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Molecular support for a sister group relationship between Pici and Galbulae (Piciformes *sensu* Wetmore 1960)

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Woodpeckers, honeyguides, barbets, and toucans form a well-supported clade with approximately 355 species. This clade, commonly referred to as Pici, share with the South American clade Galbulae (puffbirds and jacamars) a zygodactyls foot with a unique arrangement of the deep flexor tendons (Gadow's Type VI). Based on these characters, Pici and Galbulae are often considered sister taxa, and have in traditional classification been placed in the order Piciformes. There are, however, a wealth of other morphological characters that contradicts this association, and indicates that Pici is closer related to the Passeriformes (passerines) than to Galbulae. Galbulae, in turn, is considered more closely related to the rollers and ground-rollers (Coracii). In this study, we evaluate these two hypotheses by using DNA sequence data from exons of the nuclear RAG-1 and c-*myc* genes, and an intron of the nuclear myoglobin gene, totally including 3400 basepairs of aligned sequences. The results indicate a sister group relationship between Pici and Galbulae, i.e. monophyly of the Piciformes, and this association has high statistical support in terms of bootstrap values and posterior probabilities. This study also supports several associations within the traditional order Coraciiformes, including a sister group relationship between the kingfishers (Alcedinidae) and a clade with todies (Todidae) and motmots (Momotidae), and with the bee-eaters (Meropidae) placed basal relative to these three groups.

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The clade Pici consists of the woodpeckers (Picidae), honeyguides (Indicatoridae), and barbets and toucans (Ramphastides *sensu* Sibley and Ahlquist 1990). It includes approximately 355 species and members of this clade are found almost worldwide; Pici lacks representatives only in Antarctica, Madagascar, the Australo-Pacific region and in deserts. Traditional classifications have placed the barbets and toucans in separate groups, but several phylogenetic studies have shown the New World barbets to be more closely related to the toucans, than to the Old World barbets (Burton 1984, Prum 1988, Sibley and Ahlquist 1990, Lanyon and Hall 1994, Johansson et al. 2001). The barbet–toucan clade in turn is the sister group of the woodpeckers and honeyguides (Simpson and Cracraft 1981, Swierczewski and Raikow 1981, Burton 1984, Sibley and Ahlquist 1990).

Monophyly of Pici seems well established based both on morphological (Simpson and Cracraft 1981, Swierczewski and Raikow 1981, Burton 1984) and molecular data (Sibley and Ahlquist 1990, Johansson et al. 2001). The morphological synapomorphies for this clade includes a unique form of trochlea IV of the tarsometatarsus (Simpson and Cracraft 1981).

Another characteristic of the Pici is the zygodactyl foot in which digits I and IV are directed backwards, while digits II and III are directed forwards. The zygodactyl foot is not unique to Pici, but occurs also in cuckoos (Cuculidae), parrots (Psittaciformes), jacamars (Galbulidae) and puffbirds (Bucconidae), as well as in

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several fossil taxa. The zygodactyl foot seems to have evolved independently in cuckoos and parrots (Simpson and Cracraft 1981, Swierczewski and Raikow 1981). The condition in jacamars and puffbirds (Galbulae), however, has been suggested to be homologous with that in Pici (Simpson and Cracraft 1981, Swierczewski and Raikow 1981, Raikow and Cracraft 1983), despite the considerable difference in the foot morphology of Pici and Galbulae (Olson 1983). Monophyly of a Pici–Galbulae clade (Piciformes *sensu* Wetmore 1960) is further suggested by a unique arrangement of the deep flexor tendons (Gadow's Type VI; Swierczewski and Raikow 1981). In most birds digits II, III, and IV are flexed by *M. flexor digitorum longus*, whereas the hallux is flexed by *M. flexor hallucis longus*. In Pici and Galbulae the tendon of *M. flexor digitorum longus* inserts only on digit III, while digits II and IV are instead supplied by *M. flexor hallucis longus*. In addition, *M. flexor hallucis longus* originates by three heads on femur and fibula, which has been interpreted as another synapomorphy for Pici and Galbulae (Swierczewski and Raikow 1981, Raikow and Cracraft 1983) – in most other birds *M. flexor hallucis longus* originates with one or two heads, and only from femur (Raikow and Cracraft 1983). A three-headed origin of *M. flexor hallucis longus* also occurs in most passerines, but differences in the position of the lateral head of the muscle relative to the tendon of *M. iliofibularis* indicates that this condition in Passeriformes is not homologous with that in Pici and Galbulae (Swierczewski and Raikow 1981).

However, there are several characters that contradict the monophyly of Piciformes and instead support a closer affinity of both Pici and Galbulae with other groups of birds. It has often been suggested that Pici is more closely related to passerines than to Galbulae (e.g., Olson 1983, Höfling and Alvarenga 2001), and Mayr (1998) listed several derived characters shared between these taxa. Galbulae, on the other hand, have been suggested to have a closer affinity with Coracii, i.e. the rollers (Coraciidae), ground-rollers (Brachypteraciidae), and the cuckoo-roller (Leptosomatidae) (Olson 1983, Burton 1984, Höfling and Alvarenga 2001). For instance, similarities in the morphology of the quadrate have been used as evidence for grouping Pici with the passerines, and Galbulae with Coracii. In Pici and Passeriformes the orbital process of this bone is long (Mayr 1998), whereas it is broad in Galbulae and Coracii (Burton 1984). The quadrate also has a very deep medial condyle in Galbulae and Coracii, in contrast to Pici and Passeriformes (Burton 1984). Another potential synapomorphy in the cranium for a Galbulae–Coracii clade is the enlarged postorbital process in these two taxa. The postorbital process is short in both Pici and most Passeriformes, and also in other coraciiform birds (Olson 1983). Pici also shows several similarities with the Passeriformes in the postcranial skeleton. Both Pici and Passeriformes have a slender and elongated coracoid with a reduced size of the sterno-coracoid and procoracoid processes (Olson 1983). The gross morphology of the humerus is also similar between these two taxa (Olson 1983, Mayr 1998), and both have a broad and well-developed *processus intermetacarpalis* on the carpometacarpus (Olson 1983, Mayr 1998), and *processus pisiformis* of the same bone that is cranially displaced (Mayr 1998).

Galbulae and Coracii also share specializations of the hyoid apparatus, involving *M. stylohyoideus* and *M. serpihyoideus* (Burton 1984). The former muscle is reduced in many coraciiform taxa and it is completely lost in Coraciidae and Brachypteraciidae. *M. stylohyoideus* is lost also in Galbulae, and is functionally replaced by a slip of *M. serpihyoideus* in this clade, as well as in Coraciidae and Brachypteraciidae. A similar modification of *M. serpihyoideus* is apparent also in Leptosomatidae, despite that *M. stylohyoideus* is retained in this position in this taxon. Galbulae is morphologically more similar to coraciiform birds than to Pici also in other parts of the hyoid apparatus: the entoglossum is largely cartilaginous in both Galbulae and Coraciiformes (more heavily ossified in Pici), and in both groups the basihyal is very short (longer in Pici).

Morphological characters thus cannot unambiguously discriminate between the different hypotheses of the relationships between Pici and Galbulae. However, neither has molecular data been able to conclusively resolve the relationships of Pici and Galbulae. DNA–DNA hybridization data (Sibley and Ahlquist 1990) placed Pici basal in the Neoaves (*sensu* Sibley et al. 1988), a clade that consists of all living birds except the palaeognaths, galliforms and anseriforms. Galbulae, on the other hand, was placed in a clade with coraciiform birds and trogons. However, in a FITCH analysis of the same data set (Sibley and Ahlquist 1990, p. 814), Pici and Galbulae were placed as sister taxa. The FITCH analysis does not make assumptions about a "molecular-clock", but the tree is unrooted and included only *Apaloderma* (Trogonidae) and *Coracias* (Coraciidae) for comparison. A re-analysis of the original data, including a test of data consistency, resulted in an unresolved trichotomy between Pici, Galbulae and Coraciidae (Harshman 1994).

In a phylogenetic study of the "higher land-birds" based on DNA sequences from two of the nuclear genes used herein, the relationships between Pici, Galbulae, and Coracii could not be unambiguously resolved, i.e. the nodes in this part of the cladogram received no parsimony jackknifing or Bremer support (Johansson et al. 2001). Overall, few phylogenetic relationships indicated in that study received statistical support suggesting the need for more data to investigate the inter-relationships between Pici and Galbulae. In this respect, we have added approximately 1000 bp

to the RAG-1 sequences used in Johansson et al. (2001), and also sequenced approximately 700 bp of an intron of the nuclear myoglobin gene.

Material and Methods

Taxon sampling and outgroup

The taxa included in the study are listed in Table 1. In Pici, the woodpecker–honeyguide and barbet–toucan clades are represented by two and five species each in the analyses. The Galbulae is represented by three species (two puffbirds and one jacamar), and the passerines by five species (three suboscines and two oscines). Furthermore, the analyses included representatives of most families placed in the order Coraciiformes (*sensu* Wetmore 1960), with a roller (Coraciidae) representing Coracii. Due to the relatively poor knowledge of the basal phylogenetic relationships within the birds (Johansson et al. 2001), the least inclusive clade to which these birds can be assigned is Neoaves. Therefore, the tree was rooted with two representatives of the Galloanserae (*Chauna torquata* and *Alectura lathami*) – the proposed sister group of Neoaves (Groth and Barrowclough 1999, van Tuinen et al. 2000).

The genes

The DNA sequences used in the study were obtained from three gene segments located in the nuclear genome. Two of these segments are parts of the protein coding genes c-*myc* and RAG-1. These genes have proven useful in resolving the earliest divergences in the avian phylogeny (Groth and Barrowclough 1999, Johansson et al. 2001, Barker et al. 2002, Ericson et al. 2002a) and they both show low levels of homoplasy at this phylogenetic level (Groth and Barrowclough 1999, Irestedt et al. 2001, Johansson et al. 2001). The analyzed region of the c-*myc* gene is located in the exon 3 and corresponds to the region between positions 759 and 1235 in the published *Gallus* sequence (Watson et al. 1983). The analyzed portion of the RAG-1 gene is located in the single exon of this gene and includes totally 1914 bp starting at position 1054 in the *Gallus* sequence (GenBank M58530, Carlson et al. 1991).

The third gene fragment sequenced is the complete intron II of the myoglobin gene, including 13 and 10 bp of the flanking exons 2 and 3, respectively (Heslewood et al. 1998). This gene has previously proven informative in phylogenetic studies within Passeriformes (e.g., Ericson et al. 2002a, b, Irestedt et al. 2002). To evaluate the level of saturation in this intron, the observed numbers of pairwise transitions and transversions in the myoglobin intron II were plotted against the pairwise sequence divergence calculated from the combined c-*myc* and RAG-1 genes. This showed that both transitions and transversions are linearly correlated against the total sequence distances (Fig. 1), indicating that there are low levels of saturation in this gene.

DNA extraction, amplification, and sequencing

The laboratory procedures for amplifying and sequencing the different genes follow protocols described in Norman et al. (1998), Ericson et al. (2000), Irestedt et al. (2001, 2002), Johansson et al. (2001). All amplifications were carried out with Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) as 25 μl reactions following the manufacturer's recommendations with a final concentration of each primer of 0.4 μM. Before sequencing, the PCR products were cleaned with QIAquick™ PCR Purification Kit (QIAGEN®). Sequencing reactions were done with Perkin Elmer Applied BioSystems PRISM terminator cycle sequencing kits with AmpliTaq FS polymerase with BigDye terminators, following the manufacturer's protocol. Both strands were sequenced for each gene and the multiple sequence fragments obtained by sequencing with different primers were assembled to complete sequences with SeqMan II™ (DNASTAR Inc.). Primers and thermocycling conditions for amplification and sequencing are summarized in the Appendix.

For one sample, *Galbula cyaneus*, the second fragment of the RAG-1 gene were not possible to sequence under standard conditions. Sequencing of the nested PCR product consistently gave a very clean sequencing chromatogram but with an unusually high number (ca 40) of ambiguous positions. As this could be a result of a contaminated sample, a second individual (LSUMZ B10838) was amplified and sequenced, but also this specimen showed a high number of ambiguous peaks. However, sequencing of the unnested product yielded a clean, unambiguous sequence, although the external primer R63 did not yield any sequence data. As a consequence, the last approximately 400 basepairs of the *Galbula* sequence is based on reading of a single strand.

Alignment

The sequences were aligned by eye in MegAlign™ (DNASTAR inc.). No length variation was observed in the protein-coding RAG-1 gene, whereas two indels (insertions or deletions) were found in the c-*myc* gene. Compared with the outgroup, one indel represents a deletion of six basepairs (two amino acids) at position 889 in Picidae and Indicatoridae (see Johansson et al. 2001). The other indel is an insertion of twelve basepairs in *Megalaima* (Ramphastides). This insertion is

Table 1. Samples used in the study. Abbreviations: AM = Australian Museum, Sydney; ANSP = Academy of Natural Sciences of Philadelphia; LSUMZ = Louisiana State University, Museum of Natural Science; NMWM = National Museum of Namibia; NRM = Swedish Museum of Natural History, Department of Vertebrate Zoology; ZMCU = Zoological Museum, University of Copenhagen. References: 1. this study, 2. Johansson et al. 2001, 3. Irestedt et al. 2001, 4. Groth and Barrowclough 1999, 5. Ericson et al. 2001, 6. Ericson et al. 2002b, 7. Irestedt et al. 2002.

Species	Clade	Sample no.	Owner	GenBank Acc. No.					
				c-*myc*	Ref.	RAG-1	Ref.	Myoglobin	Ref.
Megalaima virens	Ramphastides	B20788	LSUMZ	AY165829	1	AY165793	1	AY165814	1
Trachyphonus usambiro	Ramphastides	P603	ZMCU	AF295156	2	AF294683	2	AY165825	1
Stactolaema olivacea	Ramphastides	P593	ZMCU	AF295157	2	AF294684	2	AY16522	1
Eubucco bourcierii	Ramphastides	P587	ZMCU	AF295158	2	AF294685	2	AY165809	1
Pteroglossus castanotis	Ramphastides	937285	NRM	AF295159	2	AF294686	2	AY165821	1
Indicator minor	Indicatoridae	2000: 66	S. Andersson	AY165830	1	AY165794	1	AY165812	1
Picumnus cirratus	Picidae	976666	NRM	AF295174	3	AF295195	3	AY165819	1
Nystalus maculatus	Bucconidae	947240	NRM	AF295153	2	AF294680	2	AY165817	1
Bucco capensis	Bucconidae		T. J. Parsons	AF295154	2	AF294681	2	AY165801	1
Galbula cyanescens	Galbulidae		T. J. Parsons	AF295155	2	AF294682	2	AY165810	1
Coracias caudata	Coraciidae	750	NMWM	AF295148	2			AY165807	1
Coracias caudata	Coraciidae					AF143737	4		
Alcedo atthis	Alcedinidae	968171	NRM	AF295143	2	AF294671	2	AY165800	1
Ispidina picta	Alcedinidae	2000: 70	S. Andersson	AY165831	1	AY165795	1	AY165813	1
Halcyon macleayii	Alcedinidae	20016589	NRM	AY165832	1	AY165796	1	AY165811	1
Chloroceryle americana	Alcedinidae	937351	NRM	AF295144	2	AF294672	2	AY165806	1
Ceryle torquata	Alcedinidae	976697	NRM	AY165833	1	AY165797	1	AY165804	1
Todus mexicanus	Todidae	B11311	LSUMZ	AF295145	2	AF294673	2	AY165824	1
Momotus momota	Momotidae	947281	NRM	AF295170	3	AF295170	3	AY165816	1
Merops viridis	Meropidae	P935	ZMCU	AF295147	2	AF294675	2	AY165815	1
Tockus erythrorhynchus	Bucerotidae	P487	ZMCU	AF295152	2	AF294679	2	AY165823	1
Trogon melanurus	Trogonidae	P494	ZMCU	AF295142	2	AF294670	2	AY165828	1
Harpactes oreskios	Trogonidae	1316	ANSP	AY165834	1	AY165798	1	AY165827	1
Pitta angolensis	Pittidae	S1027	ZMCU	AF295176	3	AF295197	3	AY165820	1
Rhinocrypta lanceolata	Rhinocryptidae	966793	NRM	AF295178	3	AF295199	3	AY065775	7
Tyrannus savana	Tyrannidae	976722	NRM	AF295182	3	AF295203	3	AY165826	1
Menura novaehollandiae	Menuridae	LAB1112	AM	AF295169	3	AF295169	3	AY064744	6
Campephaga flava	Campephagidae	O11	ZMCU	AF295162	3	AF295162	3	AY165803	1
Cuculus canorus	Cuculidae	996341	NRM	AF295127	2	AF294655	2	AY165808	1
Guira guira	Cuculidae	937391	NRM	AY165835	1	AY165799	1	AY165818	1
Chauna torquata	Anhimidae		T. J. Parsons	AY034413	5			AY165805	1
Chauna torquata	Anhimidae					AF143728	4		
Alectura lathami	Megapodidae	B20851	LSUMZ	AF296417	2	AF294687	2	AY165801	1

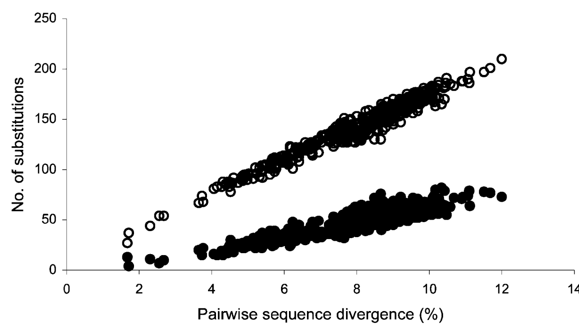


Fig. 1. The observed number of transitions (open circles) and transversions (filled circles) in intron II of the myoglobin gene plotted against the pairwise sequence divergences calculated for the combined c-myc and RAG-1 genes.

located in a region were a duplication of the twelve basepairs occurs in all investigated taxa and the insertion in *Megalaima* is yet another duplication in this region, and based on the present data it is most parsimonious to place it at position 784, although it is also possible to place at positions 772 and 796 relative to the *Gallus* sequence (Watson et al. 1983). A similar duplication has also been observed in swifts, tree-swifts, and hummingbirds (Johansson et al. 2001). In the non-coding myoglobin intron, insertions and deletions are more frequent, and the sequence lengths vary from 648 bp in *Bucco* to 807 bp in *Nystalus*. The sizes of the indels range from singletons in several species to a 158 bp long insertion in *Nystalus*. Despite the many indels, alignment was relatively straightforward and the nucleotide positions could be easily homologized in most cases. The alignment of the myoglobin gene has been deposited in the EMBL Nucleotide Sequence Database (Accession no. ALIGN_000500). The alignment of the three gene segments combined includes 3400 nucleotide positions.

Phylogenetic analysis

Maximum likelihood (ML) analyses were performed with PAUP* 4.0b10 (Swofford 1998) on both the combined data set and each of the individual genes. The model for the maximum likelihood analysis was selected with the likelihood-ratio test implemented in Modeltest 3.06 (Posada and Crandall 1998), which chooses the simplest model that cannot be rejected in favor of a more complex model. Based on the test of maximum likelihood models, the general-time reversal (GTR) model with an estimate of invariable sites (I) and a discrete (four rate categories) Γ-distribution model of among site rate heterogeneity was selected for the combined data set. This model was also chosen for the analysis of the RAG-1 gene, whereas the HKY model with Γ-distributed site rate heterogeneity was chosen for the intron sequence. The parameter values for the ML analysis were taken from the estimates made by Modeltest (Table 2).

In addition, Bayesian phylogenetic analyses were performed with MrBayes 2.01 (Huelsenbeck and Ronquist 2001) on both the combined data set and the RAG-1 and myoglobin genes separately, using the same models as in the ML analyses. The Bayesian approach to phylogeny reconstruction, as it is implemented in MrBayes 2.01, approximates the posterior probability for a phylogenetic tree by successively altering the model parameter values in a Markov chain Monte Carlo procedure (Huelsenbeck and Ronquist 2001). Initially, a random tree and parameter values are chosen as a starting point, and for each step in the chain a new combination of topology and parameter values is either accepted or rejected according to the Metropolis–Hastings–Green algorithm. At each step the log likelihood values are recorded and after these have reached a plateau and stabilized, the frequency by which a certain clade appear among the sampled trees is an approximation of its posterior probability. In order to more efficiently traverse the parameter space, several chains with different "temperatures" can be run simultaneously. A heated chain can more easily cross deep valleys and thus avoid that the chain is entrapped on a local optima (see Yang and Rannala 1997, Larget and Simon 1999, Huelsenbeck and Ronquist 2001 for a more detailed description of Bayesian inference). In the present analysis four Markov chains (three heated and one cold, temperature = 0.2) were run for 100 000 generations with trees sampled every 10th generation. The log likelihood values stabilized after approximately 20 000 generations, and the posterior probabilities were calculated from the remaining 8000 trees.

Nodal supports were also evaluated under the parsimony criterion (MP) with a bootstrap analysis in

Table 2. Models and parameter values used in the maximum likelihood analysis estimated by Modeltest (Posada and Crandall 1998).

Gene	Model	Base frequencies				Rate parameters						Shape parameter	Proportion of invariable sites
		A	C	G	T	A–C	A–G	A–T	C–G	C–T	G–T		
Combined	GTR+I+G	0.2884	0.2343	0.2502	0.2253	1.6969	6.389	0.8181	1.4375	10.5343	1.000	0.9725	0.3285
RAG-1 exon	GTR+I+G	0.2795	0.2368	0.2707	0.2130	2.1419	6.6776	0.6123	1.1963	13.2701	1.000	1.0375	0.4570
Myoglobin intron	HKY+G	0.2732	0.2318	0.2235	0.2715	transversion: transition ratio = 2.2559						2.1370	

PAUP* 4.0b10 (Swofford 1998). The support values were estimated with 1000 bootstrap replicates, each with 10 random additions of taxa.

Results

Within the ingroup the pairwise uncorrected sequence divergence in the myoglobin intron range from 2.6% between the two species of puffbirds (*Bucco* and *Nystalus*), to 20.1% between *Rhinocrypta* and *Stactolaema*. Distances between the ingroup and the outgroup taxa range from 11.1 to 20.6%, and 95% of all samples fall between 6.8% and 19.1%.

The corresponding figures for RAG-1 are generally lower, and range from 1.6% between the two puffbirds to 12.2% between *Galbula* and *Eubucco*. Distances between ingroup and outgroup taxa range from 6.3% between *Coracias* and *Chauna* to 12.2% between *Picumnus* and *Alectura*, and 95% of all observations fall between 4.5 and 11.3%.

Of the three genes studied, the lowest pairwise sequence divergences are found in c-*myc*. Within the ingroup the distances range from 0.8% between *Eubucco* and *Pteroglossus* to 8.9% between *Picumnus* and *Tockus*. Distances between the ingroup and outgroup taxa range from 3.8% between *Bucco* and *Chauna* to 11.1% between *Picumnus* and *Alectura*.

The ML and Bayesian inference of the combined data set resulted in almost identical topologies and differed only in the position of a single branch (see below). Also the bootstrap analysis under the parsimony criteria resulted in a similar tree, although not all nodes indicated by the model-based analyses were recovered with bootstrap support exceeding 50%.

Monophyly of Pici and Galbulae, respectively, were corroborated by all analyses, and both clades receive high support values (Fig. 2). Furthermore, the sister group relationship between these two clades is recovered in 90% of the bootstrap replicates and has a 100% posterior probability in the Bayesian phylogenetic analysis. Thus, the combined dataset strongly supports monophyly of the Piciformes (*sensu* Wetmore 1960). Within Pici, the woodpecker (*Picumnus*) is the sister of the honeyguide (*Indicator*) and this clade in turn, is the sister group of the barbets and toucans. In the barbet–

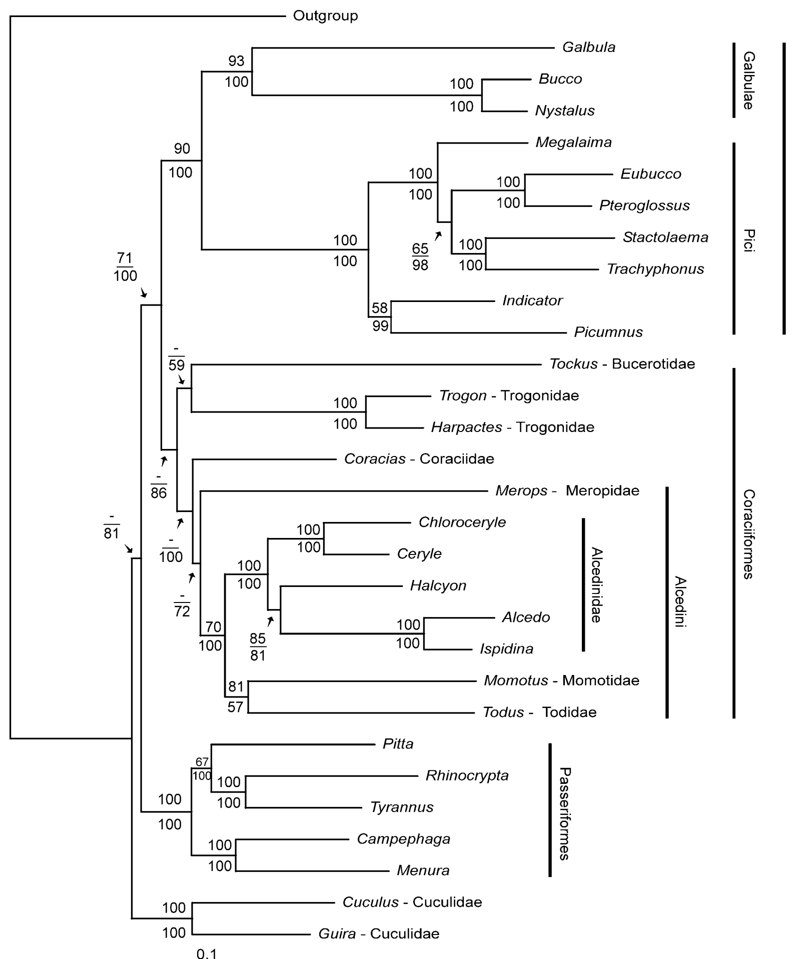


Fig. 2. The 50% majority-rule consensus tree obtained from the Bayesian inference analysis of the combined (c-*myc* exon 3, RAG-1 and myoglobin intron II) data set. Nodal supports are indicated in front of the nodes (posterior probabilities from the Bayesian analysis are given below branches and bootstrap support from the parsimony analysis above).

toucan clade, the South American taxa (*Pteroglossus* and *Eubucco*) form the sister clade to the African barbets (*Trachyphonus* and *Stactolaema*), and the Asian representative (*Megalaima*) is basal to the other. Both the African and South American clades receive a 100% bootstrap support, while the sister group relationship between these two clades and the Asian species also has a high posterior probability of 98%, but only a 69% bootstrap support.

The five kingfisher species (Alcedinidae) were recovered as monophyletic and the association of *Halcyon* (Halcyoninae) with *Alcedo* and *Ispidina* (Alcedininae) receive a relatively high bootstrap support (85%). *Chloroceryle* and *Ceryle* (Cerylininae) are placed as sister taxa basal to the other three kingfishers.

The only incongruence observed between the two model-based analyses concerns the relative position of *Todus* (Todidae). The Bayesian analysis places this taxon as the sister of *Momotus* (Momotidae), and these two in turn are placed as the sister group of the kingfishers (Fig. 2). These relationships are also supported by the bootstrap analysis under the parsimony criterion, but in the ML tree the motmot and the kingfishers are placed as sister taxa with the tody positioned basal to them. Although the inter-relationships between Momotidae, Todidae, and Alcedinidae differ between the analyses, the monophyly of these three taxa is strongly supported (Fig. 2). Associated with this clade are also the bee-eaters (*Merops*, Meropidae), indicating the monophyly of Alcedini *sensu* Sibley and Ahlquist (1990).

Monophyly of Coraciiformes (including the trogons, Trogonidae) is indicated by both the ML and the Bayesian analyses, and both place the coraciiforms as the sister group of Piciformes. Neither of these relationships are, however, supported by the bootstrap analysis under the parsimony criterion. Many of the associations within Coraciiformes suggested by the model-based analyses have high posterior probabilities, but are nevertheless unsupported by the MP analysis. For instance, the association of the roller (*Coracias*, Coraciidae) with Alcedini has a 100% posterior probability, but is unsupported by the parsimony analysis (Fig. 2).

Monophyly of the Passeriformes is highly supported and this taxon is placed as the sister group of the piciform–coraciiform clade in the model-based analyses. The two cuckoos (Cuculidae) are placed basal to all other ingroup taxa.

The individual gene trees differ markedly in resolution. The c-*myc* tree is almost completely unresolved (only a parsimony analysis was conducted for this gene). The RAG-1 and myoglobin gene trees are generally similar but some conflicts in weakly supported nodes exist. Both the RAG-1 and myoglobin gene trees support monophyly of Piciformes (Fig. 3), and within this clade both Pici and Galbulae are recovered as monophyletic. Both trees also place the cuckoos basal relative all ingroup taxa and place Passeriformes as the sister group to a clade containing the Piciformes and Coraciiformes. Relationships within this latter piciform–coraciiform clade differ, however, between the gene trees. The conflicting nodes are all weakly supported, or unsupported, by the parsimony analysis. None of the gene trees are identical to the tree obtained in the analysis of the combined data set, but the myoglobin tree, being the most similar, differs only in a few nodes. In agreement with the tree based on the combined data set, the myoglobin gene tree recognizes a clade with the piciform and the coraciiform taxa (Fig. 3a), although the coraciiform clade does not include the trogons as in the combined tree. Instead, the trogons are placed as the sister taxon to the piciform–coraciiform clade in the myoglobin tree. Monophyly of a Coracii–Alcedini clade is highly supported, and a sister group relationship between motmot (*Momotus*) and tody (*Todus*) is recovered by all three analyses. However, albeit weakly supported, the two model-based analyses of the myoglobin intron suggest that the bee-eaters (*Merops*) and the roller (*Coracias*) are sister groups, and within Passeriformes, the pitta (*Pitta*, Pittidae) is placed basal relative the other suboscines and oscines.

The RAG-1 tree (Fig. 3b) differs more from that proposed by the analysis of the combined data set. However, all nodes that differ, except one, are unsupported by the bootstrap analysis.

Discussion

The present study strongly supports a sister group relationship between Pici and Galbulae, i.e. monophyly of the order Piciformes *sensu* Wetmore (1960). Thus, it appears that the zygodactyl foot in Pici and Galbulae, despite the different morphology in the two groups, are homologous, and the Type VI arrangement of the flexor tendons have evolved only once within birds. Of the two other groups of recent birds with a zygodactyl foot, only representatives of one (cuckoos, Cuculidae) were included in the present analysis. Based on the MP analysis the position of the cuckoos is unresolved relative to Piciformes, but it is apparent from the model-based ML and Bayesian analyses that the cuckoos are not closely associated with Piciformes. In both these analyses the cuckoos are instead placed as the sister group to all other ingroup taxa (Fig. 2).

Within Pici, the woodpeckers (Picidae) and honeyguides (Indicatoridae) are sister taxa. The bootstrap support for this clade is relatively low (58%), but the monophyly of this clade is further supported by a synapomorphic deletion of two amino acids in the protein coding c-*myc*. Indels in the c-*myc* gene are rare, and those that have been observed in birds have been proven informative about phylogenetic relationships (Ericson et al. 2000, Johansson et al. 2001).

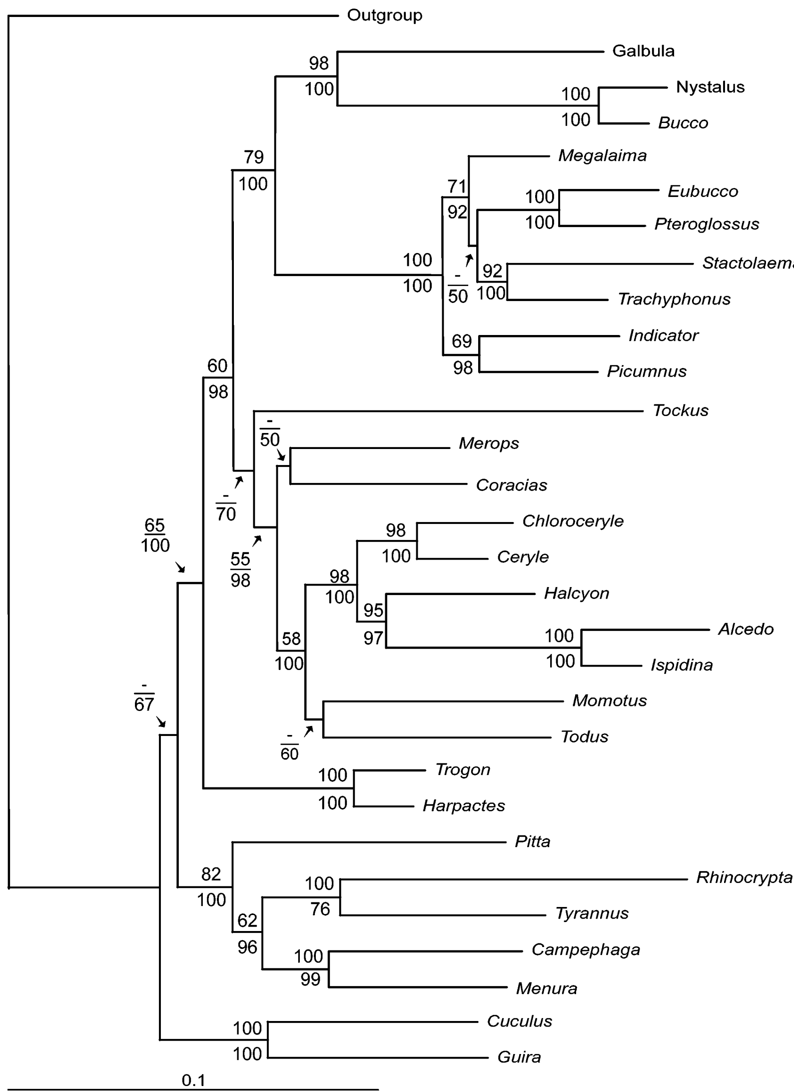
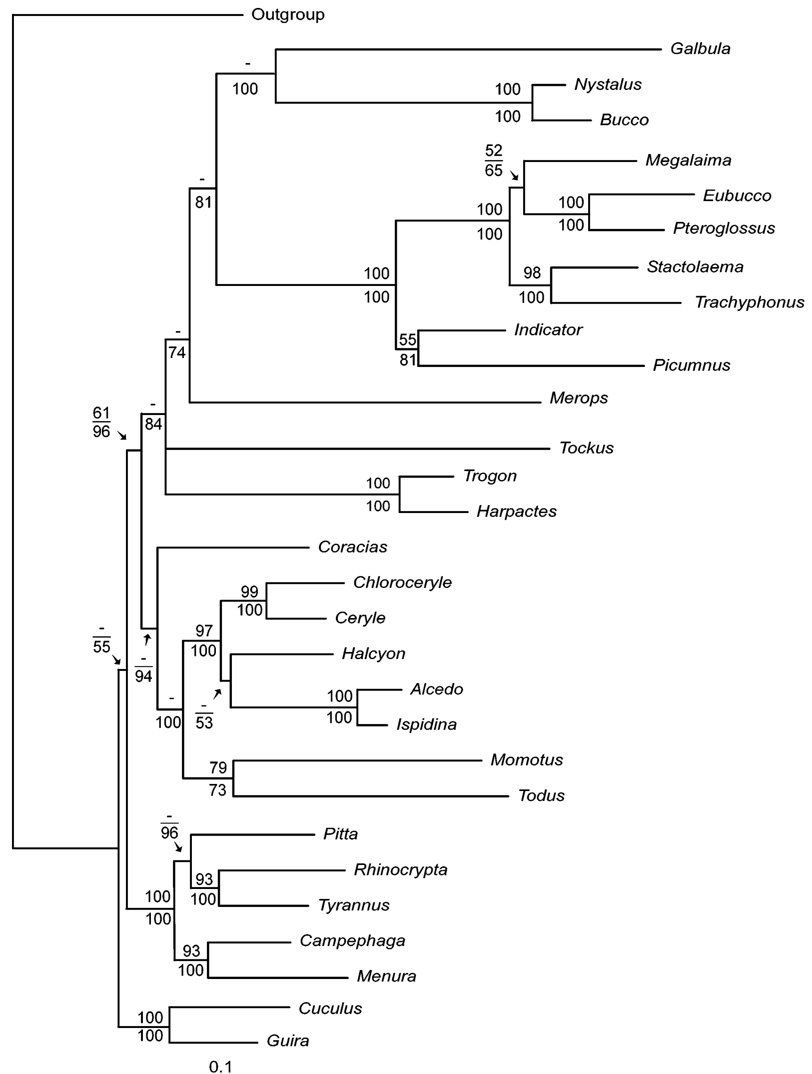


Fig. 3. The 50% majority-rule consensus tree obtained from the Bayesian inference analysis of (A) the myoglobin intron II and (B) the RAG-1 gene separately. Nodal supports are indicated in front of the nodes (posterior probabilities from the Bayesian analysis are given below branches and bootstrap support from the parsimony analysis above).

Also the barbet–toucan clade is strongly supported by the sequence data, and the South American barbets and toucans are placed as the sister group of the African barbets in the analysis of the combined data set, whereas the Asian barbets are placed basal to them. Thus, the Old World barbets do not appear to be monophyletic. The posterior probability for placing the Asian *Megalaima* basal relative to the African and South American clades is 98%, but the bootstrap support for this is, however, relatively weak (65%). This topology has, however, also been suggested by DNA–DNA hybridization data (Sibley and Ahlquist 1990). Non-monophyly of the Old World barbets has also been proposed based on morphological data by Prum (1988), but that tree is nevertheless quite different from the one presented here. In agreement with the present study and several others (Burton 1984, Sibley and Ahlquist 1990, Lanyon and Hall 1994, Johansson et al. 2001), the toucans were placed together with the South American barbets in that study, but the morphological data suggested that the South American clade is more closely related to Asian than African barbets. Furthermore, Prum (1988) placed the African genus *Trachyphonus* as the sister group of all other barbets, including *Stactolaema* and other African genera. Although more data is needed to conclusively determine how the barbets from the three geographical regions are related, the present data set indicates that *Trachyphonus* is not basal relative all other barbets, but that the two African barbets *Trachyphonus* and *Stactolaema* are monophyletic (Fig. 2).

Monophyly of a clade consisting of the kingfishers, motmots, and todies is also highly supported by the nuclear DNA sequence data. These three taxa are often

Fig. 3. Continued



placed together with the bee-eaters in the Alcedini *sensu* Sibley and Ahlquist (1990), and this relationship is suggested by both the ML and Bayesian analyses, but yields no support by the parsimony analysis. The relationships within Alcedini are not conclusively resolved by the present analysis, but both the MP and Bayesian analyses places the motmot and the tody as sister taxa, and this topology is also present in the RAG-1 and myoglobin gene trees. Only the ML analysis of the combined data set places the motmot basal to the kingfishers and the tody. Also among previous studies, both based on morphological and molecular data, there has been very little agreement about the relationships within this clade. The topology proposed by the MP and Bayesian analyses are congruent with the phylogeny of the group proposed by Burton (1984) based on a non-cladistic analysis of the feeding apparatus. Other morphological studies (Maurer and Raikow 1981, Mayr 1998) also recognizes a sister group relationship between motmots and todies, but place the bee-eaters as the sister group of the kingfishers. In addition, Maurer and Raikow (1981) place the trogons in the Alcedini, as sister the to the bee-eaters and the kingfishers. The members of Alcedini share a derived state of the columella (the stapal bone in the ear), which is shared also with trogons (Feduccia 1975). The inclusion of the trogons in the Alcedini is not corroborated by the present study, and it has also been questioned by e.g. Mayr (1998).

A basal position of the bee-eaters relative to the kingfisher–motmot–tody clade, in agreement with the result presented here, has previously been suggested by the DNA–DNA hybridization data (Sibley and Ahlquist 1990). However, different tree topologies were

recovered when the DNA–DNA hybridization data were analyzed using different clustering algorithms. In the UPGMA analysis, the motmots were placed as the sister group to the kingfishers and todies, whereas motmots and kingfishers as sisters in the FITCH analysis (Sibley and Ahlquist 1990). Furthermore, in the FITCH tree the rollers rather than the bee-eaters are the sister group of this clade. A re-analysis of the DNA–DNA hybridization data (Harshman 1994) resulted in a tree topology of the motmot-tody-kingfisher clade identical with that in the FITCH of Sibley and Ahlquist (1990). The analysis did not resolve the relationships between this clade and the bee-eaters and rollers and left them in an unresolved trichotomy. Seemingly, there are some conflicts between the different data sets, but all morphological analyses agree in placing the motmots and todies together, and this also seems to receive best support by the present data. The position of the bee-eaters is uncertain, but the two main hypotheses place them either as the sister group of the kingfishers (Maurer and Raikow 1981, Mayr 1998) or basal to a clade containing motmots, todies, and kingfishers (Burton 1984, Sibley and Ahlquist 1990). The result of the present analysis is concordant with the latter hypothesis. Furthermore, the association of the rollers with the Alcedini has a strong support by the Bayesian analysis (Fig. 2) and this association is also suggested by the ML analysis.

Although unsupported by the bootstrap analysis, the ML tree indicates that the order Coraciiformes, including the trogons, is monophyletic, and that it is the sister group to Piciformes. The passerines are placed, as sister group to these two clades and basal to all of them are the cuckoos. It should be borne in mind, however, that the taxon sampling in this study was not designed to test the monophyly of Coraciiformes, or the relationship of this group to Piciformes and Passeriformes. These three groups have often been considered as closely related but no synapomorphies have been presented to support this (Cracraft 1981, Olson 1982). Furthermore, monophyly of Coraciiformes has not been convincingly demonstrated and the three synapomorphies proposed by Maurer and Raikow (1981) are also present in groups outside of this clade.

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Appendix

RAG-1

Amplified as two separate fragments (I, II), each approx. 1000 bp. long with an overlap of approx. 100 bp.

(I) PCR

Primers: R17 and R22, R17 and R51, R50 and R22, or R50 and R51

94°C 5 min

94°C 40 s		94°C 40 s		94°C 40 s
63°C 60 s × 4	→	60°C 60 s × 4	→	55°C 60 s × 32
72°C 60 s		72°C 60 s		72°C 60 s

72°C 5 min

Sequencing

External primers: R17 or R50, R22 or R51.
Internal primers: R52, R53

Primer	Sequence (5′ to 3′)	Reference
R17	CCCTCCTGCTGGTATCCTTGCTT	Groth and Barrowclough (1999)
R22	GAATGTTCTCAGGATGCCTCCCAT	Groth and Barrowclough (1999)
R50	CTGATCTGGTAACCCCAGTGAAATCC	Irestedt et al. (2001)
R51	GACCCTCTTTCTGCTATGAGGGGGC	Irestedt et al. (2001)
R52	CAAGCAGATGAAYTGGAGGC	Irestedt et al. (2001)
R53	TCCATGTCCTTTAAGGCACA	Irestedt et al. (2001)

(II) PCR

1st amplification:	2nd amplification:
Primers: R56, R2B	Primers: R62, R63
94°C 5 min	94°C 5 min
94°C 40 s	94°C 40 s
55°C 60 s	55°C 60 s
72°C 60 s	72°C 60 s
72°C 5 min	72°C 5 min

Sequencing

External primers: R62, R63,
Internal primers: R58, R65

Primer	Sequence (5′ to 3′)	Reference
R56	CCCAGAGAAGGCTGTTCGC	This study
R2B	GAGGTATATAGCCAGTGATGCTT	Groth and Barrowclough (1999)
R62	GGCTGTTCGCTTTTCTTTCAC	This study
R63	CCAGTGATGCTTCAAGACAT	This study
R58	GAGACTGTTCCCTCCATAGATGC	This study
R65	TCACCAATCTCCATCTGGA	This study

c-*myc*

PCR

Nested PCR. Size of amplified fragment approx. 500 bp.

1st amplification:	2nd amplification:
Primers: *myc*3D, R*myc*3D	Primers: *myc*3A, R*myc*3A
94°C 5 min	94°C 5 min
94°C 40 s	94°C 40 s
50°C 40 s × 40	50°C 40 s × 30
72°C 60 s	72°C 60 s
72°C 5 min	72°C 5 min

Sequencing

External primers: *myc*EX3A, R*myc*EX3A.
Internal primers: *myc*EX3C-1, R*myc*EX3B.

Primer	Sequence (5′ to 3′)	Reference
*myc*EX3D	GAAGAAGAACAAGAAGAAGATG	Ericson et al. (2000)
R*myc*EX3D	ACGAGAGTTCCTTAGCTGCT	Ericson et al. (2000)
*myc*EX3A	CAAGAAGAAGATGAGGAAAT	Ericson et al. (2000)
R*myc*EX3A	TTAGCTGCTCAAGTTTGTG	Ericson et al. (2000)
*myc*EX3C-1	CAAAAAGGCTAAAGTTGG	Johansson et al. (2001)
*Rmyc*EX3B	CGGTTGTTGCTGATCTG	Irestedt et al. (2001)

Myoglobin Intron II

PCR

Amplified either in a single amplification with Myo2 and Myo3F or as a nested PCR with Myo2 and Myo3 in the first amplification and Myo2 and Myo3F in the second amplification. All reactions were performed under similar thermocycling conditions, but the re-amplification was carried out with only 22–25 cycles.

94°C 5 min

94°C 40 s
59°C 40 s $\times$ 40
72°C 60 s

72°C 5 min

Sequencing

External primers: Myo2, Myo3F
Internal primers: Myoint.C, Myoint.NC, Myoint.H2

Primer	Sequence (5′ to 3′)	Reference
Myo2	GCCACCAAGCACAAGATCCC	Slade et al. 1993
Myo3	CGGAAGAGCTCCAGGGCCTT	Slade et al. 1993
Myo3F	TTCAGCAAGGACCTTGATAATGACTT	Heslewood et al. 1998
Myoint.C	AGCCCTGGAGGATCCATTGG	Heslewood et al. 1998
Myoint.NC	CCAATGGATCCTCCAGGGCT	Heslewood et al. 1998
Myoint.H2	TCTAAACTTGGATATTCACAT	Irestedt et al. 2002