

# Mitochondrial Genomes and Avian Phylogeny: Complex Characters and Resolvability without Explosive Radiations

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We improve the taxon sampling for avian phylogeny by analyzing 7 new mitochondrial genomes (a toucan, woodpecker, osprey, forest falcon, American kestrel, heron, and a pelican). This improves inference of the avian tree, and it supports 3 major conclusions. The first is that some birds (including a parrot, a toucan, and an osprey) exhibit a complete duplication of the control region (CR) meaning that there are at least 4 distinct gene orders within birds. However, it appears that there are regions of continued gene conversion between the duplicate CRs, resulting in duplications that can be stable for long evolutionary periods. Because of this stable duplicated state, gene order can eventually either revert to the original order or change to the new gene order. The existence of this stable duplicate state explains how an apparently unlikely event (finding the same novel gene order) can arise multiple times. Although rare genomic changes have theoretical advantages for tree reconstruction, they can be compromised if these apparently rare events have a stable intermediate state. Secondly, the toucan and woodpecker improve the resolution of the 6-way split within Neoaves that has been called an “explosive radiation.” An explosive radiation implies that normal microevolutionary events are insufficient to explain the observed macroevolution. By showing the avian tree is, in principle, resolvable, we demonstrate that the radiation of birds is amenable to standard evolutionary analysis. Thirdly, and as expected from theory, additional taxa breaking up long branches stabilize the position of some problematic taxa (like the falcon). In addition, we report that within the birds of prey and allies, we did not find evidence pairing New World vultures with storks or accipitrids (hawks, eagles, and osprey) with Falconids.

## Introduction

Our primary interest here is using the phylogenies of birds to test questions such as whether the processes of microevolution are sufficient to explain macroevolution or how frequently major changes occur in the ecological niche a group occupies. In practice, we need to distinguish between the 5 models of Penny and Phillips (2004; see also Cooper and Penny 1997) on the extent that ecological, physiological, and taxonomic diversification occurred prior to, or after, the Cretaceous/Tertiary (K/T) boundary. Such a program of inquiry needs to be broken down into many testable steps that can be examined using specific data sets. Here we use 7 new mitochondrial (mt) genomes to consider 3 main aspects of the questions. The first is to understand why a particular change in mt gene order appears to have occurred several times during avian evolution, and therefore why (in this case) gene order may not be a useful phylogenetic character. The next aspect is that the additional taxa make it appear that resolution of the basal 6-way split among Neoaves (Cracraft 2001) will be possible, eliminating the need to postulate an “explosive radiation” (e.g., Poe and Chubb 2004). Finally, breaking up some long branches increases the stability of the tree as predicted from theory.

Over the past 30 years, the use of DNA or protein sequence data has increasingly become the main data type used to recover phylogeny in general, but there are fundamental limits on how far back sequence data will allow reliable recovery of evolutionary history (Mossel and Steel 2005). In principle, “rare genomic changes” (Rokas and Holland 2000; Boore 2006), or more evocatively, “sequence characters, uniquely derived” (SCUDs), such as

changes in gene order, can retain information for long periods of time. When the number of character states is so high that the same change is unlikely ever to be repeated, then simple parsimony is a maximum likelihood estimator (Steel and Penny 2004, 2005). Such rare DNA changes can, in principle, retain phylogenetic information even when primary sequence data must have become randomized due to the long time periods involved (see Mossel and Steel 2005). With mammals, the identification of retrotransposon insertions has been extremely valuable (Nishihara et al. 2005, 2006), a fact highlighted in the recent resolution of the placental mammal tree, including the position of the root, using only rare genomic changes (Kriegs et al. 2006). This was equivalent to giving DNA sequence data 30 years start and catching and overtaking them in a single study. Although the particular repetitive elements used to study mammalian evolution may not be so useful in birds, there is considerable potential for the use of these types of rare events in phylogenetic studies (see Snel et al. 2005). Two recent studies in birds have used the chicken repeat 1 (CR1) retrotransposon to determine relationships among closely related groups of birds, and this is promising for the future use of insertions in elucidating deeper avian phylogenetic relationships (St John et al. 2005; Watanabe et al. 2006).

Differences in mt gene order have been useful for phylogenetic resolution of some groups of species, for example, Arthropoda being monophyletic and within this Crustacea grouping with Hexapoda to the exclusion of Myriapoda and Onychophora (see Boore 2006 and references therein). Birds also have a different mt gene order compared with other vertebrates, and this reinforced their already accepted monophyly (Desjardins and Morais 1990). The difficulty in general is ensuring that the rare genomic changes are genuinely unique events. Several different arrangements of mt gene order have been observed in birds, including the likely ancestral avian gene order first found in the chicken (*Gallus gallus*, Desjardins and Morais 1990), corresponding to

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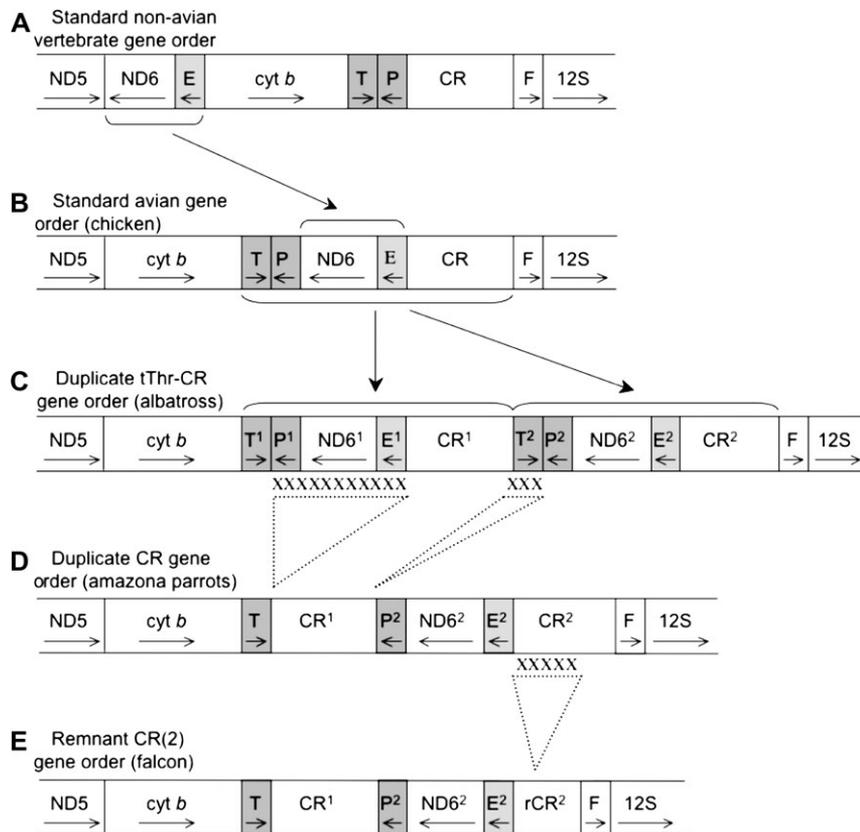


FIG. 1.—Gene orders found in avian mt genome CRs. Arrows between figure parts show one scenario for conversion between the different gene orders. Arrows underneath gene names represent gene directionality. X's and dotted triangles represent possible gene loss or reduction.

*cytb*/tThr/tPro/ND6/tGlu/Control Region/tPhe/12SrRNA (fig. 1A) and the alternative gene order reported by Mindell et al. (1998), that is, *cytb*/tThr/Control Region/tPro/ND6/tGlu/Noncoding region/tPhe/12S rRNA (fig. 1E). However, Mindell et al. (1998) pointed out that all plausible avian phylogenies implied that the alternative gene order arose independently several times within birds. Despite these parallel changes, they suggested that gene order may still be useful in certain cases, for example, to distinguish oscine from suboscine passerines, though Bensch and Härlid (2000) later reported exceptions within oscines. Species with the alternative gene order typically had a short noncoding region between tRNA Glu and tRNA Phe. However, a control region (CR) duplication has been observed in *Amazona* parrots (Eberhard et al. 2001; fig. 1D), and another alternative (fig. 1C) in albatrosses was reported by Abbott et al. (2005). It is important to understand the reasons for the multiple origins of an alternative gene order because in many cases gene order has potential for being excellent markers for phylogeny (Snel et al. 2005; Steel and Penny 2005; Boore 2006).

Turning to avian phylogeny in particular, there is support for a basal split into paleognaths (ratites and tinamou) and neognaths, with neognaths then being further split into Galloanseres (chickens and ducks) and Neoaves (a group containing 95% of avian species). This 3-way split is now found on morphological, nuclear, and mt data (e.g., Groth and Barrowclough 1999; Cracraft et al. 2004; Slack,

Delsuc, et al. 2006). In contrast, the basic divisions within Neoaves are not clear, and Cracraft (2001) suggests a 6-way split between:

passerines (or perching birds, Passeriformes),  
 parrots (Psittaciformes),  
 cuckoos (Cuculiformes),  
 woodpeckers/toucans, rollers/bee-eaters/kingfishers, jacamars/puffbirds, and mousebirds (Coliiformes, Coraciiformes, and Piciformes, jacamars and puffbirds sometimes split from Piciformes and placed in Galbuliformes),  
 owls, nightjars, swifts, and turacos (Strigiformes, Caprimulgiformes, Apodiformes, and Musophagiformes), and seabirds, shorebirds, doves, cranes, raptors, rails, penguins, storks, loons, and grebes (a very diverse group including the traditional orders Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Gaviformes, Gruiformes, Pelicaniformes, Procellariiformes, and Sphenisciformes).

Even though this division is modified somewhat in Cracraft et al. (2004), we use these 6 groups as an informal prior for evaluating results, first to see how well those groups stand up to further analysis and then to see how much resolution can be found in their branching order (if the 6 are supported). Of the 6 Neoaves groups, only 4 (passerines, an owl, a parrot, and the seabird/shorebird/raptor alliance) are currently represented in the complete mt data set.

Two members of another group (a toucan and woodpecker) are added here, leaving only cuckoos unrepresented in the avian tree from mt genomes. The largest group represented (seabird/shorebird/raptors/gruiformes) is very diverse and has various informal names such as Cracrafti or Conglomerati (Slack, Delsuc, et al. 2006) or simply “water carnivores”—because it includes the main carnivorous birds (raptors—buzzards, hawks, eagle, osprey, etc) and a large group of aquatic birds (shorebirds, seabirds, and penguins) that are carnivorous.

This 6-way split of Neoaves has been called an “explosive radiation” (see e.g., Poe and Chubb 2004). This expression raises concerns regarding the sufficiency of microevolutionary processes to explain macroevolution (Penny and Phillips 2004). As commonly used, the term “explosive radiation” implies that there are major examples where microevolution is unable to explain macroevolution. With the present example, we take “explosive radiation” to imply both

an unresolvable 6-way split, and simultaneous (geologically very fast) morphological and ecological radiation of the 6 Neoavian lineages.

Such major morphological changes would be difficult to explain using known microevolutionary processes. However, as yet we have no information regarding the rate of morphological change during the radiation. Using parrots to illustrate this example, there are 2 quite separate issues:

when the parrot lineage diverged from other Neoaves, and when the mix of morphological and ecological features arose by which we define modern parrots (the crown group).

In practice, the mix of parrot features could have occurred significantly after the divergence of the lineage. Therefore, we do not agree with the use of terms such as “explosive radiations” just because phylogeny is difficult to resolve (Poe and Chubb 2004), when evidence regarding the speed of morphological and ecological adaptation is unavailable. Instead of using explosive radiation, we use “adaptive radiation” when the divergences may be fast (in geological time), thus leading to short, difficult to resolve internodes. However, in such cases normal microevolutionary processes are sufficient to account for any adaptive component of the radiation. There are many examples of well-studied adaptive radiations (e.g., Lockhart et al. 2001). Thus, it is important when testing the 5 hypotheses of Penny and Phillips (2004) to determine the times of divergence of the Neoavian groups (see Slack, Jones, et al. 2006).

The third topic studied here is testing for increased stability of the avian tree by breaking up some long branches. Theoretical (Hendy and Penny 1989; Mossel and Steel 2005) and simulation-based (Hillis et al. 1994) as well as empirical studies (Anderson and Swofford 2004) show that breaking up long branches is important to increase the stability of a tree. Our experience with both mammalian (Lin et al. 2002; Phillips and Penny 2003) and avian (Slack, Delsuc, et al. 2006) mt genomes has strongly supported this conclusion—increased taxon sampling has increased the agreement between nuclear and mt data sets. Thus, it is important to improve taxon selection to get a reasonably stable

tree. As mentioned above, only 4 of Cracraft’s (2001) 6 Neoaves lineages are currently represented in the complete mt genome data set. A fifth proposed lineage corresponding to the group containing woodpeckers, rollers, bee-eaters, kingfishers, jacanas, and mousebirds is added here. The 2 species added are an ivory-billed aracari (a toucan, *Pteroglossus azara*) and a pileated woodpecker (*Dryocopus pileatus*). These 2 species are expected to be quite distantly related to each other but sufficiently close to lessen the effects of long-branch attraction from having just 1 member from this proposed group.

The other 5 new taxa are from the Conglomerati/Cracrafti/water-carnivore group, namely, osprey, forest falcon, kestrel, a pelican, and a heron. The novel raptors (osprey and forest falcon and American kestrel) were selected because the peregrine falcon has been difficult to place on the avian tree. Although predicted to be members of the water-carnivore group related to the other seabirds and shorebirds (Cracraft 2001), the falcon tended to come out basal to the passerines when few mt genome sequences were available (see discussion in Slack et al. 2003). With additional sequences, especially another raptor (e.g., buzzard), the falcon usually shifts into the water-carnivore group (Slack, Delsuc, et al. 2006). However, when a single parrot and/or owl sequences are included, the falcon can join with 1 of these groups, even when the buzzard is in the data set (Harrison et al. 2004). By contrast, the buzzard has never come outside the water carnivores (Slack, Delsuc, et al. 2006).

The reason for the instability on the placement of the falcon has not been identified. It could reflect some form of compositional bias (Phillips and Penny 2003) or a covarion-like shift like that reported in primates (Schmidt et al. 2005; see also Ane et al. 2005). However, given the instability of the falcon, we believed that it is important to add additional raptors into the data set. The osprey (*Pandion haliaetus*, Accipitridae) is often placed in the same family as the buzzard but is not a close relative. Similarly, a forest falcon (*Micrastur gilvicollis*, Falconidae) is expected to be deep on the falcon lineage (see Sibley and Ahlquist 1990), again breaking up a long branch. In addition, the American kestrel (*Falco sparverius*) and hawk eagle (*Spizaetus alboniger* [Asai et al., unpublished data]) fall within the falcon/forest falcon and osprey/buzzard groupings, respectively, and they would be expected to further stabilize this part of the tree.

The position of storks on the avian tree has also been uncertain (see Slack, Delsuc, et al. 2006; Slack, Jones, et al. 2006). As expected, they are within the Conglomerati, but have come closest to penguins, even when a turkey vulture (*Cathartes aura*) was included in the tree (Slack, Jones, et al. 2006). Based upon morphological/behavioral characters (Ligon 1967) and DNA–DNA hybridization (Sibley and Ahlquist 1990), it had been suggested New World (or cathartid) vultures like the turkey vulture should be grouped with the storks, rather than raptors. We have added a white-faced heron (*Ardea novaehollandiae*) and an Australian pelican (*Pelecanus conspicillatus*) to the mt data set to further examine the stork/penguin association.

Progress is made on each of the 3 questions discussed here. It appears as if a duplicated CR can be maintained for relatively long periods of time (tens of millions of years) by

gene conversion between the 2 copies, and the maintenance of a duplicated CR has the potential to explain the apparent homoplasy in mt gene order. We find evidence that the radiation of Neoaves is potentially resolvable. Although there is still methodological difficulty in resolving some parts of the tree due to short internodes, this does not mean unknown forces must be at work. Although these results need to be supported by nuclear data, unless further evidence were to come to light showing simultaneous morphological and ecological radiation also occurred, it is not necessary to postulate an “explosive radiation” (e.g., Poe and Chubb 2004) that would involve unknown evolutionary forces. Finally, adding additional taxa does seem to increase the stability of the avian tree.

## Materials and Methods

The forest falcon (*M. gilvicolis*) and aracari (*P. azara*) samples were provided by the Louisiana State University Museum of Natural Science Collection of Genetic Resources and are samples B-10720 and B-9081, respectively. The osprey (*P. haliaetus*) was provided by the Australian Museum (Sydney), sample EBU 37010, and the Australian pelican (*P. conspicillatus*) by the Museum of Victoria, sample number MV 1883. The white-faced heron (*A. novaehollandiae*) was provided by the Department of Conservation (Waikanae). The pileated woodpecker (*D. pileatus*) and the American kestrel (*Falco sparverius*) were collected in North Central Florida near Gainesville and were part of the Braun/Kimball laboratory tissue collection.

For the forest falcon, aracari, osprey, pelican, and heron, extractions of genomic DNA were taken from 25 to 50 mg of liver tissue using the High Pure PCR Template Preparation Kit (Protocol Vb; Boehringer Mannheim), according to the manufacturers instructions. To minimize the possibility of obtaining nuclear copies of mitochondrial genes, mt genomes were amplified in 2–3 long overlapping fragments (3.5–12 kb in length) using the Expand Long template PCR System (Roche Applied Science, Mannheim, Germany). The woodpecker and kestrel were also amplified in 2 long overlapping segments, although Eppendorf Triple Master Taq was used for long polymerase chain reaction (PCR). The products were excised from agarose gel using an Eppendorf gel extraction kit, and the long-range products were then used as templates for subsequent short-range PCR of overlapping fragments 0.6–3 kb in length. Primers were found by searching an electronic database maintained in our laboratory (described in Slack, Jones, et al. 2006) or by examining a list maintained by the Braun/Kimball group. Sequencing was performed using BigDye Terminator Cycle Sequencing reagents v3.1 according to the manufacturers instructions (Applied Biosystems, Foster City, CA), and the reactions sequenced on ABI 3730 automated sequencers (Applied Biosystems). Sequences were aligned in Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI) and manually edited and checked for complete agreement between sequences.

In some cases (e.g., length heteroplasmy in CRs from short nucleotide sequence repeats), PCR products were cloned using standard techniques with Promega pGemT

Easy Vector system and Invitrogen Max efficiency DH5 $\alpha$  competent cells. At least 3 clones were sequenced for each region to check for any PCR error. In all cases, overlaps between sequences were sufficient to ensure homology. Sequence identity was confirmed through a combination of Blast searches of the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/blast/>), confirmation of amino acid translation in coding regions and alignment with other species.

In addition to the 7 new avian sequences reported in this paper, 33 other complete avian mt genomes were included in the analyses (26 neognaths and 7 paleognaths). The 26 neognath taxa are chicken (*G. gallus*; GenBank accession number AP003317), Japanese quail (*C. japonica*; AP003195), Australian brush-turkey (*Alectura lathami*; AY346091), magpie goose (*Anseranas semipalmata*; AY309455), redhead (duck, *Aythya americana*; AF090337), greater white-fronted goose (*Anser albifrons*; AF363031), rifleman (New Zealand [NZ] wren, *Acanthisitta chloris*; AY325307), gray-headed broadbill (*Smithornis sharpei*; AF090340), fuscous flycatcher (*Cnemotriccus fuscatus*; AY596278), superb lyrebird (*Menura novaehollandiae*; AY542313), village indigobird (*Vidua chalybeata*; AF090341), rook (*Corvus frugilegus*; Y18522), morepork (NZ owl, *Ninox novaeseelandiae*; AY309457), kakapo (NZ parrot, *Strigops habroptilus*; AY309456), peregrine falcon (*Falco peregrinus*; AF090338), Eurasian buzzard (*Buteo buteo*; AF380305), Blythe’s hawk eagle (*S. alboniger*; AP008239), turkey vulture (*C. aura*; AY463690), blackish oystercatcher (*Haematopus ater*; AY074886), ruddy turnstone (*Arenaria interpres*; AY074885), southern black-backed gull (*Larus dominicanus*; AY293619), Oriental stork (*Ciconia boyciana*; AB026193), red-throated loon (*Gavia stellata*; AY293618), little blue penguin (*Eudyptula minor*; AF362763), black-browed albatross (*Diomedea melanophris*; AY158677), and Kerguelen petrel (*Pterodroma brevirostris*; AY158678). The 7 paleognath taxa are emu (*Dromaius novaehollandiae*; AF338711), southern cassowary (*Casuarius casuarius*; AF338713), great spotted kiwi (*Apteryx haastii*; AF338708), greater rhea (*Rhea americana*; Y16884), ostrich (*Struthio camelus*; Y12025), great tinamou (*Tinamus major*; AF338707), and elegant crested tinamou (*Eudromia elegans*; AF338710). The NZ moa (Cooper et al. 2001; Haddrath and Baker 2001) were omitted from the analyses for reasons discussed in Slack, Delsuc, et al. (2006) but do not affect this study. The issue of fine-tuning paleognath interrelationships will be readdressed once additional kiwi sequences become available (Gibb GC, in preparation).

## Phylogenetic Analysis

Sequences were aligned in SeAl v2.0a11 (Rambaut 1996), at the amino acid level for protein-coding genes, and based on stem and loop secondary structure for RNA genes. The data set has 12 protein-coding genes, 2 rRNAs and 21 tRNAs (lacking tRNA Phe). Gaps, ambiguous sites adjacent to gaps, the ND6 (light-strand encoded), and stop codons (often incomplete in the DNA sequence) were excluded from the alignment. The full data set had 13,139 bp, and the Neoaves-only data set had 13,323 bp.

In previous work (Phillips and Penny 2003; Delsuc et al. 2003; Harrison et al. 2004; Phillips et al. 2006), we found that RY coding of the most variable partitions of the nucleotide data (specifically the third-codon position) was advantageous. The recoding increases the proportion of observable changes on internal branches of the tree (tree-ness) and decreases the differences in nucleotide composition (relative compositional variability, RCV). It also increases concordance between mt and nuclear data sets. RY coding does increase the maximum likelihood (ML) scores, but because RY coding has amalgamated some nucleotide categories, the data is now different and it is not valid to compare directly the RY and nucleotide ML scores (Steel MA, personal communication). However, because of the better fit of the data to the model (higher tree-ness) and less variability in nucleotide composition (lower RCV), this is our preferred method of analysis of vertebrate mt data. Thus, the trees reported here have the third-codon positions recoded as R or Y. The full data set is available from our Web site <http://awcmee.massey.ac.nz/downloads.htm>.

ML analysis in PAUP\*4.0b10 (Swofford 1998) used likelihood settings from the best-fit model (Transversional model, TVM + I + G, both transition classes are treated equally) selected by both hierarchical and Akaike Information Criterion tests in Modeltest 3.7 (Posada and Crandall 1998). Preliminary results have shown current species fall into the expected 3 groups paleognaths, Galloanseres, and Neoaves (data not shown). Therefore, for further analyses we constrained the tree to these 3 groups as this drastically reduces analysis time (329 h reduced to 186 h for ML analysis of 40 birds). Maximum parsimony bootstrap analysis with 1,000 bootstraps was also carried out (data not shown). For MrBayes (Huelsenbeck and Ronquist 2001) analysis, the data was partitioned into 5 character sets (first codon, second codon, third codon with RY coding, RNA stems, and RNA loops, as in Harrison et al. [2004]), unlinked (except for topology), and run for  $10^7$  generations. Sampling of the Monte Carlo Markov Chain was assessed with Tracer v1.4 (Rambaut and Drummond 2003), and consensus networks (Holland et al. 2005) of MrBayes results were constructed with SplitsTree v4.3 (Huson and Bryant 2006).

## Results

The 7 new mt genome sequences have been deposited in GenBank under the following accession numbers: Ivory-billed aracari (*P. azara*: DQ780882; 18,736 bp); pileated woodpecker (*D. pileatus*: DQ780879; 16,832 bp); osprey (*P. haliaetus*: DQ780884); forest falcon (*M. gilvicolis*: DQ780881; 17,344 bp); American kestrel (*F. sparverius*: DQ780880; 17,507 bp); white-faced heron (*A. novaehollandiae*: DQ780878; 17,511 bp); and Australian pelican (*P. conspicillatus*: DQ780883; >16,846 bp [incomplete]).

Because of the potential utility of “rare genomic changes,” we first describe the gene orders in these 7 birds and give a model for the mode of transition between them. We will return to the significance of these findings in the discussion and provide explanation for the apparent high frequency of the mt gene order changes, which reduce the phylogenetic utility of this potentially highly informative

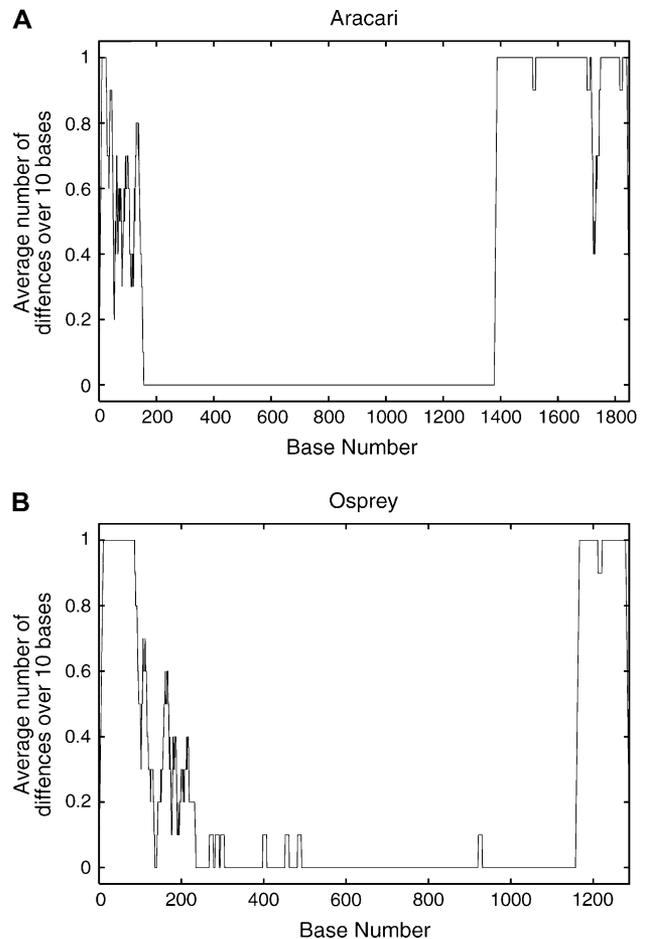


Fig. 2.—Comparison between CR(1) and CR(2) in Aracari (A) and Osprey (B) using the average number of differences in a sliding window of 10 bases.

data. The heron has the gene order first identified in the chicken (Desjardins and Morais 1990), whereas the forest falcon, kestrel, and woodpecker all have the alternative gene order where the CR lies between tRNAs Thr and Pro, and a second, unalignable, and often shorter noncoding region lies between tRNAs Glu and Phe (fig. 1E) that was first identified by Mindell et al. (1998). The noncoding regions in the forest falcon and kestrel, much like in the peregrine falcon (*F. peregrinus*, Mindell et al. 1998), are mostly repeats of a short sequence—a 4-bp repeat in the forest falcon and a 9-bp sequence in the kestrel. Both birds also have longer repeat sequences at the end of the first CR. The short woodpecker noncoding region has neither discernable repeats nor any similarity to the woodpecker CR.

The osprey and aracari both have the gene order previously described only in *Amazona* parrots (Eberhard et al. 2001), where the CR is duplicated and the repeated CRs lie between tRNAs Thr and Pro and Glu and Phe (fig. 1D). This is different to the gene order found in the falcon, as the 2 CRs are clearly duplicates and are easily alignable to each other. In both species, it is striking that the 2 CRs are nearly identical, differing only in the 5' and 3' ends (fig. 2). In the aracari, 1,230 bp are 100% identical between the 2 CRs, including a 90-bp repeat sequence at the 5' end. This

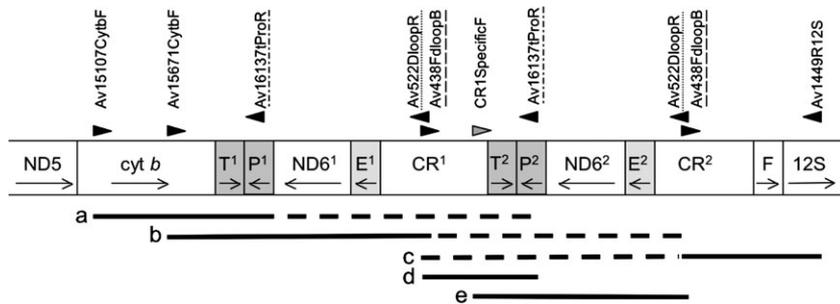


FIG. 3.—Primers used to sequence duplicate tThr–CR gene order. Arrows show the direction of the primer, and numbers in primer names refer to location relative to the chicken genome. Primers that bind twice are underlined with matching lines. Black lines labeled a–e are PCR products; dashed lines indicate longer unseen PCR products. Products a, b, and c can be aligned to completely miss the gene duplication found by d and e.

90-bp repeat occurs 6 or 7 times in the first CR, followed by a 71-bp truncated repeat (different clones contained different numbers of repeats). In the second CR, the 90-bp repeat sequence is repeated 4 times followed by a 15-bp truncated repeat, and then a 14-bp sequence repeated 7 or 8 times. The long repeats contribute to the genome length of 18,736 bp. In contrast, the osprey contains no repeat sequences but still has 99.2% similarity over 929 bp between the 2 CRs. Neither the aracari nor the osprey has identifiable remnants from ND6 or tRNA Glu repeats, as was found in *Amazona* parrots (Eberhard et al. 2001).

Abbott et al. (2005) reported that *Thalassarche* albatrosses have a duplicated region from tRNA Thr to the CR (fig. 1C). We have rechecked the *Diomedea* albatross sequence (*D. melanophris*; AY158677) reported in Slack, Jones, et al. (2006) and have identified a duplicate region in this species as well. Because 3 tRNA's plus ND6 are duplicated, as well as the CR, it is possible to miss the duplicated region using standard primer pairs (see fig. 3 and Discussion). The revised genome length for the albatross is now 18,967 bp, the longest avian mt genome reported so far. The duplicated segments are nearly identical, beginning with a 100% match for the last 51 bases of *cyt b*, followed by Thr/Pro/ND6/Glu/CR. The CRs differ by 21 mismatched bases near the start, and the last 114 bases of CR(1) are unalignable because CR(2) ends with a 22-bp sequence repeated 15 times. The pelican may also have the duplicate tThr–CR gene order (based on a sequence fragment containing CR/Thr/Pro). However, the region between CR(1) and tRNA Phe is currently incomplete, so we cannot rule out a nuclear mt copy (numt) or another gene order for this region.

The number of different mt gene order rearrangements in birds currently stands at 4. At this point, names such as “standard,” “normal,” “alternative,” “novel,” and “albatross” gene order start to lose their meaning, so a new naming system is required. Currently, only the standard avian gene order exists for paleognaths and Galloanseres (fig. 1B), so it is logical to assume this was the ancestral gene order at the root of the Neoaves. This order is only 1 rearrangement away from the presumably ancestral gene order found in many reptiles (fig. 1A). The 3 other orders require at least 2 rearrangements from the ancestral reptilian gene order. We refer to the order first found in the chicken (Desjardins and Morais 1990) as “ancestral avian,” the order first described in the falcon (Mindell et al. 1998) as

“remnant CR(2),” the order first described in the *Amazona* parrots (Eberhard et al. 2001) as “duplicate CR,” and the order first described in the albatross (Abbott et al. 2005) as “duplicate tThr–CR.” This proposal provides a systematic framework that allows the naming of any additional gene orders that might be discovered, for example, “duplicate ND6–CR,” or “remnant CR(1).” Using the term “remnant” can imply either a reduction from a full CR or a leftover part when the CR moved from 1 location to another. Either scenario is possible for the falcon, for example, so the name should not imply one over the other. We prefer this to “pseudo” or “noncoding” region, as the remnant CR has been called in the past (Mindell et al. 1998; Haring et al. 2001). In addition, duplicate sequences are labeled (1) and (2) from heavy-strand 5' to 3' for ease of notation, even if this may imply CR(1) duplicated from CR(2) (see Discussion). These different arrangements have been found in all parts of the Neoavian tree and do not uniquely define specific clades in the tree (see fig. 4).

#### Phylogenetic Analysis

We will return later to the significance of the gene order finds, but next report the ML tree for the 40 bird sequences. Slack, Delsuc, et al. (2006) reported that the improved taxon sampling has stabilized the root of the avian tree; as predicted earlier (Braun and Kimball 2002; Garcia-Moreno et al. 2003), there is now agreement between nuclear, mt, and morphological data. We have run the current data set with a reptilian outgroup, and the position of the root is again between neognaths and paleognaths (data not shown). However, excluding the more distantly related reptile species allows us to increase the total number of nucleotide positions from, approximately, 11,500 to 13,000. Therefore, we use the paleognaths as the outgroup to root the neognath tree. Figure 4 clearly resolves into the 3 main groups: paleognaths, Galloanseres, and Neoaves.

Having confirmed that including the 7 new Neoavian species does not lead to any unexpected effects, we are able to address the question of resolution within Neoaves. The groupings of paleognaths, Galloanseres, and Neoaves were constrained for further analyses in PAUP\*. We add 2 members of the fifth Cracraft (2001) group; an aracari and a woodpecker, intending to reduce problems of long-branch attraction that have hindered the placing of the morepork, kakapo, and falcon. As can be seen from

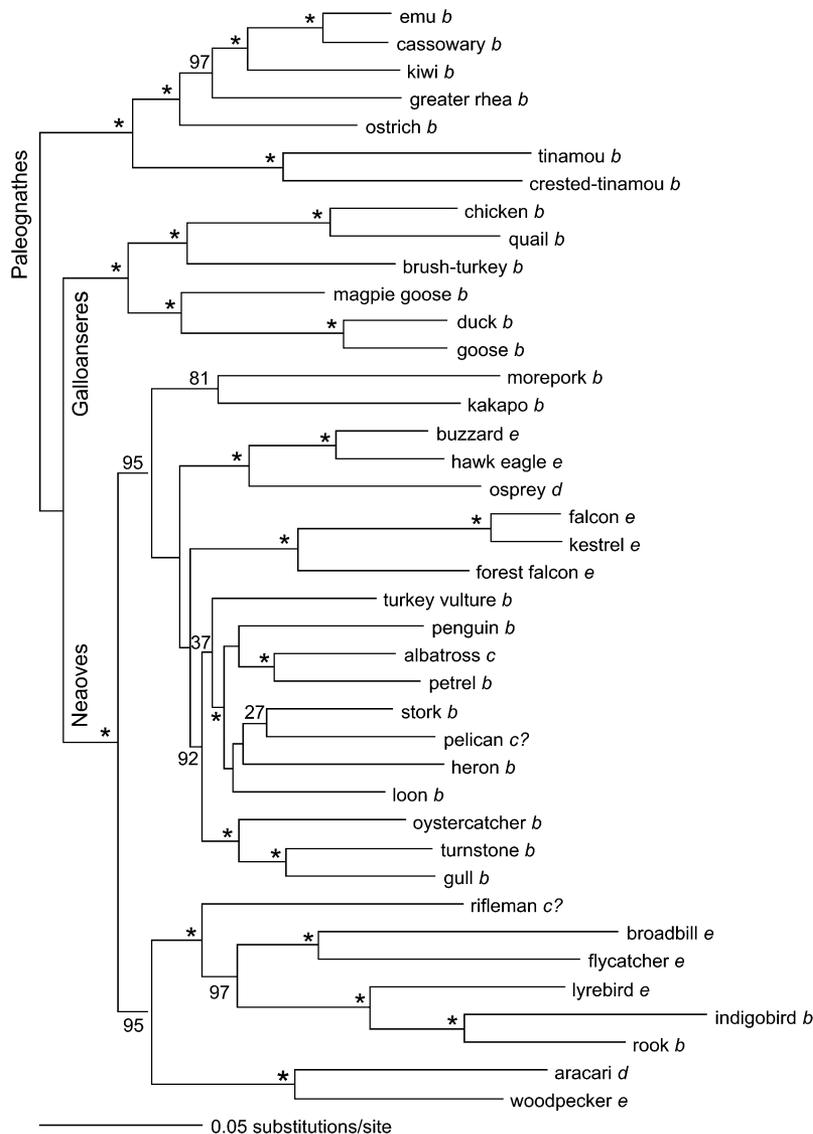


FIG. 4.—ML tree of 40 birds. Asterisks represent 100% support in Bayesian MCMC analysis in MrBayes, with support over 25% shown. The letters in italics after each species name refer to gene orders shown in figure 1.

figure 4, the aracari and the woodpecker pair together and join basal to the Passeriformes. The fifth Cracraft (2001) group has long been placed in a hypothetical grouping called the “higher land birds” that contains the passerines (e.g., Mayr et al. 2003). Although the exact set of avian taxa that should be included in this higher land bird group is unclear and some suggestions conflict with the Cracraft (2001) 6-way split, the proposed higher land-bird group suggests that finding Piciformes sister to passerines should not be viewed as unexpected. In fact, these results could be viewed as validating earlier morphological work suggesting that Piciformes and passerines are related (e.g., Shufeldt 1900; Livezey and Zusi 2001). Neither ML nor Bayesian analyses show any conflict in this placing. Given the congruence with other data, this result provides evidence that at least some of Cracraft’s (2001) 6-way split can be resolved and is initial evidence against any “explosive radiation” hypothesis (e.g., Poe and Chubb 2004).

The kakapo and the morepork (a parrot and an owl) fall between the aracari/woodpecker/passersine grouping and the “Conglomerati.” Although they currently come together, we expect this is in part because of a long-branch attraction because both are long, isolated branches. We are currently sequencing a lovebird and a barn owl to help resolve this grouping. It is interesting to note that when the parrot is omitted, the owl moves more toward the aracari/woodpecker/passersine grouping (MrBayes analysis, data not shown). Conversely, in the absence of the owl, the parrot (kakapo) is closer to the Conglomerati. There has been speculation based on morphological data that owls are closely related to the Falconiformes (e.g., Mayr and Clarke 2003; though not in Livezey and Zusi 2001); however, our molecular evidence does not appear to support this. The limited stability of the morepork and kakapo in the present tree combined with the very long branches involved suggests their current position may not reflect their

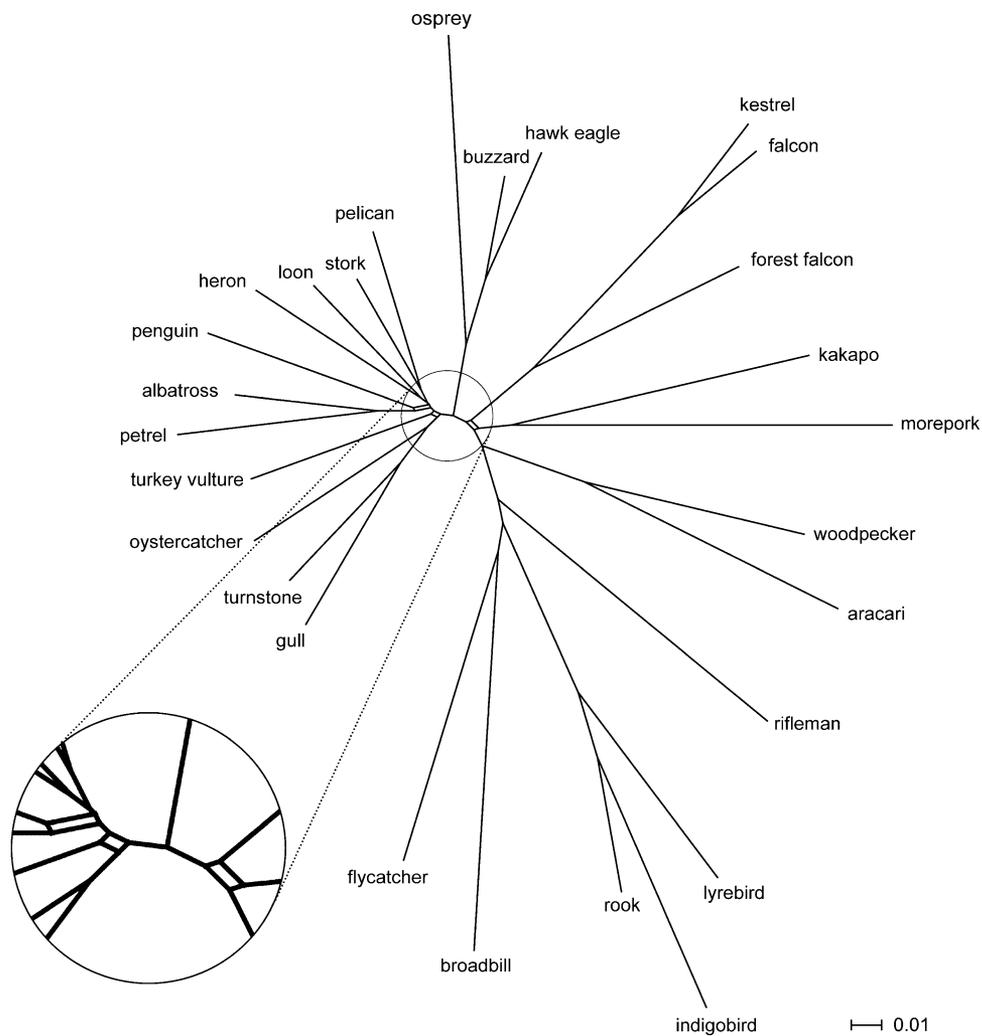


FIG. 5.—Consensus network for 27 Neoavian birds based on trees sampled by the Bayesian MCMC analysis using MrBayes. There is no conflict in splits with over 25% support; this network shows support greater than 20%.

phylogenetic position, so we feel it is best to defer judgment regarding these taxa until taxon sampling has been improved further.

Turning to the raptors, the falcon joins to the kestrel at a fairly shallow node, with the forest falcon joining deep on this branch, forming the Falconidae. The hawk eagle joins the buzzard, with the osprey joining deeper on this branch, forming the Accipitridae. Both groups are well supported in our data set and are expected from previous studies (e.g., Mayr and Clarke 2003; Cracraft et al. 2004). The addition of these 4 species has helped to stabilize the position of the falcon and the buzzard but interestingly have not joined the 2 groups into a strictly monophyletic group, though they may be paraphyletic. Considering the consensus network from MrBayes analysis (fig. 5), it appears the kakapo and morepork are still creating conflict for the Falconidae, pulling them out of the Conglomerati toward the passerine/Piciformes. When both the morepork and kakapo are removed from MrBayes analysis, releasing the Falconidae, they move further into the Conglomerati, and shorebirds (Charadriiformes) actually fall between Falconidae and

Accipitridae (data not shown). Although the positions of raptors and shorebirds are variable on a local scale on the tree, they all support the conclusion of Paton et al. (2002) that shorebirds are not deep in the avian tree—they are not “transitional” to modern birds. At present, we favor the suggestion that the tree is indicating an ancestral raptor group, members of which diverged toward new marine habitats.

The position of the turkey vulture is not completely resolved either. It falls within the seabird/shorebird part of the Conglomerati, rather than joining deep on the Accipitridae branch as predicted by some studies (Cracraft et al. 2004). However, the addition of further species in the seabird/shorebird group has shown that the turkey vulture also does not come within the Ciconiiformes (storks), as is sometimes suggested by current taxonomy (e.g., The American Ornithologists' Union Check-list, <http://www.aou.org/checklist/index>) and Sibley and Ahlquist (1990). Even though MrBayes analysis places the turkey vulture within the seabird/shorebird grouping with some conflict regarding its location, it does not fall closer to the stork than other

species (fig. 5). Again, it is a single isolated branch on the tree.

We have added 2 further members to the seabirds/shorebirds portion of the Conglomerati: a heron and a pelican. The most significant change is that the penguin/stork association (see Slack, Delsuc, et al. 2006; Slack, Jones, et al. 2006) was not seen in our analyses. The penguin (Sphenisciformes) and the albatross/petrel (Procellariiformes) are now united, though the relationship of these taxa to the loons (Gaviiformes) and other groups is not well defined (figs. 4 and 5). Although it may seem surprising that the pelican and storks are relatively close, examining the positions of these species in Sibley and Ahlquist (1990) shows these were in fact probably the closest relatives present in the data set (excluding the turkey vulture). In standard taxonomies, the heron and the stork should group together in Ciconiiformes, perhaps with the turkey vulture (discussed earlier), but separate from the loon, penguin, and pelican. The MrBayes analysis still reveals conflict in the placement of these species, so it is unsurprising that sometimes these species group in slightly different conformations, depending on the analyses conducted. Now that the heron has been added, the loon does not pair with the stork or penguin in either ML or Bayesian analyses. However, there are very short internal edges on these branches suggesting that additional species, improved methods, and nuclear sequences could all help here. Despite discrepancies in positioning within this group, the group as a whole (albatross, petrel, penguin, stork, loon, heron, and pelican) has 100% Bayesian support (fig. 4). As a caveat to this, it is important to note that high Bayesian or bootstrap support does not necessarily mean the tree is “right,” and phylogenetic inferences need to take this into account (Phillips et al. 2004). There is still work to be done in untangling the deeper phylogenetic resolution within seabirds and shorebirds.

## Discussion

The most commonly suggested model for gene rearrangement involves duplication, followed by the reduction or loss of 1 copy (e.g., Bensch and Härlid 2000; Sano et al. 2005). Although it is impossible to re-create exactly how the gene rearrangements took place, 1 scenario is that gene rearrangements began as the result of a duplication of tThr/tPro/ND6/tGlu/CR (to give fig. 1C) and that the duplicate tThr–CR, duplicate CR, and remnant CR(2) are intermediates in the reduction of 1 of each of these duplicates (see fig. 1D and E). For example, duplicate tThr–CR gene order shows little reduction in either copy; the duplicate CR order still has 2 CRs, but only 1 tRNA Thr, and small pseudofragments of ND6(1) and tRNA Glu(1), and finally, the remnant CR(2) gene order has only one copy of each gene-coding region, with CR(2) reduced to a short noncoding fragment. Continuing with this scenario, it would also be possible that the first duplication reduced again instead of the second, returning to the ancestral avian order. A different model for gene rearrangement is that gene region duplication is sloppy, sometimes duplicating the whole fragment tThr–CR, at other times just the CR, or something in between. This method would make each gene order the result of a dif-

ferent type of gene duplication. This second scenario requires the duplication to be reinserted between tRNA Thr and tRNA Pro, rather than adjacent to the first copy. However gene rearrangement has occurred, it has still happened more than once. Each time a gene duplication or rearrangement has occurred, it could (in principle) have been by a different pathway.

Even more so than the *Amazona* parrots (Eberhard et al. 2001), the duplicate CRs are very similar in both the aracari and osprey. The aracari is particularly striking as the first 142 bp of the 2 CRs do not align (about 50% of bases are mismatched), and then the following 1,230 bp are 100% identical (fig. 2). The last 90 bp of CR(1) similarly does not align to CR(2), which has a 14-bp sequence repeated 7 or 8 times. It is possible that this main central region reflects very recent gene duplication, with no time for mutations in 1 of the CRs. More likely, given our knowledge of concerted evolution, it is concerted evolution that has kept both copies identical. This could be tested by examining additional toucans because the existence of multiple taxa with virtually identical CRs would be more parsimoniously explained by concerted evolution rather than multiple independent duplications, each without sufficient time for the duplicated sequences to diverge. However, even when our attention is limited to the aracari, we observed that the first scenario (recent duplication) does not explain the few hundred mismatched bases at start and end of the CRs given that the average size of the intergenic spacer between tRNAs Thr and Pro in Neoaves is 6–14 bases (Slack et al. 2003). The second scenario (concerted evolution) would explain the unalignable nature of the ends of the CRs, which could be remnants accumulating as 1 CR replaced the other. Eberhard et al. (2001) also suggested concerted evolution when they showed that paralogous CRs were more alike than orthologous copies with nearest phylogenetic neighbors (the scenario we predict for toucans).

Many authors have suggested mechanisms that would cause gene rearrangements in a circular genome, although the exact mechanism is unknown. These include recombination, slipped-strand mispairing, errors in synchronizing the points of initiation and termination, and illicit priming of replication by tRNAs near the replication origin (e.g., Mindell et al. 1998; Mueller and Boore 2005). The gene regions that are rearranged in birds are also predominantly coded by the heavy strand and are near the origin of heavy-strand replication. It is possible that any combination of these mechanisms could be responsible for the gene orders seen in birds (Mueller and Boore 2005).

Bensch and Härlid (2000) also discuss how the CR gene rearrangements may have occurred and suggest duplication of a region followed by multiple deletions. In their figure 1C, they show a hypothetical reconstruction of an intermediate stage between ancestral avian gene order and the remnant CR(2) gene order found in the willow warbler. This hypothetical intermediate shows a duplication of tPro/ND6/tGlu/CR that is reduced to the remnant CR(2) gene order. This is very similar to the gene order that has now been found in albatross and possibly pelicans (except without the duplication of tRNA Thr). Even more interestingly, in preliminary work we have found this gene

order in the Hihi (NZ stitchbird, *Notiomystis cincta*), probably a basal corvid (Meliphagidae—Honeyeater) (Gibb GC, in preparation).

Because of the duplicate nature of the tThr–CR repeat, it is possible to completely miss the second duplication using standard primer pairs (see fig. 3). PCR primers will be more likely to amplify the shorter fragment, with the longer fragment going undetected. For example, Cyt *b*–CR primer pairs may preferentially amplify CR(1), rather than all the way to CR(2) (fig. 3, b) and CR–12S primer pairs may preferentially amplify CR(2), not the longer CR(1)–12S (fig. 3, c). Additionally, because CR(1) and CR(2) can be nearly identical, it is possible to align the first part of CR(1) to the second half of CR(2) and miss an entire duplication. To correctly determine whether a duplication exists, primer pairs that have not traditionally been used are required, for example, CR forward with tRNA Pro reverse (fig. 3, d). A positive sequence result, crossing gene boundaries, will indicate the existence of a duplicated gene region (rather than just primers binding incorrectly). Of course, a negative PCR result does not completely disprove the existence of a duplicated region! We recommend adding this diagnostic primer combination when sequencing any avian genome. Currently, we are checking all bird species sequenced in this lab for the possibility of previously undetected gene duplications.

Mindell et al. (1998) tested 137 bird species representing 13 orders for the 2 gene orders known in 1998 (ancestral avian and remnant CR(2)). It is not clear whether all DNA regions mentioned in their paper were analyzed or just the gene regions shown with positive results in their Table 1. If all regions shown in their figure 1 were tested in all their species, then we can discount the presence in those species of the other gene orders discovered since 1998. However, if DNA regions were tested sequentially, stopping after encountering a positive result, then gene orders such as duplicate tThr–CR or duplicate CR may have been overlooked, for the reasons discussed above (see fig. 3).

The significance of multistate characters (such as gene order) in phylogeny is still being developed. Elementary logic indicates that shared character states that are genuinely unique must be informative for phylogeny, and indeed, under such models parsimony is a ML estimator (see Steel and Penny 2004, 2005). The positions of insertions/deletions in genes (as used in Fain and Houde 2004 and others, e.g., Kimball et al. 2001; Allen and Omland 2003; Kawakita et al. 2003) are potentially such unique characters. Others include gene order (Henz et al. 2005), gene fusions (e.g., Stechmann and Cavalier-Smith 2002), the presence or absence of repetitive elements (e.g., SINEs—short interspersed nuclear elements, Shedlock and Okada 2000, or long interspersed nuclear elements, Kriegs et al. 2006). The use of such characters is reviewed in Rokas and Holland (2000) and Boore (2006) under the grouping of “rare genomic changes.” In contrast, for primary sequence data the state space is 4 for nucleotides (or 2 after RY coding) and 20 for amino acids characters, and it is both expected with such a small state space that the same character state will arise multiple times and the standard maximum average likelihood is the preferred estimator (Steel and Penny 2000).

Thus, there is a good theoretical basis for using rare genomic changes in the resolution of phylogeny, if the number of character states is so large that parallel changes and/or reversions are unlikely. However, these multistate characters are fraught with difficulty, and care will always be required to check for reversals. For example, Stechmann and Cavalier-Smith (2002) proposed an alternative with the rooting between (animals, fungi, and choanazoa/choanoflagellates) and all other eukaryotes. This is based on a gene fusion between dihydrofolate reductase and thymidylate synthase genes. These genes are fused in most eukaryotes but not in the animal/fungi/choanazoa group mentioned above. However, it is also known that reversals (fissions, when 2 genes end up separate) do occur (Snel et al. 2000), and so relying on a single gene fusion is risky. With SINEs, sequences around the insertion can be checked that the insertion is at precisely the same place (Shedlock and Okada 2000). We refer to such genomic characters with an extremely large state space as SCUDs. They may be extremely effective but used carelessly that can be highly damaging to the user! If there is a genuine very large character space, then they can be highly effective for phylogenies. Waddell et al. (2001) concluded that 3 SINEs were able to give 95% confidence limits to polytomies of 3 taxa, even when groups were so closely related that lineage sorting was the difficulty with sequence data. In the future, it will be very interesting to integrate SCUDs with likelihood values from sequence data (Steel and Penny 2005).

On a more positive note, although mt gene order in birds is not yet useful as a multistate character, it appears that the Neoavian radiation will be resolvable, ending the suggestion that it represents an “explosive radiation” (e.g., Poe and Chubb 2004). Currently, we are not getting the raptors as a monophyletic group. Although this may be unfortunate for a taxonomist who might like organisms in neatly arranged boxes, it may be of more importance to an ecologist or evolutionary biologist. The implication from figures 4 and 5 is that there was an early group of raptors (in the late-Cretaceous to fit with the timing from Slack, Delsuc, et al. 2006) from which a variety of other carnivore groups have adapted to a more aquatic environment. The present data set, with 7 raptors and 10 sea and shorebirds, shows this clearly. Similarly, grouping the woodpecker/aracari clade (Piciformes) with the passerines is consistent with expectation based upon prior data (e.g., Mayr et al. 2003) and indicates that the grouping reflects evolutionary history. This suggests resolution of Cracraft’s 6 Neoaves groups, and rigorous testing of the monophyly of those groups is possible. This is strong evidence that there is not an irresolvable 6-way split at the base of Neoaves. The addition of the sixth group (cuckoos) along with taxa that can subdivide the long terminal edges corresponding to the morepork (owl) and kakapo (parrot) are likely to further resolve the avian tree. Because an evolutionary tree is not an end in itself but a guide toward answering biologically significant questions, we assert that the present tree provides evidence against an “explosive radiation” at the base of the Neoaves and suggests that birds with a terrestrial raptor phenotype may be ancestral to a wide range of other carnivores, especially marine carnivores. This tree just represents a starting point for biological studies, and further

resolution of the tree will increase the insights from those biological studies.

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