2), or they have a weak association (as in fig. 3, see later). The respective positions of owl and parrot are only preliminary until taxon sampling is increased. In the meantime, however, it is noteworthy that both come within Neoaves as ancient and distinctive lineages.

This leaves the six representatives of the large Neoavian group that includes seabirds, shorebirds, and raptors. Four taxa that come together on nearly all trees are penguin, stork, and the shore birds (oystercatcher and turnstone). The buzzard is usually adjacent to this group of four (although it might be expected to be one step closer to the stork/penguin; Sibley and Ahlquist 1990). However, the position of the falcon is quite variable; only with

the RNA data set does it come strongly with the buzzard (as in fig. 2), although both have the same duplication/rearrangement of gene order (Mindell, Sorenson, and Dimcheff 1998; Haring et al. 2001). In earlier work, with smaller numbers of mt genomes, the falcon even came with passerines (see Discussion in Slack et al. 2003). Given our emphasis on increased taxon sampling as solving many problems (Hendy and Penny 1989; Lin et al. 2002), the frequent separation of falcon and buzzard, especially on protein-coding genes, is unexpected. At the moment we can only fall back on the "still better taxon sampling" argument and perhaps a kite, osprey, or especially a forest-falcon would strengthen the position of falcon and buzzard on the tree. Whether this then moves the raptors closer to penguin and stork could then be evaluated.

Finally, we use an asterisk to indicate those aspects of the tree that are well supported by these and other data. With the ever-increasing number of analysis methods available, it appears arbitrary selecting one set of, say, bootstrap or posterior probability values. Our approach is to identify the groupings that have strong support values from several methods and which are supported by other data (nuclear and/or morphological). The weakest association we have marked with an asterisk is the stork/penguin pairing, which has only 95% bootstrap support under ML, but was in our prior tree (Cracraft 2001). In addition, although we would be surprised if the buzzard and falcon continued to be separate when more falconiformes are available, it is hard to recover this grouping from our data. Consequently, falconiformes is indicated by a question mark. For other weaker groupings, we simply show results from the MrBayes tree. However, we would not be surprised, with more data, if these groupings changed on the tree—though usually not by more than a single interchange on the tree.

Rooted Trees

As in Slack et al. (2003), we used six reptilian sequences to root the avian tree, two turtles, two crocodilians, and two lizards. Figure 3 shows a MrBayes analysis on the combined protein and RNA data, with third codon positions reduced to RY-coding (that is, $12_n3_rSL_n$). Again, the model was optimized for each of the five partitions. Unlike previous analyses of avian mt genomes, this straightforward analysis gave the root between paleognaths and neognaths (as expected from morphological and nuclear data). The bootstrap value for this position of the root is 96% with ML, but less with minimum evolution (78% with ML distances). Reducing other partitions to RY-coding also placed the root in the same position (and increased the treeness/RCV ratio, indicating a higher signal-to-noise ratio). Because the position of the root in figure 3 is found from mitochondria data with analyses giving the strongest signal-to-noise ratio, and because nuclear and morphological data give the same rooting, we consider this the accepted rooting for birds. This is basically the argument of congruence between independent data sets (Penny, Foulds, and Hendy 1982). In addition, finding crocodilians closest to birds (Archosauria) has been difficult to obtain with mitochondrial data (see Cao et al. 2000) but is recovered easily with the present RY-coding.

Apart from the position of the owl, the ingroup is unchanged from figure 2. Among the paleognaths, the same relationship holds between ratites and tinamous, although again deeper divergences within ratites are not highly stable. The passerines still come together and rifleman can still occur as the deepest division within Passerines. The penguin/stork/shorebird group is unchanged, but the owl has moved across to the parrot, and they come within the expected association with the seabird/shorebird/raptor group. However, the placement of owl and parrot is not strong, and a Shimodaira/Hasegawa test (1999) shows that at least 10 trees involving the deeper Neoavian lineages cannot be rejected (even at $P = 0.50$). The 10 trees all have either owls or passerines as the deepest division within Neoaves. Irrespective of the placement of the root, the passerines appear to be a very old Neoavian group, and this point needs more emphasis (see Boles 1997). As in figure 2, we indicate with an asterisk the groupings that are both expected on prior information and are stable over a variety of analyses; we would be surprised if these changed with additional data.

Analyses of the present data without RY-coding of the third position can still place the root within passerines. In such trees the oscine songbirds were separated from suboscines, and they are usually the first avian branch (although the rest of the tree was virtually unchanged). In such trees the passerines are at best paraphyletic, at worst polyphyletic. One possibility is that in earlier analyses of the avian mitochondrial data, the outgroup came into the traditional position, and that the long edge to the oscines (rook and indigobird) was secondarily attracted to the long edge of the outgroup. In any case, adding the outgroup can lead to a rearrangement of the unrooted avian tree. We have reported such effects in mammals (Lin et al. 2002) and in simulations (Holland, Penny, and Hendy 2003). Although it is possible in simulations for the addition of the outgroup to correct an error in the ingroup, it is much more common for the outgroup to disrupt a correct ingroup (Holland, Penny, and Hendy 2003). This is additional grounds for accepting the root in figure 3 as highly likely to be correct. Finding a taxon (such as lyrebird) that breaks up the long branch to the oscines is a priority, and our prediction is that, even without RY-coding, the root will then come between paleognaths and neognaths.

Some preliminary results on dating are given here, basically comparing results with two new calibration points. The first is a new penguin date taken at 62 MYA (Jones and Mannering 1997; Slack et al. in preparation). Good fossils (Jones and Mannering 1997) of at least two species of early penguins dated at between 61 and 63 MYA have been found in North Canterbury, New Zealand. This calibration point may be conservative because the closest bird to penguin in the present data set is a stork. The second calibration point is the Presbyornis/magpie goose divergence estimated at either 60 MYA (Ericson 1997) or (with the discovery of new fossils on Vega Island, Antarctica) 66–67 MYA (Case and Tambussi 1999). This latter site was discovered relatively recently and has the remains of at least five different species that fall within modern birds (J. Case, personal communication). Our aim here is to compare the divergence times estimated from the

two potential Presbyornid dates with those found using the penguin date.

Divergence times estimated by the Sanderson (1997) method that allows rate variation, are given in table 2. We have deliberately omitted confidence intervals to focus on the issue of the two Presbyornis/magpie goose divergences; the older divergence (66 MYA) is in agreement with the penguin/stork date. Although this is encouraging, it is preferable that these Vega Island fossils (Case and Tambussi 1999) be fully described, because they will give additional calibration points, including burhinid (stone curlew/thick knees) shorebirds. In general, our results are about 10% younger than those of Van Tuinen and Hedges (2001). They used an external calibration point approach with the avian/mammalian divergence at 310 MYA as their primary point. A combination of internal and external calibration points may be preferable, because interpolating between points can give an unbiased estimate (M.A. Steel and M.D. Hendy, personal communication).

Our preliminary analyses support at least 13 lineages of modern birds surviving from the Cretaceous to the present. These include two lineages each of ratites, anseriformes, and passerines; plus at least one lineage each of tinamou, galliformes, owls, parrots, shorebirds, falconiformes, and stork/penguin. In Cooper and Penny (1997) we report 22 lineages, none of which are contradicted on this present data set with fewer taxa but longer sequences. Although several methods of estimating divergence have been tried in the present work, our preference at present is to resolve the avian tree further before returning to date estimates.

Table 2
Dating Estimates for Early Avian Divergences Based on Two Calibration Points

	Penguin/Stork @ 62 MYA	Magpie Goose/Duck @ 60 MYA
Within birds		
Paleognaths/neognaths	101	92
Ratites/tinamou	84	77
Ostrich/other ratites	75	69
Gallianseres /Neoaves	90	82
Galliforms/ anseriforms	76	70
Magpie goose/duck+goose	66	**60** (fixed)
Owl/other neoavian birds	80	73
Passerines/other neoavians	78	71
Oscines/suboscines	70	64
Falconiforms+parrot/rest	74	68
Falconiforms/parrot	72	66
Shorebirds/penguin,stork	74	68
Penguin/stork	**62** (fixed)	57
Outside birds		
Birds/crocodilians	183	167
Archosaurs/turtles	199	182
Turtles (green/painted)	79	72
Iguana/skink	146	134

NOTE.—Calibration points (boldface) are a penguin/stork divergence of 62 MYA (left column) and a Presbyornis/magpie goose divergence of 60 MYA (the conservative estimate for Presbyornis, right column). The less conservative Presbyornis/magpie goose divergence (66 MYA) gives the same estimates as the penguin calibration point. Standard errors are omitted to permit focus on the comparisons.

Discussion

An important reason to establish a good phylogeny of modern birds (the crown group) and then estimate divergences times is that fundamental evolutionary models can be tested (see fig. 4). Our underlying interest here is whether the diversifying lineages of modern birds were competing with (and possibly outcompeting) pterosaurs and earlier avian groups during the Late Cretaceous. In other words, can we use dated trees to infer evolutionary processes? If all lineages of modern avian orders only diverged and diversified in the Tertiary (after the extinction of the earlier groups) then modern birds cannot have affected these earlier groups, either directly or indirectly. This example, basically the Feduccia model (1995; 2003), is Model A in figure 4—all modern birds have a common ancestor in the Tertiary. On this model, all ecological, morphological, and taxonomic differentiation of birds (ratites and raptors, swifts and seabirds, penguins and parrots, owls and oystercatchers) occurred early within the Tertiary, and by unknown genetic mechanisms.

There is a range of alternative models. One (4B) is that many lineages of modern birds diverged in the Cretaceous, but diversification into the range of forms and niches we see today only occurred in the Tertiary. Here we distinguish *divergence* of lineages, and *diversification* into a range of ecological, taxonomic, and morphological forms. This includes both short fuse and long fuse models (Cooper and Fortey 1998; Springer et al. 2003). Under this model, there may have been little competition during the Late Cretaceous between modern birds and earlier birds; each could still be in a separate niche. In contrast, the third model (4C) proposes that phylogenetic divergence and ecological/morphological diversification both occurred in the Late Cretaceous. This does not mean that all orders of birds diverged and diversified in the Late Cretaceous, but that most of them did. In 4C, major ecological transitions occurred during the Cretaceous, and we expect that modern birds were competing in the same niche as some earlier birds (such as enantiornithines, *Hesperornis*, and *Ichthyornis*) and pterosaurs. There is a range of intermediates between 4A, 4B, and 4C.

It is premature to decide which model is closest to being correct, and more detailed treatments are needed (Phillips and Penny 1998; Penny and Phillips, in preparation). Although results clearly contradict model A—all divergences in the Tertiary—the present evidence is insufficient to decide between B and C. Both models have early divergences, but current results do not tell directly about diversification. The eventual goal is to understand interactions in the Late Cretaceous between modern birds and the earlier groups mentioned above. It is helpful to separate the process into three steps: the phylogeny, comparison of divergence times based on molecular and fossil data, and ecological transformations (if any) in the Late Cretaceous. For the first, we do not require a complete phylogeny of all modern birds; just a robust phylogeny of major avian groups, especially those for which the oldest fossils are available. Our priority is to improve taxon

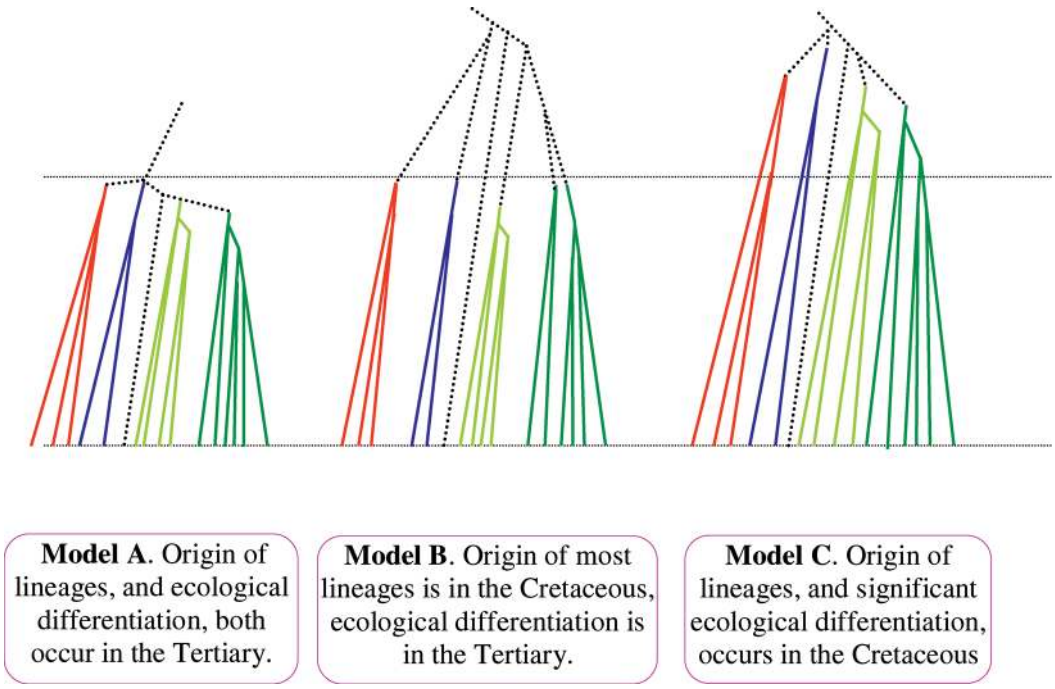

FIG. 4.—Three general models that need to be evaluated for both avian and mammalian evolution. In model A, modern orders of birds both originate and diversify ecologically in the Tertiary. In model B, many lineages diverge in the Cretaceous, but ecological diversification is in the Tertiary. In model C, both the origin of lineages and significant ecological diversification occurs in the Cretaceous. The models differ in their implications about mechanisms of evolution leading to extinctions, and they illustrate how trees can be used to study evolutionary mechanisms. (In each model, dashed lines represent a group still within the ancestral niche.)

sampling until a more stable tree is obtained for both nuclear and mitochondrial data. This will allow stronger estimates of the divergence of major avian lineages. Comparing divergence times with molecular and fossil data (Bromham and Penny 2003; Smith and Peterson 2002) still requires careful work, but there is a large body of evidence for fossil of modern birds toward the end Cretaceous (Hope 2002). This includes newer fossil discoveries in Antarctica such as reported in Case and Tambussi (1999). The final aspect is evaluating the fossil record for the ecological role of modern birds in the Late Cretaceous (Chiappe and Dyke 2002), including evidence from fossil footprints (Lockley and Rainforth 2002). Only after this analysis can our models be evaluated thoroughly.

So far the discussion has been on general issues of avian evolution, not specifically on the other claim in Feduccia (2003), the relatively recent origin of passerines. There is no fossil information available to substantiate that claim. Recent molecular work places early oscine evolution in Australia (Barker, Barrowclough, and Groth 2002; Edwards and Boles 2002), and this is consistent with the earliest known passerine fossil being Australian (Boles 1995). Unfortunately, no land vertebrate fossil beds are known from Australia between the early Eocene (54 MYA) until the Early Cretaceous (105 MYA). Gurnis, Müller, and Moresi (1998) report that plate tectonic processes raised the Australian continent during the mid-late Cretaceous by up to 250 m, leaving few areas for net deposition and fossilization. The absence of fossil beds means that it is unlikely there will be fossil evidence, for or against, the older origin of passerines, and so the molecular data stand alone.

There appears to be sufficient information in the mitochondrial data to recover a good avian phylogeny, especially with RY-coding. Although our results support the avian root between paleognaths and neognaths, it can appear arbitrary if some analyses are favored over others, even if the rooting is supported by prior information. For this reason the treeness/RCV ratio is helpful in evaluating which method of analysis gets the most phylogenetic signal. There are many signals in DNA sequence data (Penny et al. 1993). There is no guarantee that the largest signals are always the correct phylogeny, and in the present data there is some signal from a particular form of nucleotide bias (such as within pyrimidines) which has to be reduced.

There is always a tendency to "blame the data" if a predicted result is not obtained. On the contrary, we suggest the data are neutral; it is the methods of analysis that are inadequate. It is important to develop improved methods that more accurately reflect the underlying mutational mechanisms; an "optimal" model can still give a wrong tree. Thus we require methods that determine which aspects of the mutational mechanism and/or selection are accounted for, and which are not. With both mammals and birds it appears that improved taxon sampling and RY-coding are key factors in obtaining highly congruent trees between different data types (nuclear and mitochondrial). Of course, there will be cases where the appropriate taxa no longer exist and improved taxon sampling will not be

possible. Overall, the results are extremely encouraging that the avian tree is being resolved, and will then allow improved estimates of the survival of bird lineages through the Cretaceous/Tertiary boundary (Cooper and Penny 1997).

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