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# Diversification of Neoaves: integration of molecular sequence data and fossils

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**Patterns of diversification and timing of evolution within Neoaves, which includes almost 95% of all bird species, are virtually unknown. On the other hand, molecular data consistently indicate a Cretaceous origin of many neoavian lineages and the fossil record seems to support an Early Tertiary diversification. Here, we present the first well-resolved molecular phylogeny for Neoaves, together with divergence time estimates calibrated with a large number of stratigraphically and phylogenetically well-documented fossils. Our study defines several well-supported clades within Neoaves. The calibration results suggest that Neoaves, after an initial split from Galloanseres in Mid-Cretaceous, diversified around or soon after the K/T boundary. Our results thus do not contradict palaeontological data and show that there is no solid molecular evidence for an extensive pre-Tertiary radiation of Neoaves.**

**Keywords:** Neoaves; phylogeny; nuclear DNA; fossils; molecular clock; divergence times

## 1. INTRODUCTION

Birds are used as model organisms in many fields of biology, and the lack of a thorough understanding of their systematics has often compromised interpretations of experiments and observations. The DNA–DNA hybridization studies of Sibley & Ahlquist (1990) have repeatedly been criticized for methodological reasons (Harshman 1994; Cracraft *et al.* 2004), and the few cladistic analyses of Neoaves with dense taxon sampling show poor resolution of

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the deep divergences (Livezey & Zusi 2001; Cracraft *et al.* 2004; Fain & Houde 2004). DNA sequence data have begun to clarify interfamily relationships for a handful of higher level groups such as some aquatic birds (van Tuinen *et al.* 2001), ‘higher land birds’ (Johansson *et al.* 2001; Mayr *et al.* 2003), shorebirds (Ericson *et al.* 2003; Paton *et al.* 2003) and passerines (Barker *et al.* 2004). Recent analyses of morphological and molecular data support a sister group relationship between Galloanseres (land- and waterfowl) and all other neognathous birds, the Neoaves (Livezey & Zusi 2001; Mayr & Clarke 2003; Cracraft *et al.* 2004; Fain & Houde 2004). However, there are no hypotheses concerning the most basal neoavian divergences, except for the proposed division of the group into Metaves and Coronaves based on an analysis of  $\beta$ -fibrinogen sequence data (Fain & Houde 2004).

Molecular clock analyses have suggested that the earliest diversification of Neoaves had already occurred in the Cretaceous (Hedges *et al.* 1996; Cooper & Penny 1997; Cracraft 2001; van Tuinen & Hedges 2001). However, there are few neoavian fossils from the Cretaceous (Hope 2002; Feduccia 2003) and instead the palaeontological record suggests that only a few neoavian lineages existed at the end of the Cretaceous, 65 Myr ago (Feduccia 2003). The considerable diversity of stem group representatives of modern neoavian taxa, which is evident in the Early Eocene 50 Myr ago (Mayr 2005), would thus result from a rapid diversification of taxa, which filled the many vacant ecological niches after the K/T boundary (Feduccia 2003). There is an apparent conflict between earlier molecular datings and the palaeontological record—but is this conflict real? The molecular dating methods must be correctly calibrated to yield reliable data, and this has not previously been done in studies including Neoaves. Since all Cretaceous fossils of neornithine birds are very fragmentary (Hope 2002) and their identification is often uncertain (Hope 2002), most calibrations have so far used a calculated age for the split between galliforms and anseriforms (90 Myr ago) which is in turn based on the diapsid/synapsid split age at 310 Myr ago (Hedges *et al.* 1996). However, Graur & Martin (2004) have argued convincingly that this estimate is not reliable, and nor are any of the calibration points that are based on it. Here, we employ an alternative strategy and use multiple fossils of more recent neoavian groups as internal calibration points in order to test the different diversification models suggested by Penny & Phillips (2004).

## 2. MATERIAL AND METHODS

Traditional classification recognizes 145 families in Neoaves (Morony *et al.* 1975). We obtained genomic DNA from blood or tissue samples of 87 neoavian species representing 75 families. Charadriiformes (shorebirds and allies, 19 families in total) and Passeriformes (passerines, 57 families in total), which have been shown to be monophyletic (Ericson *et al.* 2002, 2003; Paton *et al.* 2003; Barker *et al.* 2004), are represented by four and two families, respectively. At least one genus was sampled from the remaining neoavian families. Two palaeognaths (*Rhea* and *Apteryx*), one megapode and one screamer, were used as outgroups following the well-established hypothesis that Palaeognathae are the sister taxon of Neognathae, and that Galloanseres, in turn, are the sisters of Neoaves (Groth & Barrowclough 1999). The sample information and GenBank accession numbers are given in the electronic supplementary material.

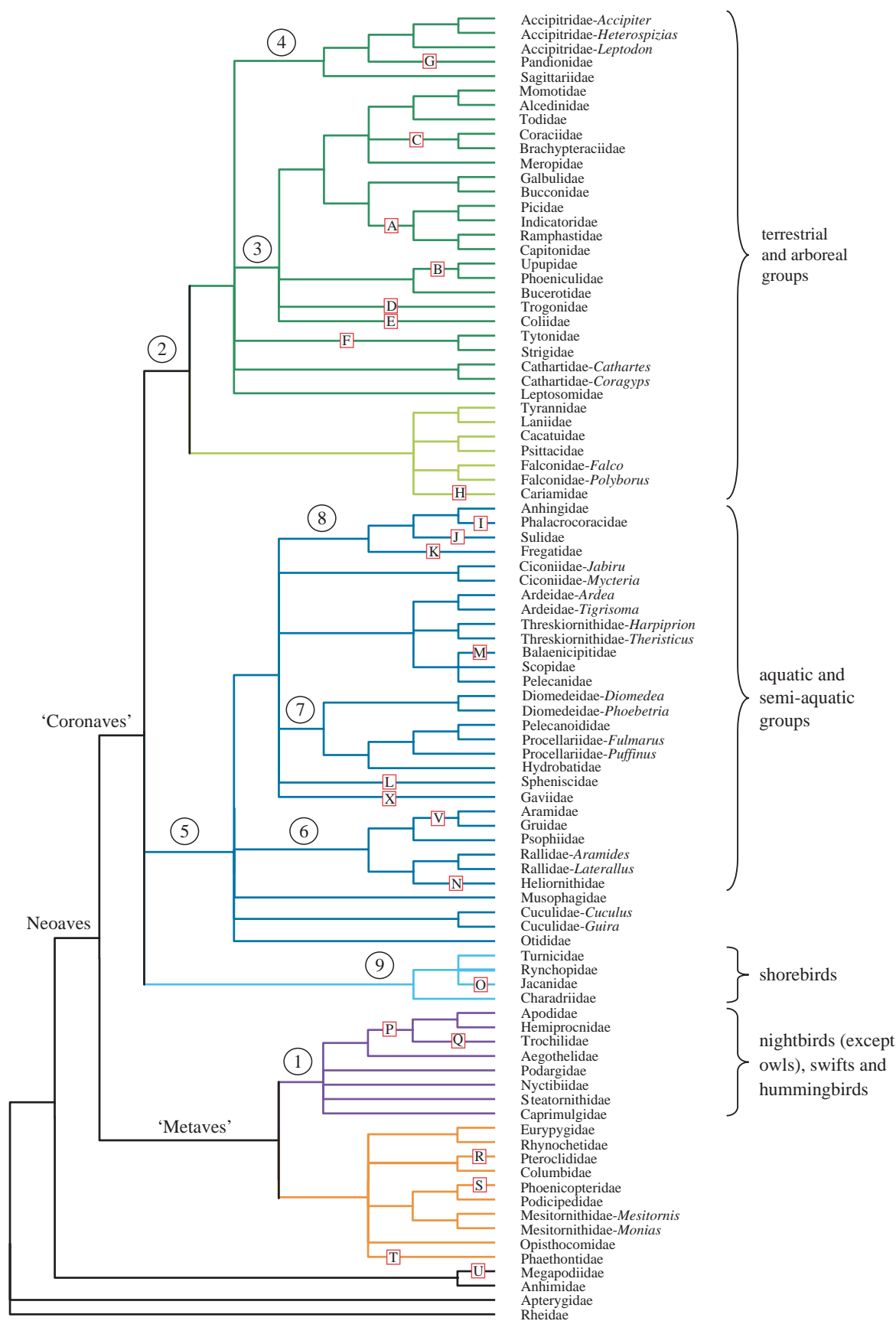


Figure 1. Family-level relationships within Neoaves estimated by Bayesian analysis of five nuclear genes (5007 nucleotide positions). Nodes that received a posterior probability value of less than 95% have been collapsed. Note that the branch lengths are not proportional to the number of nucleotide substitutions along each branch. Neoavian families fall into a few reciprocally monophyletic clades (coloured) that roughly correspond to ecological adaptations of extant taxa. Nodal numbers correspond to clades discussed in the text. Letters in boxes, referring to table 3 in the electronic supplementary material, indicate fossil calibration points.

The aligned dataset consists of 5007 bp obtained from five gene regions: *c-myc* (exon 3), *RAG-1*, myoglobin (intron 2),  $\beta$ -fibrinogen (intron 7) and ornithine decarboxylase (introns 6 and 7, along with the intercepting exon 7). For laboratory procedures, alignments, selection of models for nucleotide substitutions, parsimony analysis and Bayesian analyses of individual gene regions, see electronic supplementary material.

Divergence times were estimated using two rate-smoothing methods, penalized likelihood (PL; Sanderson 2002) and PATHd8 (Britton *et al.* 2006). PL combines a model that overfits the data with a penalty for fast-rate changes between mother and daughter lineages. PATHd8 smoothes substitution rates between sister groups, instead of mother–daughter lineages, by sequentially taking averages over path lengths from an internode to all its descending terminals. Both PL and PATHd8 need one fixed calibration point. For this purpose, we used a 47.5-Myr-old stem group representative of hummingbirds. We also constrained the root of the tree (the stem species of extant birds) to a maximum age of 100 Myr. An additional set of 21 stratigraphically and phylogenetically well-studied fossils were used as minimum age constraints. All the fossils used for calibrations (see electronic supplementary material) are stem group representatives of extant higher level taxa and provide a minimum age for the divergence of the total group (stem and crown group) from its sister taxon.

### 3. RESULTS AND DISCUSSION

Our Bayesian analysis of the dataset resulted in a well-resolved and strongly supported topology defining several clades within Neoaves (figure 1). The obtained tree topologies, one from the combined data, one without  $\beta$ -fibrinogen and one based on  $\beta$ -fibrinogen only, are similar in many respects, providing further evidence for a strong phylogenetic signal in the analysed data (see electronic supplementary material).

The combined data support a basal dichotomy into Metaves and Coronaves as proposed by Fain & Houde (2004). However, monophyly of Metaves (doves, sandgrouse, mesites, flamingos, grebes, kagu, sunbittern, hoatzin, tropicbirds, swifts, treeswifts, hummingbirds and nightbirds) is retained only if the  $\beta$ -fibrinogen data are included. Moreover, our Bayesian analysis of the  $\beta$ -fibrinogen data alone did not provide a strong support for Metaves even though this group was originally defined in an analysis based on this gene (Fain & Houde 2004). We obtained strong support for Metaves only after the inclusion of all genes, which shows that all or some other genes also contain a phylogenetic signal for Metaves, albeit this signal seems to be weak. Our data also strongly support the recently suggested flamingo–grebe clade (van Tuinen *et al.* 2001; Cracraft *et al.* 2004; Mayr 2004).

All trees support a clade including nightbirds (the traditional ‘caprimulgidiforms’ but not owls) and apodiform birds (swifts and hummingbirds; figure 1, node 1). Our results confirm a sister group relationship between the owl–nightjars and Apodiformes (Mayr 2002; Cracraft *et al.* 2004), and for the first time, suggest monophyly of a clade that includes all the taxa traditionally placed in ‘Caprimulgidiformes’ and Apodiformes. The obtained topology suggests that the diurnal Apodiformes evolved within a radiation of nocturnal birds, indicating a nocturnal ancestor of Apodiformes.

Strongly supported is a previously unrecognized major clade (Johansson *et al.* 2001; Mayr *et al.* 2003), which includes diurnal birds of prey, seriemas, parrots and the ‘higher landbird assemblage’ (figure 1, node 2). For the majority of families, traditionally included in the orders Coraciiformes and Piciformes

(figure 1, node 3), the same internal relationships have been recovered as in other recent molecular analyses (Johansson *et al.* 2001; Johansson & Ericson 2003; Mayr *et al.* 2003).

In concordance with other recent analyses (Sibley & Ahlquist 1990; Cracraft *et al.* 2004; Fain & Houde 2004), our data recover a clade (figure 1, node 4) that includes the secretarybird and accipitrid diurnal birds of prey (osprey, hawks and allies) to the exclusion of falcons. This grouping is recovered in separate analyses of four of the five investigated genes. The New World vultures clearly have their affinity with other raptors and not with storks (contra, e.g. Sibley & Ahlquist 1990).

Another well-supported clade includes birds with various aquatic or semi-aquatic adaptations (figure 1, node 5), as well as, in unresolved basal positions, the terrestrial turacos, bustards and cuckoos. The well-supported groupings within this clade are the ‘core-gruiforms’ (i.e. cranes, limpkin, rails, finfoots and trumpeters; figure 1, node 6), procellariiforms (albatrosses, storm-petrels, diving petrels, petrels and shearwaters; figure 1, node 7) and a group consisting of the anhingas, cormorants, gannets and frigatebirds (figure 1, node 8). As suggested previously, pelicans group not only with shoebill and hamerkop (Cottam 1957; Livezey & Zusi 2001; van Tuinen *et al.* 2001; Cracraft *et al.* 2004), but also with herons and ibises. Penguins, loons and storks also belong to this clade. The results confirm that the traditional Pelecaniformes and Ciconiiformes are not monophyletic. The shorebirds (figure 1, node 9) are in an unresolved position relative to the two major clades of terrestrial/arboreal and aquatic/semi-aquatic groups, respectively.

The PATHd8 analysis suggests that although the earliest diversification of Neoaves took place in the Late Cretaceous, the majority of higher level phylogenetic splits in Neoaves occurs after the K/T boundary (figure 2). The pattern of divergence obtained from PATHd8 and PL is similar with both the methods. However, PL adds an average ‘ghost range’ of 21 Myr to all the fossil records, and hence provides systematically older ages. We therefore consider the PATHd8 result to be the more reliable one (a comparison between the PATHd8 and the PL chronograms, and age estimates for major bird groups, are placed in the electronic supplementary material). The differences between the results of the PATHd8 and PL analyses leave open the question of how many stem lineages of neoavian birds existed before the K/T boundary. While the PATHd8 analysis suggests that there were only a few (model 2 of Penny & Phillips 2004), the PL analysis (see electronic supplementary material; Figure 9) indicates that there may have been more lineages of which some may have already obtained the ecological adaptation of their crown group representatives (model 4 of Penny & Phillips 2004). The present reconstruction of the phylogeny and divergence times of Neoaves accounts for both molecular and palaeontological data. It disagrees with the claim that molecular data indicate a deep Cretaceous diversification of neoavian birds (cf. Hedges *et al.* 1996; van Tuinen & Hedges 2001; corresponding to model 5 of Penny & Phillips 2004).

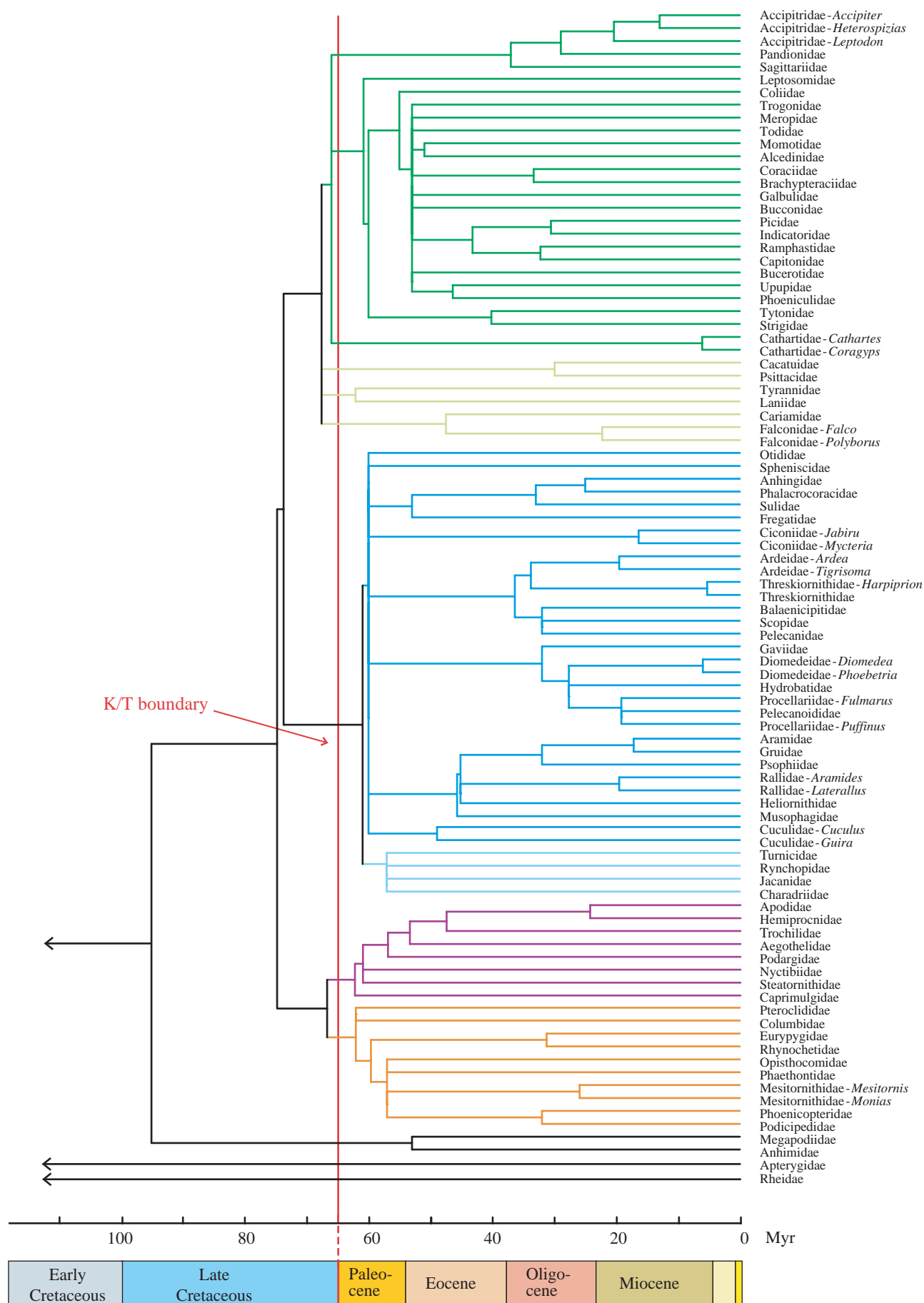


Figure 2. Chronogram (calibrated ultrametric tree with branch lengths proportional to time) for Neoaves estimated using PATHd8. Note that the split between Palaeognathae (represented by Rheidae and Apterygidae) and Neognathae is not shown, but estimated to be 177 Myr ago. We do not consider this age to be reliable due to difficulties in aligning the intron sequences of palaeognaths with those of the other taxa.

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