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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 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 β-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.

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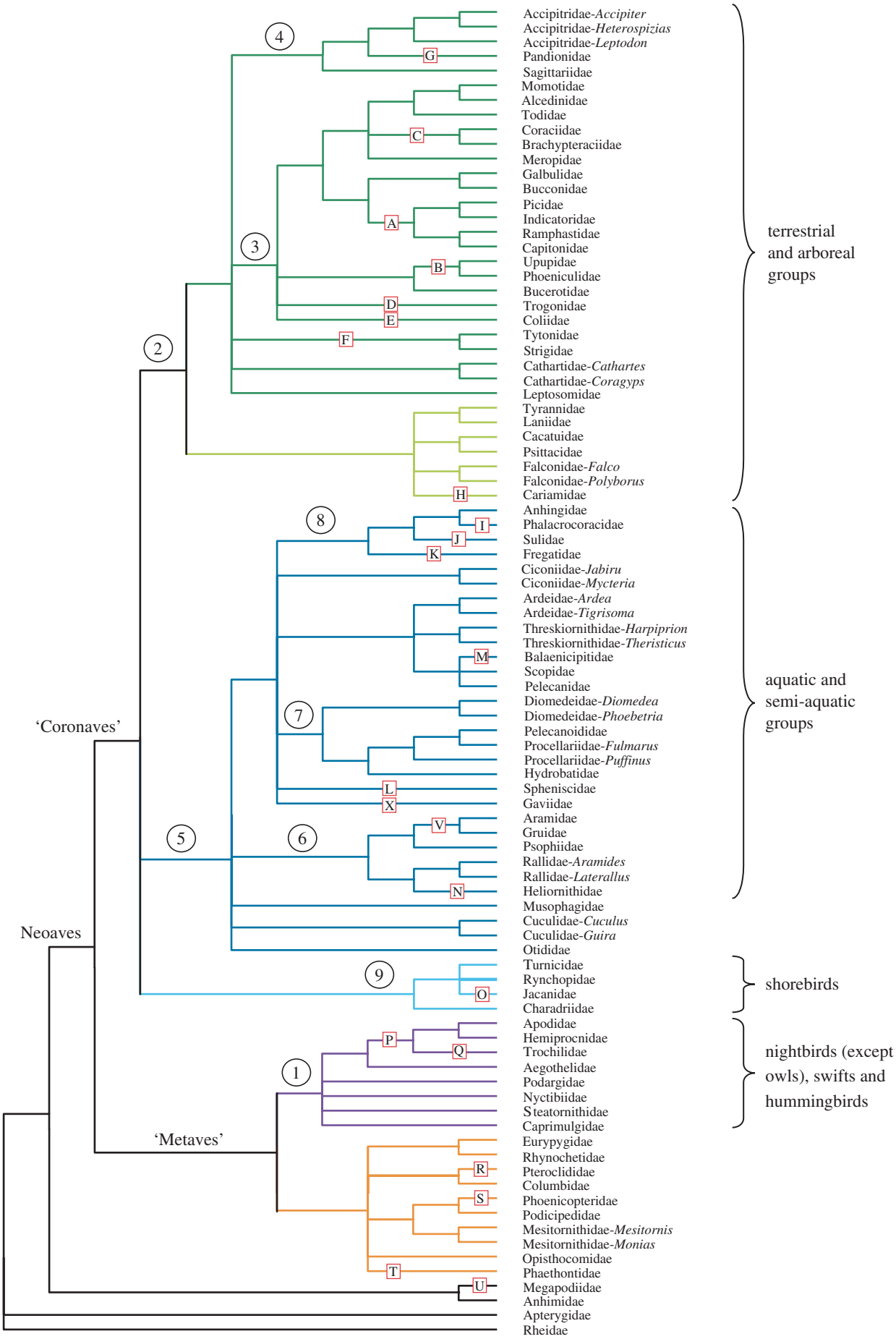

Figure1. (*Caption Opposite.*)

Figure 1. (*Opposite*.) 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), β-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 β-fibrinogen and one based on β-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 β-fibrinogen data are included. Moreover, our Bayesian analysis of the β-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 'caprimulgiforms' but not owls) and apodiform birds (swifts and hummingbirds; figure 1, node 1). Our results confirm a sister group relationship between the owlet-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 'Caprimulgiformes' 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

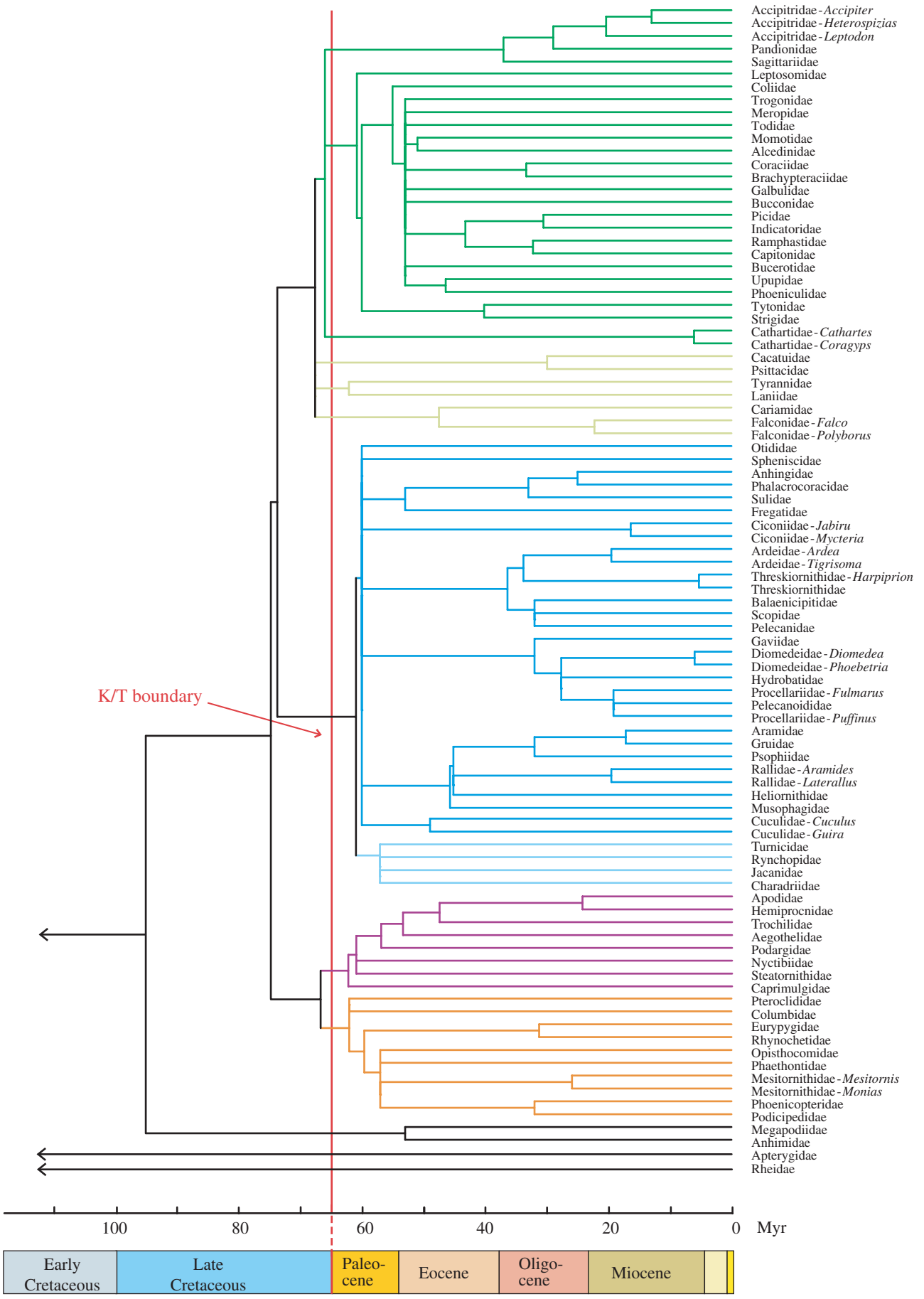

Figure 2. Chronogram (calibrated ultrametric tree with branch lengths proportional to time) for Neoaves estimated using PATHd8. Note that the split between Palaeognathae (represented by Rheidae and Apterygidae) and Neognathae is not shown, but estimated to be 177 Myr ago. We do not consider this age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

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).

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Electronic Supplementary Material for:

Diversification of Neoaves: integration of molecular sequence data and fossils

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Samples and sequence data

DNA was obtained from blood or tissue samples of 87 species in Neoaves representing 73 non-passerine and two passerine families. Two paleognaths, one megapode and one screamer were used as outgroups. Sample information and GenBank accession numbers are given in table ESM-1. The same species and individuals were sequenced when possible, but in a few cases when amplification failed we had to use sequences from other individuals or species.

Five gene regions were sequenced: c-*myc* (exon 3), RAG-1, myoglobin (intron 2), β-fibrinogen (intron 7) and ornithine decarboxylase (ODC) (introns 6 and 7, along with the intercepting exon 7). Laboratory procedures for the extraction, PCR-amplification, and sequencing follow published protocols (Prychitko & Moore 1997; Ericson et al. 2000; Irestedt et al. 2001; Johansson et al. 2002; Irestedt et al. 2002; Allen & Omland 2003). For myoglobin no sequences were obtained for *Cacatua*, *Rhinopomastus* and *Upupa*, and only the 3´ end could be sequenced for *Fregata*. For β-fibrinogen we could not obtain sequences for *Upupa epops.* The β-fibrinogen sequences of the two parrot species were highly autapomorphic. *Indicator*, *Megalaima* and *Picumnus* could not be sequenced for ornithine decarboxylase. Especially for β-fibrinogen we used sequences published in GenBank (table ESM-1). Most often we could use sequences obtained from individuals of the same species or genus as our own samples, but in a few cases we instead used a representative of another genus of the same family. The effect of this on tree topology is probably negligible given the short genetic distances between extant members within a family compared to the distance to the closest taxon outside the family.

Alignment of the protein-coding genes (c-*myc* and RAG-1) was unproblematic with few indels observed. Also the myoglobin, β-fibrinogen and ODC introns could be aligned by eye although the number of indels was larger. The preferred alignments can be downloaded at http://www.nrm.se/inenglish/researchandcollections/zoology/vertebratezoology/birds. The concatenated, aligned data set consist of 5007 bp (after the exclusion of ambiguously aligned regions) of which 2400 (48%) are potentially phylogenetically informative.

Phylogenetic analysis

The models for nucleotide substitutions were selected for each gene individually using the Akaike Information Criterion (Akaike 1973). The program MrModeltest 2.2 (Nylander 2002) in conjunction with PAUP* (Swofford 1998) was used to evaluate the fit of the data to different models for nucleotide substitutions. The same models and parameter settings chosen for the individual genes were also used for these partitions in the analysis of the combined data set. The posterior probabilities for tree, branch lengths and model parameters were approximated with a Metropolis-coupled Markov chain Monte Carlo (MCMCMC) Bayesian analysis as implemented in the computer program MrBayes 3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). For each gene region, we ran duplicate analyses of 4 million generations each with trees sampled every 100 generation. The saved trees from each

analysis were pooled and the posterior probabilities were calculated after discarding the trees saved during the "burn-in phase" (as estimated graphically). The analysis of the combined data set was based on the 70,000 trees saved after "burn-in". Substitution models, base frequencies, and parameter settings used in the analysis (after reaching stability) are listed in table ESM2.

In most analyses we used one galliform, one anseriform, and two palaeognathous birds as outgroup following the well-established understanding that Galloanseres and Neoaves are sistergroups within Neognathae, and that Palaeognathae is the sister of Neognathae (Groth & Barrowclough 1999). The five gene regions were analyzed individually (figures ESM-1 to ESM-5). The β-fibrinogen data yielded the most resolved tree and in order to investigate the influence of this gene region upon the results we also analyzed the data set with β-fibrinogen excluded (figure ESM-6). To investigate the influence by the choice of outgroup we also excluded the paleognaths and only used the two representatives of Galloanseres. All parts of the tree topology and the respective node supports remained essentially identical with one important exception; "Metaves" was not recovered as monophyletic when using only the galliform and anseriform birds as outgroups (figure ESM-7).

We also estimated the phylogenetic relationships within Neoaves by parsimony jack-knifing (Farris et al. 1996) as implemented in *XAC: Parsimony Jackknifer* (Farris 1997). The analysis was performed with 1,000 replicates, each with 10 random additions of taxa and branch swapping. The overall topology and clade support values in the resulting tree (figure ESM-8) do not contradict those obtained in the Bayesian analysis.

We have searched preliminary neighbour-joining trees and likelihood phylograms for cases where long-branch attractions may be suspected to have caused artificial groupings of taxa, but we have found no obvious such case.

Divergence time estimations

Divergence times were estimated using two rate smoothing methods, PATHd8 (Britton et al. 2006) and penalized likelihood (PL) (Sanderson 2002), the latter as implemented in the software r8s 1.7 (Sanderson 2003). Both methods need a topology with branch lengths, plus an arbitrary number of calibrations for calculation of absolute ages. The same topology and constraints were used as input for both methods.

PATHd8 is a non-parametric method, which smoothes substitution rates sequentially by taking averages over paths lengths from an internode to all its descending terminals. The smoothing is thereby done between sister groups, as opposed to most other methods, where rate smoothing is done between mother and daughter lineages. This has the effect of preserving more of the pattern of heterogeneous branch lengths that we find in the phylogram. An other property of the method is that zero or near-zero branchlengths collapse, which seems reasonable, considering that these branch lengths probably represents short time or uncertainties in the phylogeny (or both).

PL is a semi-parametric method, which smoothes substitution rate variation simultaneously over the whole tree. A maximum likelihood model, that overfits the data, is combined with a smoothing factor, and a penalty for fast rate changes between mother and daughter lineages. The optimal smoothing value for the data set is chosen objectively by a statistical cross-validation procedure. In this data set, the smoothing value was set to 13, which is of the same magnitude as most non clock-like data sets.

Both PL and PATHd8 need one calibration point to be fixed in age. For this purpose we used a 47.5 MY old fossil assigned to the stem group of hummingbirds. The r8s program also requires the root node to be constrained either by a fixed age or a maximum age. We chose to set a maximum age of 95 MY on the divergence of the Neoaves, based on the age obtained from the PATHd8 analysis. An additional set of 21 stratigraphically and phylogenetically

well-studied fossils were used as minimum age constraints. All fossils 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).

All fossils used for calibrations are placed in table ESM-3. Chronograms from PATHd8 and r8s are compared in figure ESM-9. Age estimates for major bird groups, obtained from both dating methods, are found in table ESM-4.

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Table ESM-1. Samples used in the study. Taxonomy follows Morony et al. (1975). Museum acronyms: AM, Australian Museum, Sydney; ANSP, Academy of Natural Sciences, Philadelphia; FMNH, Field Museum, Chicago; LSUMZ, Louisiana Museum of Natural Science, Baton Rouge; MV, Museum Victoria, Melbourne; NMWN, National Museum of Natural History, Windhoek; NRM, Swedish Museum of Natural History, Stockholm; PFIAO, Percy Fitzpatrick Institute of African Ornithology, Cape Town; SMF, Senckenberg Museum, Frankfurt; UWBM, Burke Museum of Natural History and Culture, Seattle; ZMCU, Zoological Museum, University of Copenhagen. NS = not sequenced. References: 1) Lerner & Mindell 2005, 2) Fain & Houde 2004, 3) Ericson et al. 2001, 4) Groth & Barrowclough 1999, 5) Johansson & Ericson 2003, 6) Pereira et al. 2002, 7) Johansson et al. 2001, 8) Thomassen et al. 2005, 9) Moyle 2004, 10) Ericson et al. 2003a, 11) Johnson & Clayton 2000, 12) Ericson & Johansson 2003, 13) Ericson et al. 2003b, 14) Mayr et al. 2003, 15) Johansson & Ericson 2005, 16) Mayr & Ericson 2004, 17) Prychitko & Moore 2003, 18) Johansson et al. 2002, 19) Fjeldså et al. 2003, 20) Chesser 2004, 21) Ericson et al. 2006.

Family	Species	Sample used for new sequences	c-*myc*	RAG-1	Myoglobin	Beta-fibrinogen	ODC
Accipitridae	*Accipiter gentilis*	NRM 966488	DQ881882	DQ881796	DQ881837	DQ881938	DQ881709
Accipitridae	*Heterospizias meridonalis*	NRM 947034	DQ881904	AY233359 (14)	AY233365 (14)	DQ881964	DQ881743
Accipitridae	*Leptodon cayanesis*	NRM 947125	DQ881909	DQ881814	DQ881858	DQ881970	DQ881750
Aegothelidae	*Aegotheles albertsii*	MV E044	DQ881883	AY233362 (14)	AY233375 (14)	DQ881939	DQ881710
Alcedinidae	*Alcedo atthis*	NRM 968171	AF295143 (7)	AF294671 (7)	AY165800 (5)		DQ881712
Alcedinidae	*Ceryle alcyon*					AY695158 (2)	
Anhimidae	*Chauna torquata*	J. P. Parson uncat	AY034413 (3)	AF143728 (4)	AY165805 (5)	AY140702 (6)	DQ881793
Anhingidae	*Anhinga anhinga*	NRM 947129	DQ881885	DQ881797	DQ881838	DQ881941	DQ881713
Apodidae	*Apus apus*	ZMCU P3	AF295136 (7)	AF294664 (7)	DQ881839	AY513096 (8)	DQ881714
Apterygidae	*Apteryx australis*	NRM uncat.	DQ881936	DQ881835	DQ881880	DQ882004	DQ881794
Aramidae	*Aramus guarauna*	NRM 947123	DQ881887	DQ881798	DQ881840	AY695250 (2)	DQ881716
Ardeidae	*Ardea cocoi*	NRM 947128	DQ881888	DQ881799	DQ881841	DQ881943	DQ881717
Ardeidae	*Tigrisoma lineatum*	NRM 937362		DQ881833	DQ881878	DQ881999	DQ881785
Ardeidae	*Tigrisoma lineatum*	LMS B 1212	AY034420 (3)				
Balaenicipitidae	*Balaeniceps rex*	LSUMZ B-13372	DQ881889	DQ881800	DQ881842	AY695218 (2)	DQ881719
Brachypteraciidae	*Brachypteracias leptosomus*	FMNH 345686	AF295149 (7)	AF294676 (7)	DQ881843	DQ881945	DQ881720
Bucconidae	*Nystalus maculatus*	NRM 947240	AF295153 (7)	AF294680 (7)	AY165817 (5)	DQ881946	DQ881721
Bucerotidae	*Tockus erythrorhynchus*	ZMCU P487	AF295152 (7)	AF294679 (7)	AY165823 (5)	DQ882000	DQ881786
Cacatuidae	*Cacatua sulphurea*	NRM 20026154	DQ881890	DQ881801	NS	DQ881947	DQ881722
Capitonidae	*Megalaima virens*	LSUMZ B-20788	AY165829 (5)	AY165793 (5)	AY165814 (5)	AY279227 (9)	NS
Caprimulgidae	*Podager nacunda*	NRM 947016	AF295132 (7)	AF294660 (7)	AY233373 (14)	DQ881984	DQ881766
Cariamidae	*Cariama cristata*	LSUMZ B-8656	DQ881891	DQ881802	DQ881844	DQ881948	DQ881723
Cathartidae	*Cathartes aura*	NRM 956732	DQ881892	DQ881803	DQ881845	DQ881949	DQ881724
Cathartidae	*Coragyps atratus*	NRM 947124	DQ881896	DQ881804	DQ881847	DQ881951	DQ881729
Charadriidae	*Charadrius collaris*	NRM uncat.	DQ881893	AY339106 (10)	AY339079 (10)	DQ881950	DQ881725
Ciconiidae	*Jabiru mycteria*	NRM 947193	DQ881906	DQ881812	DQ881856	DQ881967	DQ881746
Ciconiidae	*Mycteria americana*	NRM 956665	DQ881913	DQ881816	DQ881860	DQ881974	DQ881756
Coliidae	*Colius striatus*	ZMCU P398	DQ881894	AF294669 (7)	DQ881846	AY695164 (2)	DQ881726
Columbidae	*Scardafella squammata*	NRM 956728	DQ881895	AY339121 (10)	AY339094 (10)	AF182651 (11)	DQ881727

Coraciidae	*Coracias caudata*	NMWN 750	AF295148 (7)	AF143737 (4)	AY165807 (5)	AY695155 (2)	DQ881728
Cuculidae	*Cuculus canorus*	NRM 996341	AF295127 (7)	AF294655 (7)	AY165808 (5)	DQ881953	DQ881731
Cuculidae	*Guira guira*	NRM 937391	AY165835 (5)	AY165799 (5)	AY165818 (5)	DQ881961	DQ881740
Diomedidae	*Diomedea nigripes*	LSUMZ B-13340	DQ881897	DQ881805	DQ881848	DQ881954	DQ881732
Diomedidae	*Phoebetria palpebrata*	MV 979	DQ881920	DQ881822	DQ881866	DQ881981	DQ881764
Eurypygidae	*Eurypyga helias*	LSUMZ B-20749	DQ881898	DQ881806	DQ881849	DQ881955	DQ881733
Falconidae	*Falco rufigularis*	NRM uncat.		DQ881807			
Falconidae	*Falco subbuteo*	NRM 986329	DQ881899		DQ881850	DQ881956	DQ881734
Falconidae	*Polyborus plancus*	NRM 947200	DQ881923	AY233358 (14)	AY233364 (14)	DQ881987	DQ881770
Fregatidae	*Fregata magnificens*	NRM uncat.	DQ881900	DQ881808	DQ881851	DQ881957	DQ881735
Galbulidae	*Galbula cyanescens*	NRM uncat.	AF295155 (7)	AF294682 (7)	AY165810 (5)	AY695154 (2)	DQ881737
Gaviidae	*Gavia arctica*	NRM 976202	DQ881902	AF143733 (4)	DQ881853	DQ881959	DQ881738
Gruidae	*Grus canadensis*	NRM uncat.	DQ881903	AY339110 (10)	AY339083 (10)		
Gruidae	*Grus grus*	NRM 976209				DQ881960	DQ881739
Heliornithidae	*Podica senegalensis*	NMWN 1827	DQ881921	DQ881824	DQ881868	DQ881985	DQ881768
Hemiprocnidae	*Hemiprocne longipennis*	ANSP 1273	AF295137 (7)	AF294665 (7)	AY233376 (14)	DQ881963	DQ881742
Hydrobatidae	*Hydrobates pelagicus*	ZMCU P734	DQ881905	DQ881811	DQ881855	DQ881965	DQ881744
Indicatoridae	*Indicator minor*	S.A. 2000:66	AY165830 (5)	AY165794 (5)	AY165812 (5)	AY279222 (9)	NS
Jacanidae	*Jacana jacana*	NRM 937364	DQ881907	AY339112 (10)	AY339085 (10)	DQ881968	DQ881747
Laniidae	*Lanius collurio*	NRM 986403	AY228000 (12)	AY228042 (12)	AY228328 (12)	AY395617 (13)	DQ881748
Leptosomidae	*Leptosomus discolor*	FMNH uncat.	DQ881910	AY233361 (14)	AY233370 (14)	DQ881971	DQ881751
Megapodiidae	*Alectura lathami*	LSUMZ B-20851	AF296417 (7)	AF294687 (7)	AY165801 (5)	DQ882003	DQ881792
Meropidae	*Merops viridis*	ZMCU P935	AF295147 (7)	AF294675 (7)	AY165815 (5)	AY600483 (15)	DQ881752
Mesitornithidae	*Mesitornis unicolor*	FMNH 345610	DQ881911	AY756082 (16)	AY756085 (16)	DQ881972	DQ881753
Mesitornithidae	*Monias benschi*	SMF 3734-AL	DQ881912	DQ881815	DQ881859	DQ881973	DQ881755
Momotidae	*Momotus momota*	NRM 947281	AF295170 (7)	AF295192 (7)	AY165816 (5)	AY695159 (2)	DQ881754
Musophagidae	*Corythaixoides leucogaster*	ZMCU P509	AF295126 (7)	AF294654 (7)	AY233368 (14)	DQ881952	DQ881730
Nyctibiidae	*Nyctibius aethereus*	LSUMZ B-11236	AF295131 (7)	AF294659 (7)	AY233374 (14)	DQ881975	DQ881757
Opisthocomidae	*Opisthocomus hoazin*	LSUMZ B-10753	DQ881914	AY233357 (14)	AY233363 (14)	AY695134 (2)	DQ881758
Otididae	*Afrotis atra*	LSUMZ B-8672	DQ881884	AY339100 (10)	AY339073 (10)	DQ881940	DQ881711
Pandionidae	*Pandion haliaetus*	NRM 956317	DQ881915	DQ881817	DQ881861	DQ881976	DQ881759
Pelecanidae	*Pelecanus onocrotalus*	LSUMZ B-18956	DQ881917	DQ881819	DQ881863	DQ881978	DQ881761
Pelecanoididae	*Pelecanoides urinatrix*	MV 1870	DQ881916	DQ881818	DQ881862	DQ881977	DQ881760
Phaethontidae	*Phaethon rubricauda*	UWBM 68951	DQ881918	DQ881820	DQ881864	DQ881979	DQ881762
Phalacocoracidae	*Phalacrocorax carbo*	NRM 937348	DQ881919	DQ881821	DQ881865	DQ881980	DQ881763
Phoenicopteridae	*Phoenicopterus chilensis*	NRM P5	AY034424 (3)	DQ881823	DQ881867	DQ881982	DQ881765
Phoeniculidae	*Rhinopomastus cyanomelas*	ZMCU P916	AF295150 (7)	AF294677 (7)	NS	DQ881993	DQ881776
Picidae	*Picumnus cirratus*	NRM 976666	AF295174 (7)	AF295195 (7)	AY165819 (5)	DQ881983	NS
Podargidae	*Podargus strigoides*	S. Dunham uncat.	AF295134 (7)	AF294662 (7)	AY233372 (14)	AY082408 (17)	DQ881767
Podicepitidae	*Podiceps cristatus*	ZMCU P726	DQ881922	DQ881825	DQ881869	DQ881986	DQ881769

Procellariidae	*Fulmarus glacialis*	ZMCU P760	DQ881901	DQ881809	DQ881852	DQ881958	DQ881736
Procellariidae	*Puffinus lherminieri*	NRM uncat.	DQ881926	DQ881827	DQ881871	DQ881991	DQ881774
Psittacidae	*Pyrrhura frontalis*	NRM 966989	DQ881927	AY233360 (14)	AY233367 (14)	DQ881992	DQ881775
Psophidae	*Psophia leucoptera*	LSUMZ B-10733	DQ881924	DQ881826	DQ881870	DQ881988	DQ881771
Pteroclidae	*Pterocles gutturalis*	PFIAO 37 YtS	DQ881925	AY339116 (10)	AY339089 (10)	DQ881989	DQ881772
Rallidae	*Aramides ypecaha*	NRM 937389	DQ881886	AY756084 (16)	AY756087 (16)	DQ881942	DQ881715
Rallidae	*Laterallus albigularis*	NRM uncat.	DQ881908	DQ881813	DQ881857	DQ881969	DQ881749
Ramphastidae	*Pteroglossus castanotis*	NRM 937285	AF295159 (7)	AF294686 (7)	AY165821 (5)	DQ881990	DQ881773
Rheidae	*Rhea americana*	LSUMZ B-5799	DQ881937	DQ881836	DQ881881	DQ882005	DQ881795
Rhynochetidae	*Rhynochetus jubatus*	C. Lambert uncat.	DQ881928	DQ881828	DQ881872	AY695140 (2)	DQ881777
Rynchopidae	*Rynchops niger*	LSUMZ B-2457	DQ881929	AY339119 (10)	AY339092 (10)	AY695191 (2)	DQ881778
Sagittariidae	*Sagittarius serpentarius*	NMWN 2610F	DQ881930	DQ881829	DQ881873	DQ881994	DQ881779
Scopidae	*Scopus umbretta*	LSUMZ B-16327	AY034419 (3)	DQ881830	DQ881874	DQ881995	DQ881780
Spheniscidae	*Spheniscus humboldti*	NRM uncat.	DQ881931	AF143734 (4)	DQ881875	DQ881996	DQ881781
Steatornithidae	*Steatornis caripensis*	LSUMZ B-7474	AF295135 (7)	AF294663 (7)	AY233371 (14)	AY695135 (2)	DQ881782
Strigidae	*Asio flammeus*	S. Dunham uncat.	AF295129 (7)	AF294657 (7)	AY233366 (14)	DQ881944	DQ881718
Sulidae	*Sula bassana*	ZMCU P570	DQ881932	DQ881831	DQ881876	DQ881997	DQ881783
Threskiornithidae	*Harpiprion caerulescens*	NRM 937350	AY034416 (3)	DQ881810	DQ881854	DQ881962	DQ881741
Threskiornithidae	*Theristicus caudatus*	NRM 966880	DQ881933	DQ881832	DQ881877	DQ881998	DQ881784
Todidae	*Todus mexicanus*	LSUMZ B-11311	AF295145 (7)	AF294673 (7)	AY165824 (5)	DQ882001	DQ881787
Trochilidae	*Hylocharis chrysura*	NRM 937161	AF295139 (7)	AF294667 (7)	AY233377 (14)	DQ881966	DQ881745
Trogonidae	*Trogon melanurus*	ZMCU P494	AF295142 (7)	AF294670 (7)	AY165828 (5)	AY600479 (15)	DQ881788
Turnicidae	*Turnix pyrrhothorax*	AM O.64938					DQ881789
Turnicidae	*Turnix varia*	AM LAB1062	DQ881934	AY756083 (16)	AY756086 (16)	DQ882002	
Tyrannidae	*Todirostrum cinereum*	NRM 947036	AF453782 (18)	AF453796 (18)	AY338740 (19)	AY489421 (20)	DQ435506 (21)
Tytonidae	*Tyto alba*	NRM uncat.	DQ881935	DQ881834	DQ881879	AY082407 (17)	DQ881790
Upupidae	*Upupa epops*	ZMCU P502	AF295151 (7)	AF294678 (7)	NS	NS	DQ881791

Table ESM-2. Mean Bayes estimates of parameters for the five studied genetic markers. Substitution rate parameters are calculated with the rate for G <–> T set to 1. The models for nucleotide substitutions for the individual genes were used also in the analysis of the concatenated data set.

	c-myc	RAG-1	myoglobin	fibrinogen	ODC
No. of sites (aligned sequence)	510	930	1061	1524	982
No. of variable sites (%)	180 (35%)	476 (51%)	663 (62%)	980 (64%)	601 (61%)
No. of informative sites (%)	131 (26%)	382 (41%)	502 (47%)	852 (56%)	515 (52%)
ML model selected	GTR+I+G	GTR+I+G	GTR+G	GTR+I+G	GTR+I+G
r(AC)	1.29	1.97	1.13	1.14	1.00
r(AG)	10.19	7.53	4.93	3.56	3.81
r(AT)	0.67	0.72	0.69	0.63	0.67
r(CG)	1.34	1.49	1.69	1.48	1.33
r(CT)	20.68	12.65	5.16	3.59	3.22
r(GT)	1.00	1.00	1.00	1.00	1.00
Shape	0.3259	0.9247	1.8110	5.8062	2.7865
Proportion of invariant sites	0.4269	0.3979	N/A	0.0054	0.1522

Table ESM-3. Fossils used for the calibration of the molecular clock in the analyses. Note that “stem group” means that the taxon is outside the clade including the stem species of the modern taxa and its descendants, “crown group” means that it is within. All fossils provided minimum ages for the clades, except Q that was used as a fixed age for the split between hummingbirds and the swifts/treeswifts lineage in the PATHd8 analysis.

Symbol in tree	Taxon	Geological dating	MYA	Reference
A	Crown group Pici (Ramphastidae, Indicatoridae, Picidae]	early Oligocene	30-34	Mayr 2005a
B	Stem group (Upupidae + Phoeniculidae)	early Eocene	47.5	Mayr 2000
C	Stem group (Coraciidae + Brachypteraciidae)	early Eocene	47.5	Mayr & Mourer-Chauviré 2000
D	Stem group Trogoniformes	early Eocene	53	Mayr 2005b
E	Stem group Coliiformes	latest Paleocene/earliest Eocene	55	Houde & Olson 1992
F	Stem group Strigiformes	Paleocene	55-65	Rich & Bohaska 1976
G	Crown group Pandionidae	late Eocene	37-38	Harrison & Walker 1976
H	Stem group Cariamidae	early Eocene	47.5	Peters 1995
I	Stem group Phalacrocoracidae	late Oligocene	25	Mayr 2001
J	Crown group Sulidae	early Oligocene	33-34	Mayr 2002
K	Stem group Fregatidae	early Eocene	53	Olson 1977
L	Stem group Sphenisciformes	Paleocene	55-65	Fordyce & Jones 1986
M	Crown group Balaenicipitidae	early Oligocene	30-34	Rasmussen et al. 1987
N	Crown group Heliornithidae	middle Miocene	14	Olson 2003
O	Stem group Jacanidae	early Oligocene	30-34	Rasmussen et al. 1987
P	Stem group Apodiformes (Apodidae, Hemiprocnidae, Trochilidae)	early Eocene	53	Mayr 2003
Q	Stem group Trochilidae	early Eocene	47.5	Mayr 2005c
R	Crown group Pteroclidae	early Oligocene	30-33	Mourer-Chauviré 1993
S	Stem group Phoenicopteriformes	early Oligocene	30-34	Mayr 2005d
T	Stem group Phaethontidae	late Paleocene	55-60	Bourdon et al. 2005
U	Stem group Galliformes	early Eocene	53	Mayr & Weidig 2004
V	Stem group (Gruidae + Aramidae)	early Oligocene	30-34	Mayr 2005e
X	Stem group Gaviiformes	early Oligocene	30-34	Mayr 2004

Table ESM-4. Comparison of age estimates of major groups, obtained from the PL and PATHd8 analyses. Note that the maximum age for Neoaves is set to 95 MYA based on the result in the initial PATHd8 analysis. Ages in MYA, rounded to nearest integer.

Clade name	Colour coding in Figure 1	PL - crown group ages	PATHd8 - crown group ages	Difference absolute age, PL - PATHd8
"Metaves"	purple + orange clades	81	67	14
"Caprimulgiforms", hummingbirds and swifts	purple clade	70	62	8
"Coronaves"	green + blue clades	87	74	13
Terrestrial and arboreal birds	green clades	86	68	18
Parrots, passerines, falcons, seriemas	light green clade	85	68	17
Aquatic and semi-aquatic birds (plus Cuculidae, Otididae and Musophagidae)	dark blue clade	83	60	23
Shorebirds	light blue clade	79	57	22

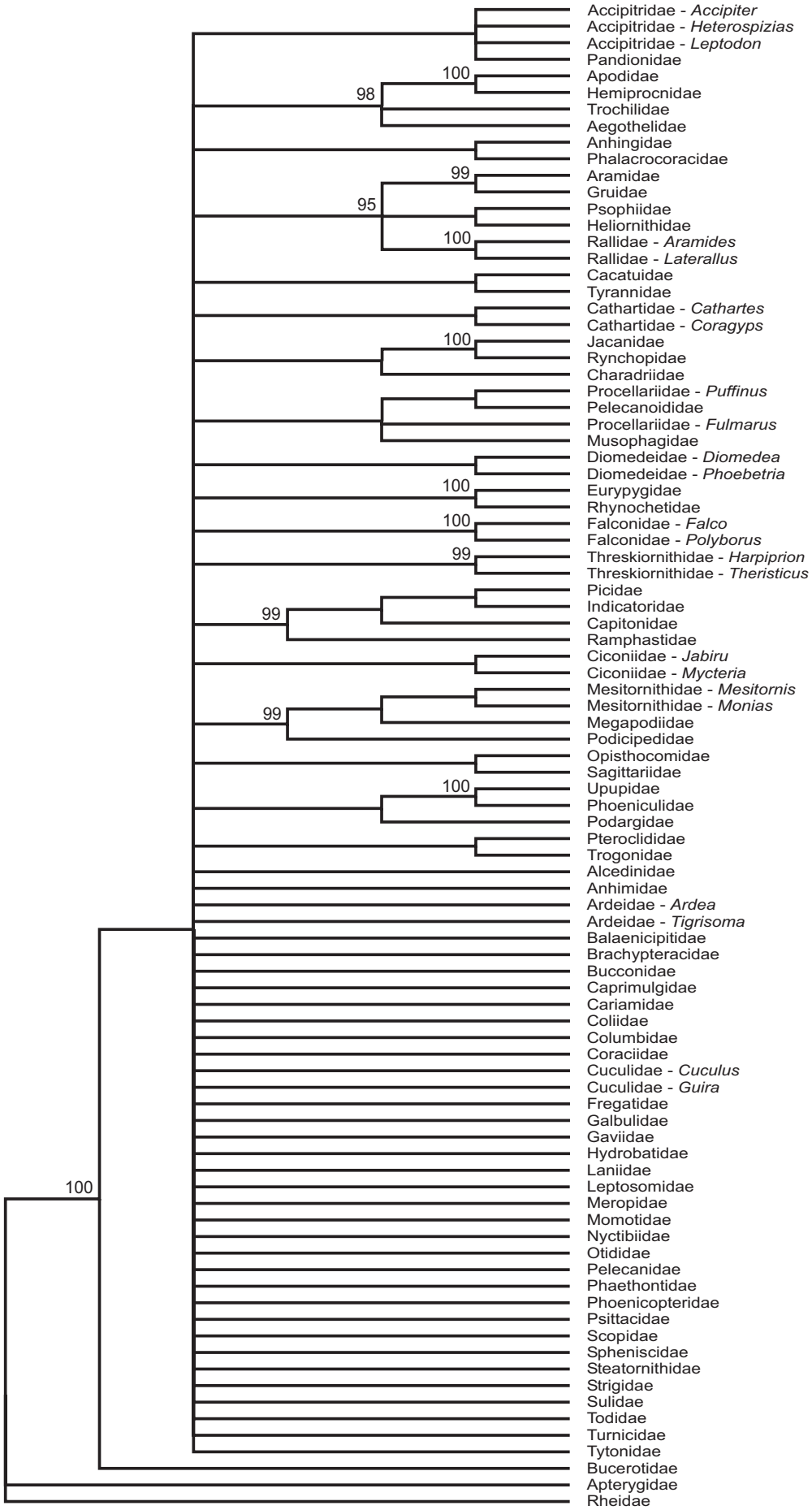

Figure ESM-1. Bayesian tree based on c-*myc* exon 3. All nodes recovered in 50% or more of the sampled trees are shown (only nodes with 95% or larger posterior probabilities are labeled).

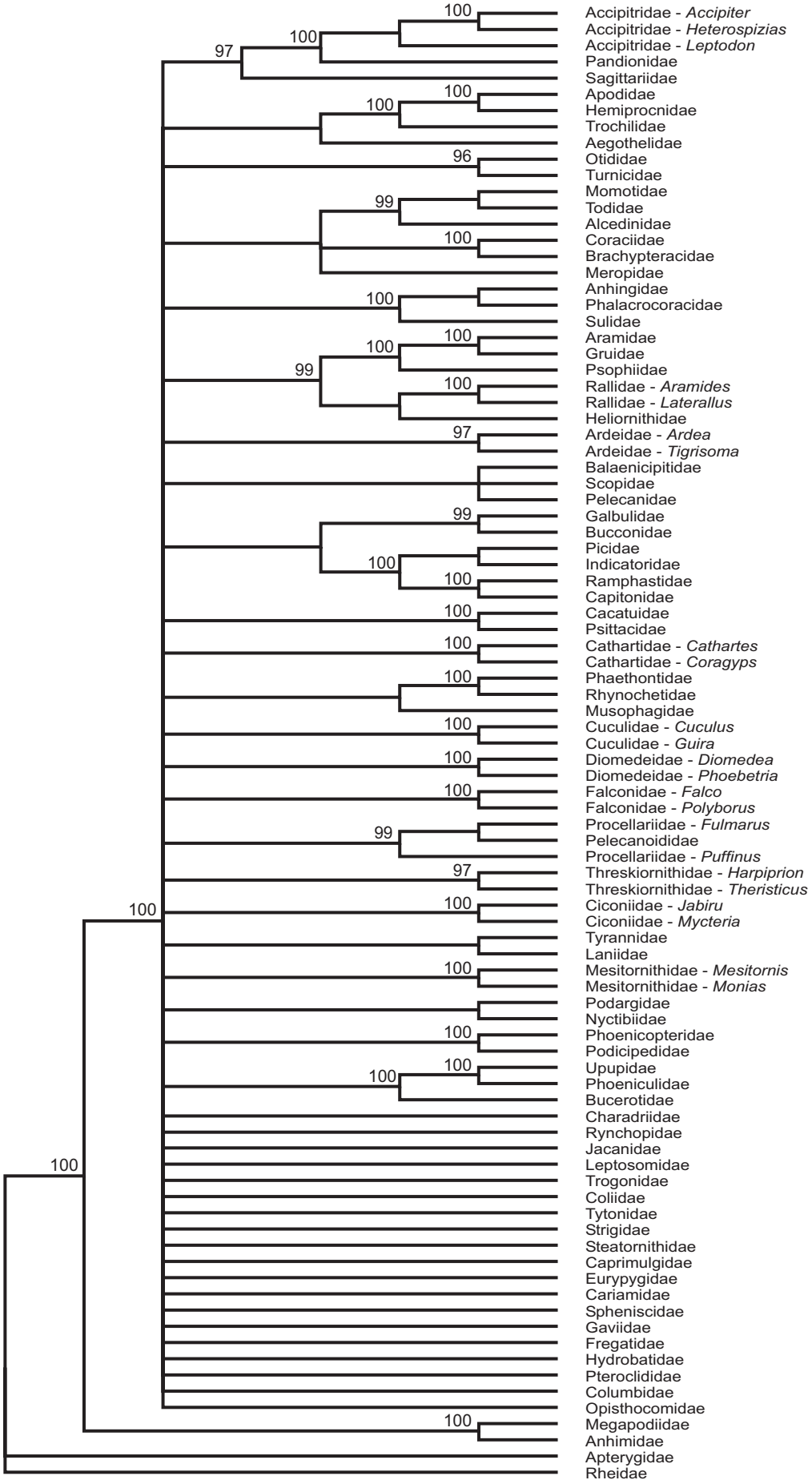

Figure ESM-2. Bayesian tree based on RAG-1. All nodes recovered in 50% or more of the sampled trees are shown (only nodes with 95% or larger posterior probabilities are labeled).

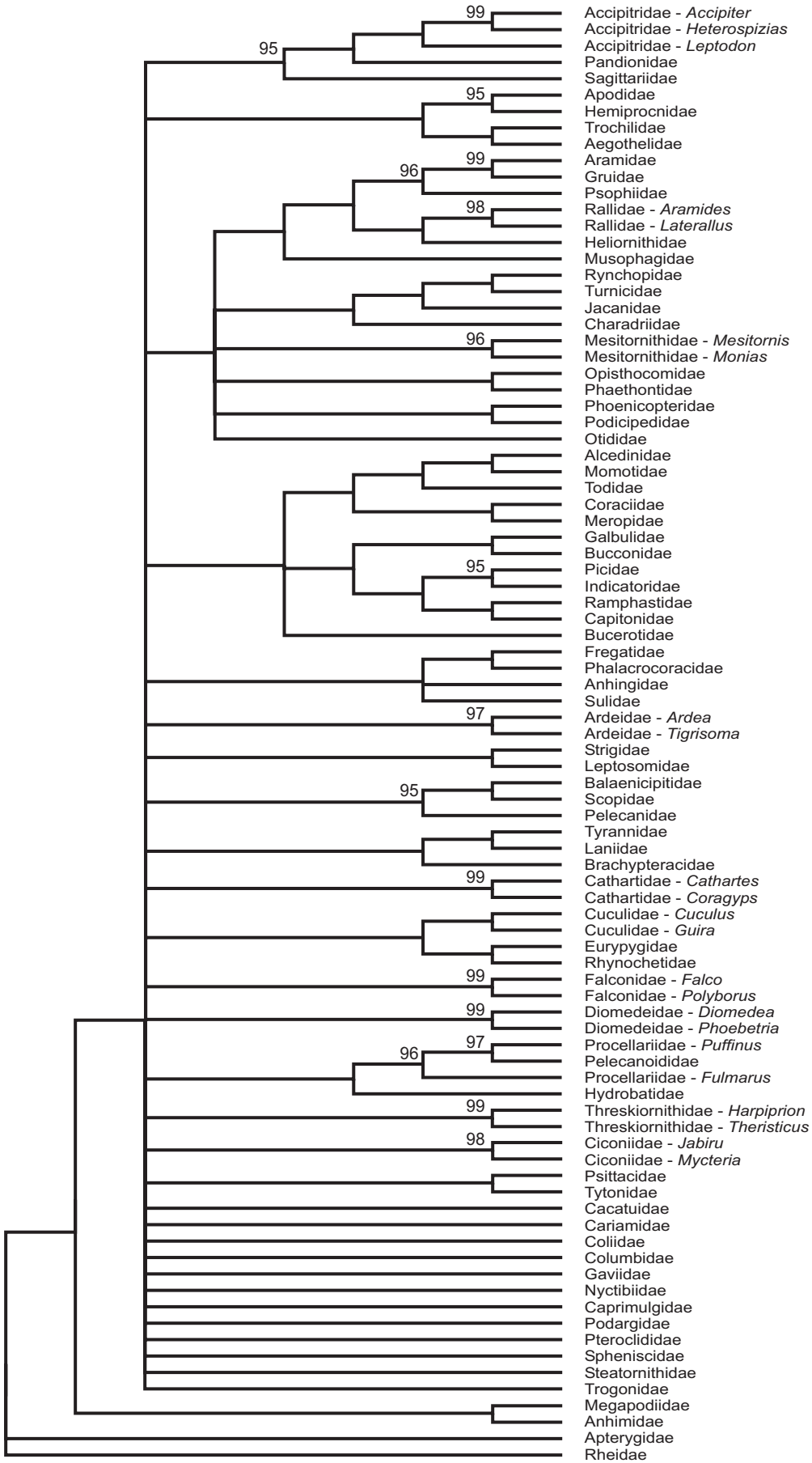

Figure ESM-3. Bayesian tree based on myoglobin intron 2. All nodes recovered in 50% or more of the sampled trees are shown (only nodes with 95% or larger posterior probabilities are labeled).

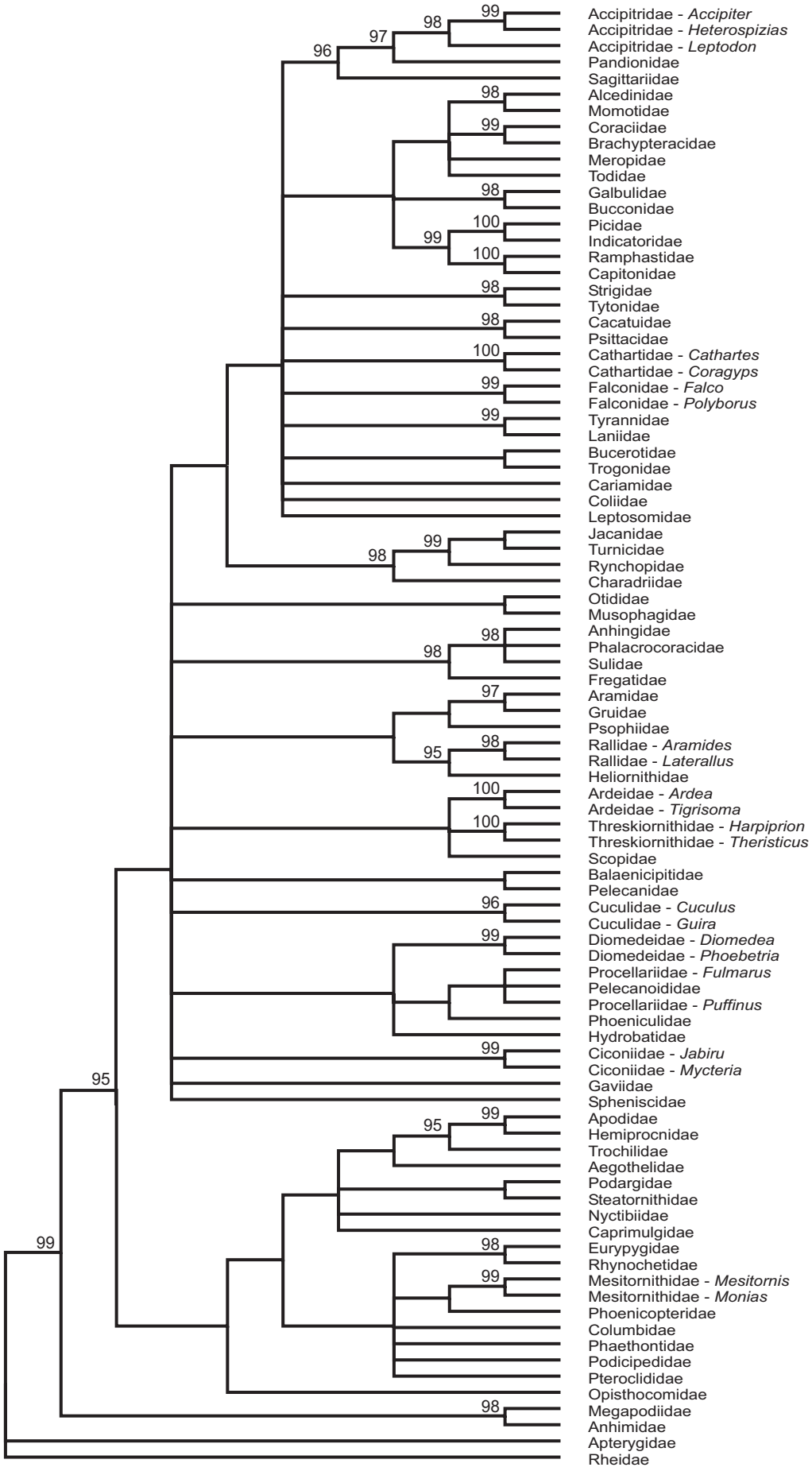

Figure ESM-4. Bayesian tree based on â-fibrinogen intron 7. All nodes recovered in 50% or more of the sampled trees are shown (only nodes with 95% or larger posterior probabilities are labeled).

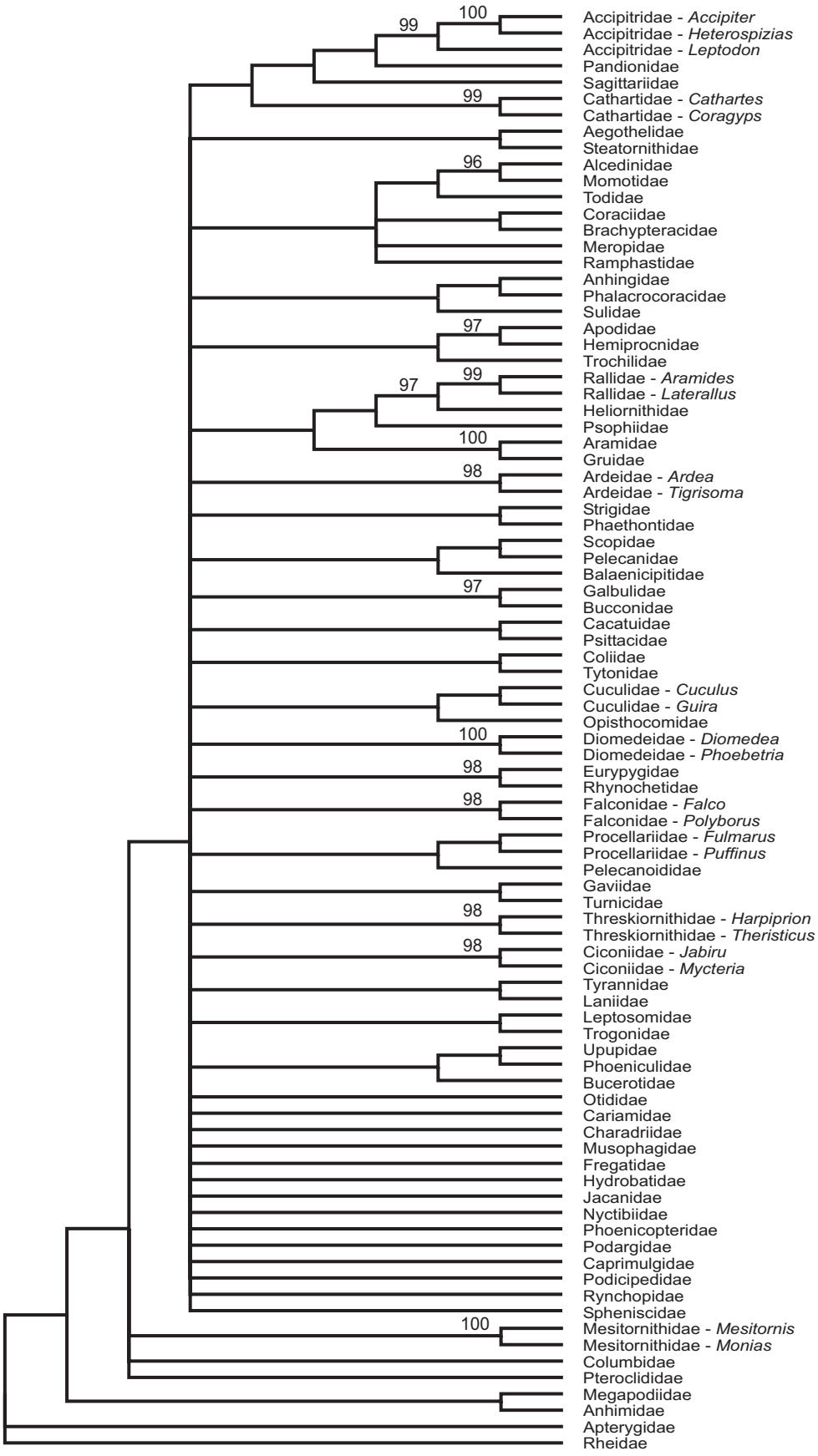

Figure ESM-5. Bayesian tree based on ornithine decarboxylase (ODC) introns 6 and 7, along with the intercepting exon 7. All nodes recovered in 50% or more of the sampled trees are shown (only nodes with 95% or larger posterior probabilities are labeled).

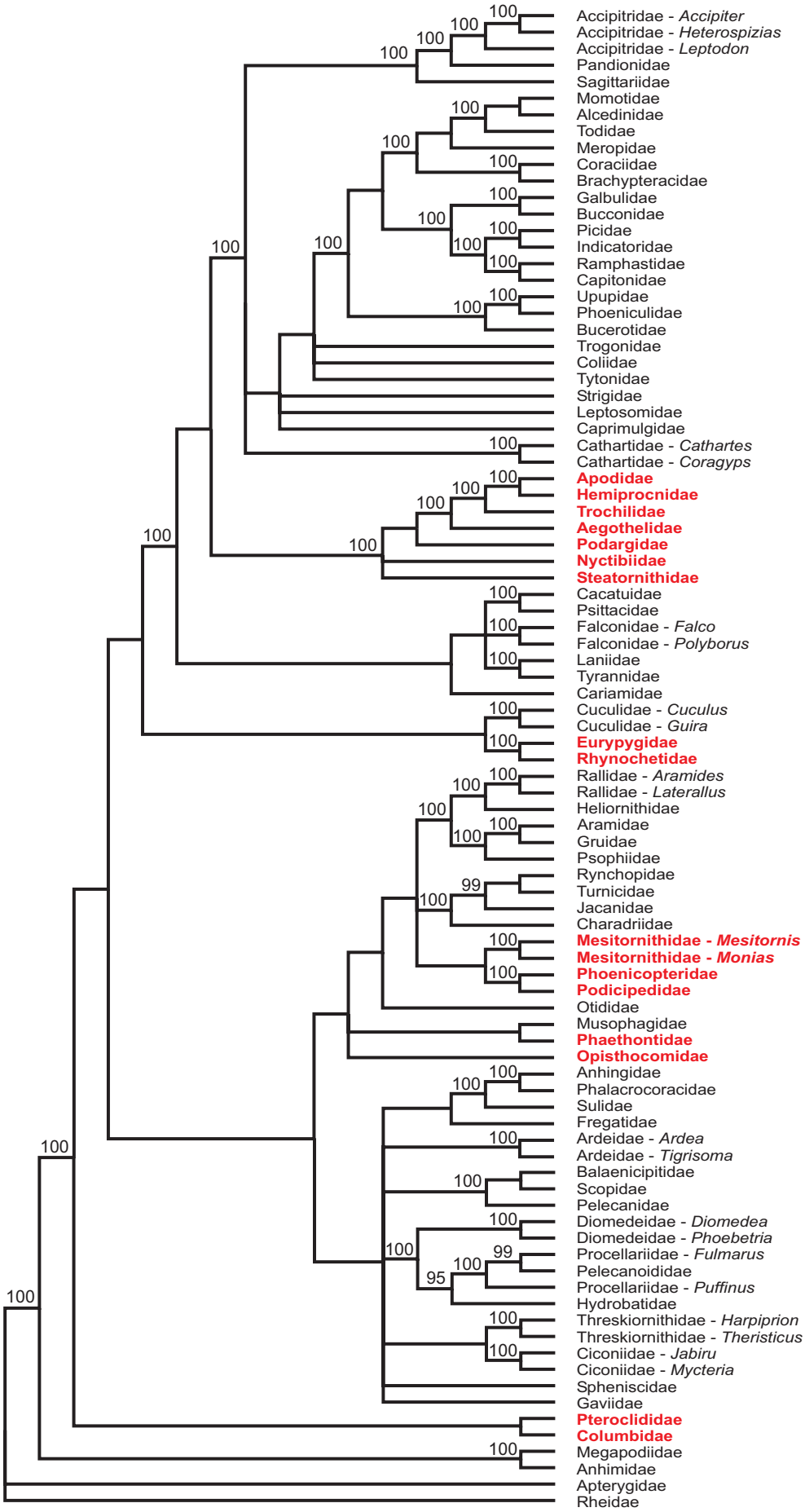

Figure ESM-6. Bayesian tree based on all genes except ß-fibrinogen in order to assess the influence of this gene upon the tree topology. Taxa included in Metaves are marked with red. All nodes recovered in 50% or more of the sampled trees are shown (only nodes with 95% or larger posterior probabilities are labeled).

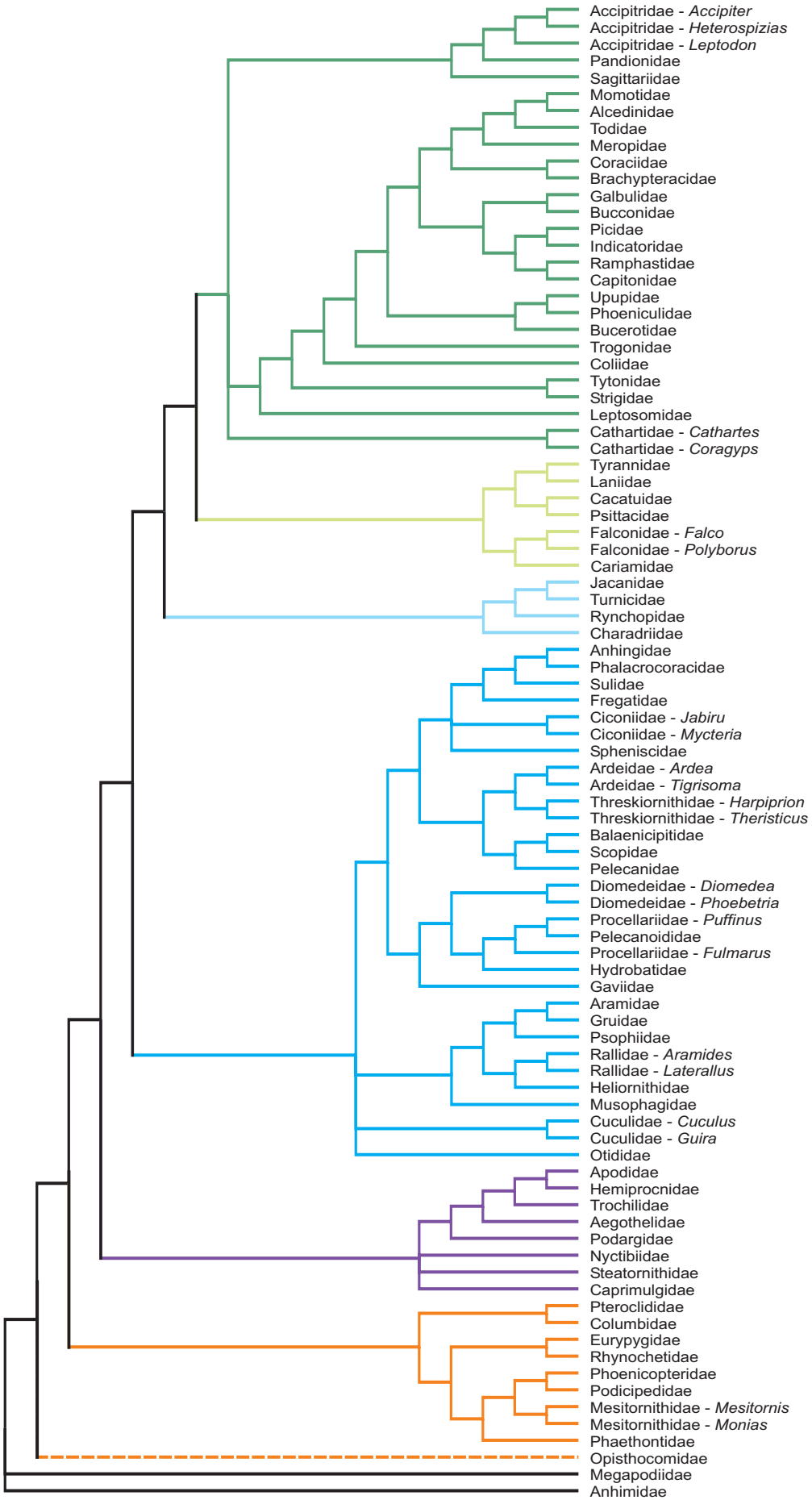

Figure ESM-7. Bayesian tree based on all genes, but with the paleognaths excluded and rooted with Galloanseres. All nodes recovered with 95% (or larger) posterior probabilities are shown.

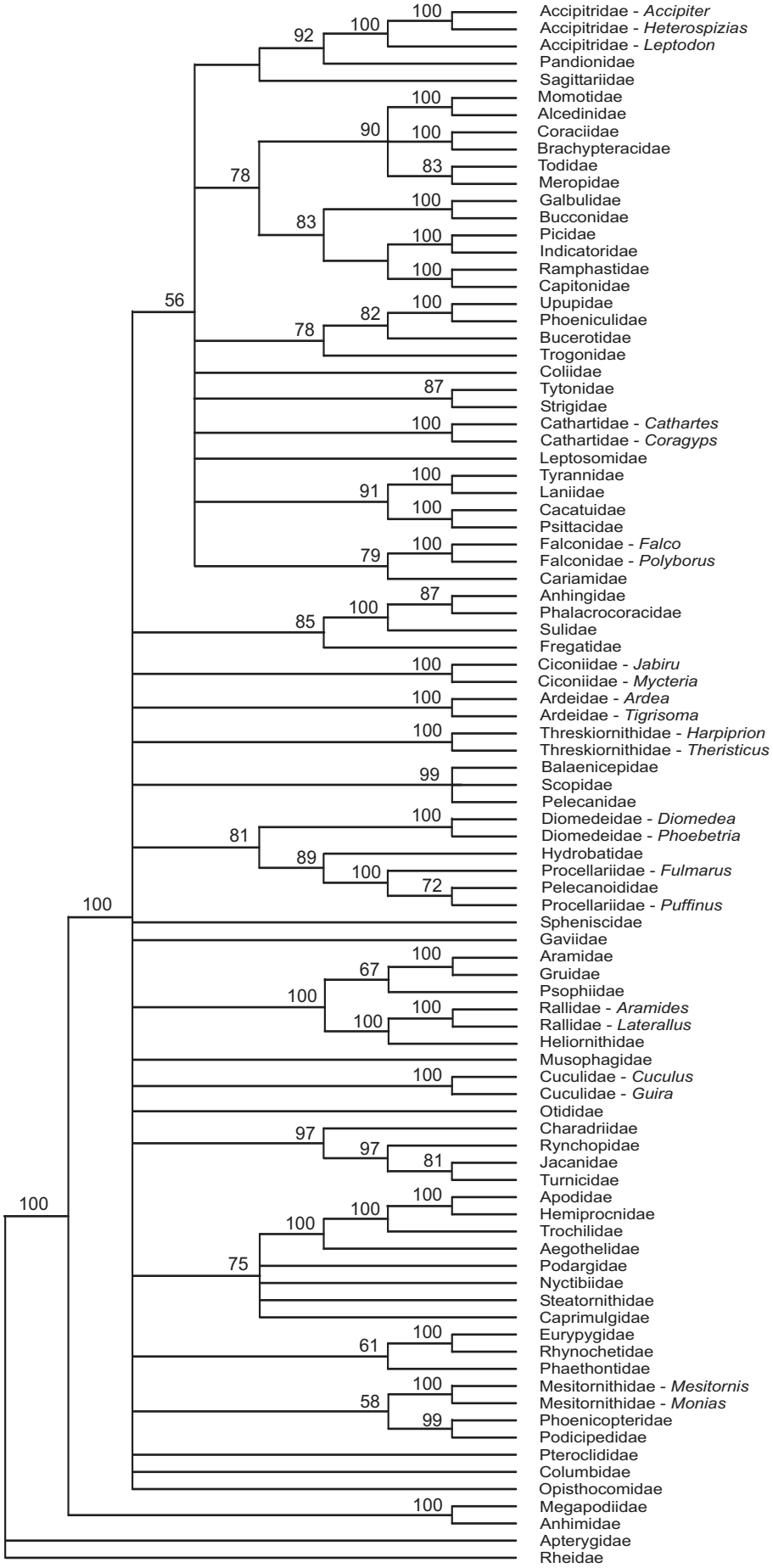

Figure ESM-8. Parsimony jack-knifing tree (Farris et al. 1996) as implemented in *XAC: Parsimony Jackknifer* (Farris 1997). The analysis was performed with 1,000 replicates, each with 10 random additions of taxa and branch swapping.

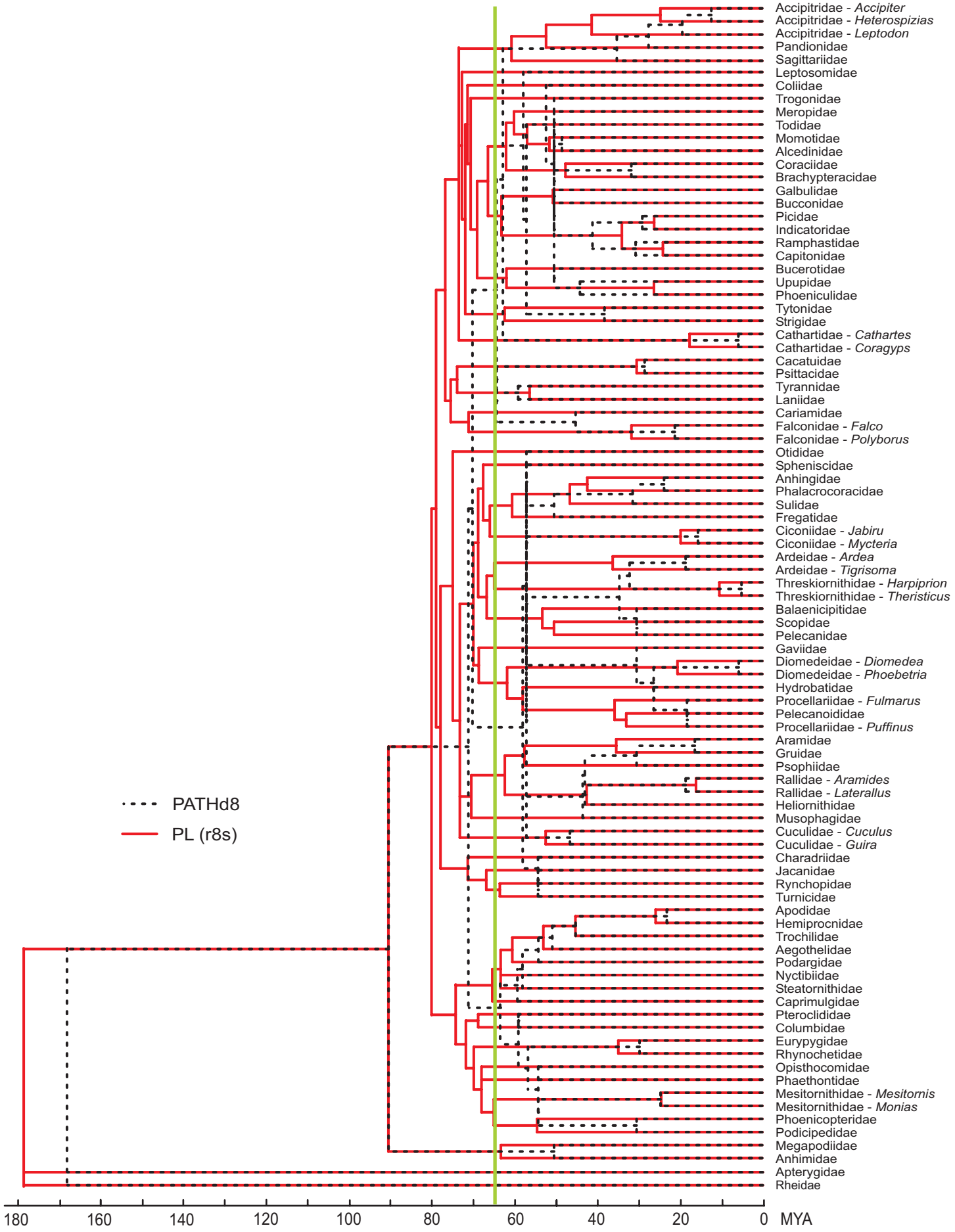

Figure ESM-9. Comparison between the results obtained from the PATHd8 and the PL analyses. The red PL chronogram suggests an earlier divergence, than the black, dotted chronogram from the PATHd8 analysis. The PL tree is less consistent with the fossil record, in the sense that it adds a "ghost range" of in average 21 MY to the fossil constrained nodes. The boundary between the Cretaceous and Tertiary at 65 MYA is marked with a green line.