
Nuclear DNA from old collections of avian study skins reveals the evolutionary history of the Old World suboscines (Aves, Passeriformes)

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Accepted: 10 July 2006
doi:10.1111/j.1463-6409.2006.00249.x

Irestedt, M., Ohlson, J. I., Zuccon, D., Källersjö, M. & Ericson, P. G. P. (2006). Nuclear DNA from old collections of avian study skins reveals the evolutionary history of the Old World suboscines (Aves: Passeriformes). — *Zoologica Scripta*, 35, 567–580.

Museum study skins represent an invaluable source of DNA for phylogenetics, phylogeography and population genetics. This becomes evident by comparing the number of study skins of birds housed in museums worldwide (c. 10 million) with the corresponding number of tissue samples (probably fewer than 500 000). While the laboratory techniques used hitherto have primarily allowed PCR-based studies of mitochondrial genes from museum skins, we present here the first avian phylogeny based on a large number of nuclear sequences. The targeted fragment sizes and the properties of the primers used are important contributory factors to obtain good amplification results. In this study we routinely amplified fragments of c. 350 bp nuclear DNA. This advance in methodology opens up a new avenue for the use of avian museum skins, as nuclear DNA is especially useful when studying ancient patterns of diversification. The phylogenetic hypothesis of the Old World suboscines (Eurylaimides) presented herein strongly supports a monophyletic origin of the pittas (Pittidae). The phylogeny further suggests that pittas could be divided into three major groups, in agreement with the external morphological variation found in this group. The broadbills (Eurylaimidae) as currently defined are, on the other hand, found to be a paraphyletic family, as both *Sapayoa aenigma* and the asities (Philepittidae) are nested among them. Based on the phylogenetic results we suggest a revised classification of the Old World suboscines (Eurylaimides).

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Introduction

Although fresh samples are always preferred in molecular studies, tissue and blood of critical avian taxa are often not available for study. In such cases museum collections of avian study skins can provide an invaluable source of DNA, as it has been estimated that museums hold nearly 10 million bird specimens of the 10 000 known species (Roselaar 2003). However, after an initial enthusiasm for the recovery of DNA from museum specimens (Houde & Braun 1988; Ellegren 1991), the use of avian study skins in molecular systematics has been rather limited (but see e.g. Cooper *et al.* 1992, 1996, 2001; Leeton *et al.* 1993; Cracraft & Feinstein 2000; Vallianatos *et al.* 2002; Payne & Sorenson 2003; Sefc *et al.* 2003; Ericson

et al. 2005), chiefly because degenerated DNA from old avian collections requires considerable extra labour in the laboratory.

In addition, the DNA regions have hitherto been almost exclusively of mitochondrial origin (for exceptions see e.g. Mundy *et al.* 1997; Eggert *et al.* 2004; Dumbacher & Fleischer 2001), and the general opinion has been that nuclear DNA sequences are extremely difficult to obtain from avian study skins. This is especially unfortunate for the study of more ancient systematic relationships in birds, as these often have proven to be difficult to resolve by fast evolving mitochondrial gene sequences, and this has certainly hampered the general use of study skins in phylogenetic analyses. By careful primer design and by excising PCR-products from

agarose gels we are here able to present the first avian phylogeny that has extensively used avian study skins, collected in the early 20th century, as the source for nuclear DNA. Three nuclear gene regions have been sequenced in order to obtain a hypothesis of the evolutionary relationships of the Old World suboscines (Eurylaimides). Our approach has enabled us to include in our phylogeny representatives of all genera, and as many as 28 out of the 30 species of pittas currently recognized (Erritzoe 2003).

The Old World suboscines consist of three groups of birds that are seldom numerous and often have elusive habits and attractive plumages. Besides pittas, the group also consists of the asities (Philepittidae) and the broadbills (Eurylaimidae). In spite of its Neotropical distribution the broad-billed sapayoa (*Sapayoa aenigma*) has recently been added to this group (Fjeldså et al. 2003; Chesser 2004).

The taxonomic history of the Old World suboscines is complicated and reflects the lack of morphological synapomorphies for the group. It has long been recognized that the asities, broadbills and pittas share the so-called suboscine type of the syrinx (the vocal apparatus in birds) with a number of passerines in the New World (Müller 1847; Forbes 1880a,b). Nevertheless, monophyly of the suboscines was not demonstrated convincingly until Feduccia (1974) showed that all suboscines (i.e. Eurylaimides and Tyrannides *sensu* Ericson et al. 2003) share a derived morphology of the stapes (a bone in the ear). In early classifications broadbills were often considered as the most 'primitive' passerine group based on anatomical evidence (e.g. Forbes 1880b), and were therefore set apart in their own suborder, the Desmodactyli. Olson (1971) showed that these anatomical characters are either not consistently found in all broadbills or are shared with other suboscine families.

The monophyly of the suboscines is now well established. Both DNA–DNA hybridization data (Sibley & Ahlquist 1990) and analyses of various kinds of DNA sequence data (e.g. Irestedt et al. 2001; Chesser 2004) have confirmed their common ancestry. The DNA sequence data have further shown that the suboscines can be divided into an Old World and a New World radiation (e.g. Irestedt et al. 2001; Chesser 2004). Nevertheless, the most complete study to date, that of Fjeldså et al. (2003), lacks several broadbill genera and contains only one pitta species. Consequently, much of our current understanding of the phylogenetic relationships among Old World suboscines largely rests on interpretations of morphology. For the pittas this means that taxonomic considerations are mainly based on overall similarity (e.g. Whitehead 1893; Erritzoe & Erritzoe 1998). Phylogenetic relationships among broadbills and asities have, on the other hand, been studied by cladistic analyses of hindlimb myology (Raikow 1987) and syrinx morphology (Prum 1993), although the results of the latter two studies conflict with

each other. For more detailed reviews of the history of the classification of the Old World suboscines see e.g. Olson (1971), Sibley & Ahlquist (1990), Lambert & Woodcock (1996), Erritzoe & Erritzoe (1998), and del Hoyo et al. (2003).

Materials and methods

Taxon sampling, amplification and sequencing

The ingroup includes representatives of all genera and more than 80% of the species of Old World suboscines recognized by del Hoyo et al. (2003). We have studied the DNA of 28 species of pittas, 11 broadbills and two asities, along with that of the broad-billed sapayoa (*Sapayoa aenigma*) (cf. Fjeldså et al. 2003; Chesser 2004). A selection of New World suboscines has also been included, in order to test the monophyly of the Old World suboscines and the affinity of *S. aenigma*. The phylogenies have been rooted with a tree sparrow (*Passer montanus*), a representative of the oscine passerines.

Three nuclear gene regions – myoglobin intron 2, ornithine decarboxylase (ODC) introns 6–7, and glyceraldehyde-3-phosphodehydrogenase (G3PDH) intron 11 — have been sequenced and used for the phylogenetic interpretations. For each gene and taxon, multiple sequence fragments were obtained by sequencing with different primers. These sequences were assembled to complete sequences with SEQMAN II™ (DNASTAR Inc.). Positions where the nucleotide could not be determined with certainty were coded with the appropriate IUPAC code. GenBank accession numbers are given in Table 1. For extractions, amplifications, and sequencing procedures for fresh tissue/blood samples see Irestedt et al. (2001, 2002, 2004a), Fjeldså et al. (2003), and Allen & Omland (2003).

DNA was extracted from footpads of museum study skin from 18 of the 42 species in the ingroup using the DNeasy Tissue Kit (Qiagen) following the manufacturer's instructions. Some minor modifications were made; the quantity of buffers and enzymes during the lysis stage was doubled, with rather large pieces (c. 10–20 mm²) of footpads used whenever possible. As the lysis procedure is prolonged (normally 24–48 h) due to the firm texture of the footpads (compared to fresh tissue), additional proteinase K (5–10 µL) was added two to four times. The amount of elution buffer during the final stage of the extraction was decreased to give a total volume of 100 µL extract. The extractions from the footpads were always performed in a room dedicated to working with old DNA material, with appropriate facilities such as a UV-bench used for sterilizing equipment. When available, 2–3 individuals from each taxon were extracted and the sample that produced the best PCR products was selected for sequencing. The chosen samples were then used throughout the study.

For amplifying and sequencing the DNA from the footpads a number of new primers were designed. Several

Table 1 Specimen data and GenBank accession numbers for samples used in the study. Acronyms: ANSP, Academy of Natural Science, Philadelphia; FMNH, Field Museum of Natural History, Chicago; MNHN, Muséum National d'Histoire Naturelle, Paris; MV, Museum Victoria, Melbourne; NRM, Swedish Museum of Natural History, Stockholm; RMNH, National Museum of Natural History, Leiden; UWBM, University of Washington, Burke Museum; ZMUC, Zoological Museum of the University of Copenhagen; YPM, Yale University, Peabody Museum, New Haven. References: 1, Irestedt *et al.* (2002); 2, Ericson *et al.* (2002b); 3, Ericson & Johansson (2003); 4, Fjeldså *et al.* (2003); 5, Johansson & Ericson (2003); 6, Fjeldså *et al.* (2005); 7, Ericson *et al.* (2006).

Species	Origin and collection years for study skin samples	Sample no.	Myoglobin	ODC	G3PDH
<i>Pitta (Hydromis) phayrei</i>	Siam, 1960	YPM 68280	DQ785995	DQ785959	DQ785920
<i>Pitta (Hydromis) soror</i>		NRM 20047038	DQ785998	DQ785962	DQ785923
<i>Pitta (Hydromis) nipalensis</i>		ZMUC 130577	DQ785992	DQ785956	DQ785917
<i>Pitta (Hydromis) oatesi</i>	Tonkin, 1939	NRM 566874	DQ785994	DQ785958	DQ785919
<i>Pitta (Hydromis) caerulea</i>	1884	ZMUC	DQ785980	DQ785944	DQ785905
<i>Pitta (Hydromis) cyanea</i>	Siam, 1914	NRM 569388	DQ785981	DQ785945	DQ785906
<i>Pitta (Hydromis) gurneyi</i>	Siam, 1916	ZMUC	DQ785987	DQ785951	DQ785912
<i>Pitta (Hydromis) guajana</i>	Java, 1900	NRM 569386	DQ785986	DQ785950	DQ785911
<i>Pitta (Hydromis) elliottii</i>		NRM 20026663	DQ785983	DQ785947	DQ785908
<i>Pitta (Hydromis) baudii</i>		ANSP 1224	AY064256 (2)	DQ785942	DQ785903
<i>Pitta (Erythropitta) erythrogaster</i>		FMNH 358346	DQ785984	DQ785948	DQ785909
<i>Pitta (Erythropitta) kochi</i>		ZMUC 5489	DQ785989	DQ785953	DQ785914
<i>Pitta (Erythropitta) venusta</i>		ZMUC 130560	DQ786001	DQ785965	DQ785926
<i>Pitta (Erythropitta) granatina</i>	Malaysia, 1912	NRM 569389	DQ785985	DQ785949	DQ785910
<i>Pitta (Erythropitta) arquata</i>	Borneo, 1910	NRM 569387	DQ785978	DQ785941	DQ785902
<i>Pitta angolensis</i>		ZMUC S1027	AY165820 (ref. 5)	DQ785940	AY336596 (ref. 4)
<i>Pitta reichenowi</i>	Gabon, 1981	MNHN uncat.	DQ785996	DQ785960	DQ785921
<i>Pitta brachyura</i>	India, 1954	YPM 32016	DQ785979	DQ785943	DQ785904
<i>Pitta moluccensis</i>		UWBM 67456	DQ785991	DQ785955	DQ785916
<i>Pitta nympha</i>		Yao Cheng-te uncat.	DQ785993	DQ785957	DQ785918
<i>Pitta versicolor</i>		MV C534	DQ786002	DQ785966	DQ785927
<i>Pitta elegans</i>		ZMUC 131938	DQ785982	DQ785946	DQ785907
<i>Pitta anerythra</i>		UWBM 60310	DQ785977	DQ785939	DQ785901
<i>Pitta sordida</i>		UWBM 67452	DQ785997	DQ785961	DQ785922
<i>Pitta iris</i>		MV MG23	DQ785988	DQ785952	DQ785913
<i>Pitta superba</i>	Manus Islands, 1934	RMNH 128428	DQ786000	DQ785964	DQ785925
<i>Pitta steerii</i>	Philippines, 1958	YPM 47872	DQ785999	DQ785963	DQ785924
<i>Pitta maxima</i>	Halmahera, 1985	RMNH 84745	DQ785990	DQ785954	DQ785915
<i>Calyptomena viridis</i>		ZMUC 548	AY338734 (ref. 4)	DQ785930	AY336588 (ref. 4)
<i>Smithornis rufolateralis</i>		FMNH 391675	AY065789 (ref. 1)	DQ785972	AY336589 (ref. 4)
<i>Smithornis capensis</i>		ZMUC 5967	DQ786004	DQ785971	DQ785929
<i>Pseudocalyptomena graueri</i>	Congo, 1935	NRM 569390	DQ786003	DQ785968	DQ785928
<i>Cymbirhynchus macrorhynchos</i>		UWBM 67461	AY338736 (ref. 4)	DQ785932	AY336592 (ref. 4)
<i>Serilophus lunatus</i>		MNHN uncat.	AY338738 (ref. 4)	DQ785970	AY336594 (ref. 4)
<i>Psarisomus dalhousiae</i>		MNHN uncat.	AY338735 (ref. 4)	DQ785967	AY336595 (ref. 4)
<i>Corydon sumatranus</i>	Siam, 1938	NRM 569391	DQ785973	DQ785931	DQ785897
<i>Eurylaimus ochromalus</i>		UWBM 67481	AY338737 (ref. 4)	DQ785934	AY336593 (ref. 4)
<i>Eurylaimus javanicus</i>	Siam, 1938	NRM 569392	DQ785974	DQ785933	DQ785898
<i>Eurylaimus (Sarcophanops) steerii</i>	Philippines, 1957	YPM 41189	DQ785975	DQ785935	DQ785899
<i>Sapayoa aenigma</i>		ZMUC 126529	AY338733 (ref. 4)	DQ785969	AY336590 (ref. 4)
<i>Philepitta castanea</i>		ZMUC 5458	AY065790 (ref. 1)	DQ785938	AY336591 (ref. 4)
<i>Neodrepanis coruscans</i>	Madagascar, 1924	NRM 569385	DQ785976	DQ785936	DQ785900
<i>Furnarius cristatus</i>		NRM 966772	AY064255 (ref. 2)	DQ435482 (ref. 7)	AY590066 (ref. 6)
<i>Rhinocrypta lanceolata</i>		NRM 966793	AY065775 (ref. 1)	DQ435499 (ref. 7)	DQ438953 (ref. 7)
<i>Conopophaga aurita</i>		ZMUC S1245	AY065784 (ref. 1)		
<i>Conopophaga lineata</i>		NRM 956653		DQ435478 (ref. 7)	AY336577 (ref. 4)
<i>Thamnophilus caeruleus</i>		NRM 967007	AY065783 (ref. 1)	DQ435504 (ref. 7)	AY336587 (ref. 4)
<i>Pyroderus scutatus</i>		NRM 967030	AY065786 (ref. 1)	DQ435498 (ref. 7)	AY336582 (ref. 4)
<i>Pipra fasciicauda</i>		NRM 947271	AY065787 (ref. 1)	DQ435495 (ref. 7)	AY336583 (ref. 4)
<i>Tityra cayana</i>		NRM 956584	AY338742 (ref. 4)	DQ435505 (ref. 7)	AY336580 (ref. 4)
<i>Tyrannus savana</i>		NRM 976722	AY165826 (ref. 5)	DQ435507 (ref. 7)	AY336579 (ref. 4)
<i>Passer montanus</i>		NRM 976359	AY228311 (ref. 3)	DQ785937	AY336586 (ref. 4)

primer-designing programs accessible via the internet, e.g. cybergene (<http://www.cybergene.se/primer.html>), were used to check the primer's melting temperatures, indication of palindrome or hairpin loop structures, etc. To avoid primer combinations that form primer dimers, all primer combinations were finally checked with the program Amplify (Engels 1993). We tried to use primers with similar melting temperatures (c. 60–65 °C) that amplified fragments of 300–350 bp. We used hot-start touchdown PCR, with annealing temperatures for the first cycles generally just 1–2 °C below the melting temperature of the primer with the lowest melting temperature. A representative thermocycling program for a given primer combination started with initial denaturation at 95 °C for 5 min, followed by four cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C, and another four-cycle phase and one 36-cycle phase with identical temperatures and intervals, except that the annealing temperatures were reduced to 58 °C and 56 °C, respectively. The thermocycling program ended with 72 °C for 5 min. The primer sequences are listed in Table 2 and their locations in the gene regions are shown in Fig. 1.

Although the primers used for the skin samples were carefully designed, the PCR products often contained primer dimers or non-specific bands of shorter size than the desired fragment. To remove these, the PCR products were routinely

loaded on to an agarose gel while the proper band was excised and cleaned of gel material using QG buffer in conjunction with the QIAquick PCR Purification Kit (Qiagen). Distinct bands were sequenced directly, while faint bands were re-amplified using identical primer combinations and PCR-program.

Phylogenetic inference and model selection

As there were relatively few insertions in the introns, the combined sequences could easily be aligned by eye. All gaps have been treated as missing data in the analyses. Bayesian inference (see e.g. Holder & Lewis 2003; Huelsenbeck *et al.* 2001) was used to estimate the phylogenetic relationships. The models for nucleotide substitutions used in the analyses were selected for each gene individually by applying the Akaike Information Criterion (AIC, Akaike 1973) and the program MrModeltest 2.2 (Nylander 2005) in conjunction with PAUP* (Swofford 1998).

Posterior probabilities of trees and parameters in the substitution models were approximated with MCMC and Metropolis coupling using the program MrBayes 3.1.1 (Ronquist & Huelsenbeck 2003). In this version of MrBayes, two chains starting from different randomly chosen trees run simultaneously in order to calculate the standard deviation of split frequencies; this ensures that the individual runs have converged on the same target distribution (each chain is

Primer name	Primer sequence (5'–3')	Reference
Myoglobin		
Myo2	GCCACCAAGCACAAAGATCCC	Slade <i>et al.</i> (1993)
Myo344H	CCTCCAGGGCTTACTCTAAAATTGTA	
Myo309L	CATAAGACCTGTGTCAGTGGCTGGA	
Myo3f	TTGAGCAAGGACCTTGATAATGACTT	Heslewood <i>et al.</i> (1998)
Myo030L	ATCTGGAGGTATGGAAAAGGGCA	
Myo177L	AGAAAGGGCTCATGGTATGTGA	
Myo218h	GCATGTGGTGTGGAAATGGGAA	
Myo491L	GCAGATCAGCGTCAGAGCTAGGA	
Myo549h	GAGACAGTGAGGTCTAGTATGCA	
ODC		
<i>OD6</i>	GACTCCAAGCAGTTTGTCTCAGTGT	Allen & Omland (2003)
<i>OD8r</i>	TCTTCAGAGCCAGGGAAGCCACCAAT	Allen & Omland (2003)
ODintF1	ATGCCCGCTGTGTGTTG	
ODintF2	CACCTAAGACTAGCAGGCTTCTTCTGGA	
OdintF3	CAGATGCAGTAAAATTGGCAGCT	
OdintF4	TTGTTGGGGTTAGGTGAGTTGA	
ODintR3	CAAACACACAGCGGCATCAGA	
ODintR4	CATATTGAAGCCAAGTTCAGCCTA	
OdintR5	GCCAATTTTACTGCATCTGTAGA	
OdintR6	CCAACATGGAACTATGAAAAGA	
G3Pdh		
G3P13b	AAGTCCACAACACGGTTGCTGTA	Irestedt <i>et al.</i> (2002)
G3P14b	TCCACCTTTGATGCGGGTGTGGCAT	Irestedt <i>et al.</i> (2002)
G3PosoL1	TGCTGGTGATCCAGGCAGAT	
G3PosoH1	GCAGTAAGCATCCCATCCACC	
G3PintL1 (sequencing only)	GAACGACCATTTGTCAAGCTGGTT	Irestedt <i>et al.</i> (2002)

Table 2 Primers used in this study. Primers in bold are those used to amplify DNA from a majority of the avian study skins samples, while those in italic have only been used for fresh DNA samples. The remaining primers have been used for a few study skin samples where it was necessary to amplify some DNA regions in shorter pieces. G3PintL1 was not used for amplifications but during sequencing instead of G3P13b. The primers relative locations in the gene regions used in this study are shown in Fig. 1.

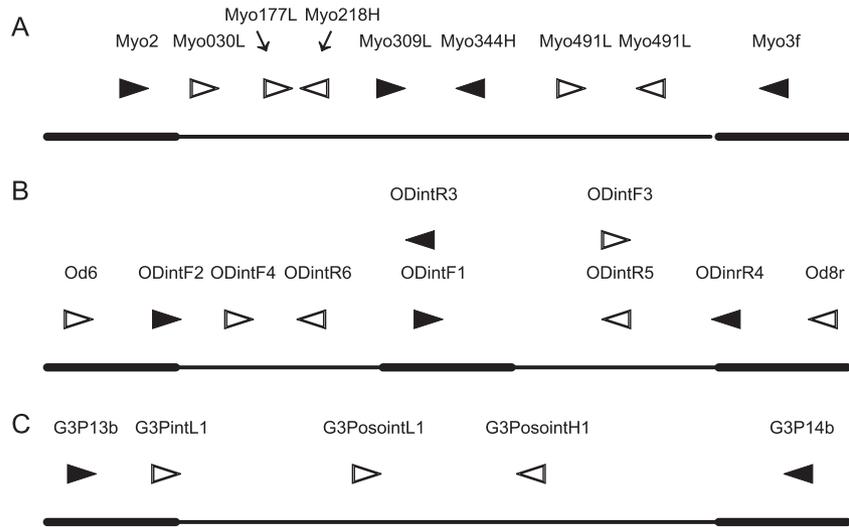


Fig. 1 A–C. Relative positions of the PCR and sequencing primers at —A. myoglobin intron 7, —B. ODC intron 6 and 7, and —C. G3PDH. Broad lines indicate exons and narrow lines introns. Solid arrows specify the primers that were generally used to amplify DNA extracted from footpads of museum study skins.

actually complemented by three incrementally ‘heated’ chains to ensure that the run performs a better search of the parameter space). Analyses were performed for both the individual gene partitions and the combined data set. In the analysis of the combined data set, the models selected for the individual gene partition were used, but the topology was constrained to be the same. We used an unconstrained, exponential branch length prior. All chains were run for 10 million generations, with trees sampled every 100th generation. The trees sampled during the burn-in phase (i.e. before the chain had reached its apparent target distribution) were discarded, and after checking for convergence, final inference was made from the concatenated output from the two runs.

The combined data set was also analysed with parsimony jackknifing (Farris *et al.* 1996) using the software Xac (Farris 1997) with the following settings: 1000 pseudoreplicates, each with branch swapping and 10 random addition sequences. All trees were drawn using MrEnt 1.2 (Zuccon & Zuccon 2006).

Results

Amplification and sequencing of nuclear genes from museum study skins

We were able to sequence all three gene regions almost completely for all taxa amplified from degraded DNA extracted from the footpads. For most samples we amplified and sequenced ODC and myoglobin in two fragments and G3PDH in one; the primer combination used to amplify these fragments is indicated by solid arrows in Fig. 1. In the ODC region all these taxa lack a short fragment of 22 bp, as we used overlapping primers in exon 7 to amplify the two ODC fragments. For a few taxa it was necessary to divide some of the fragments into two pieces. These extra primers

are marked with open arrows in Fig. 1. These taxa also lack a short fragment in the ODC, and/or a short fragment in the myoglobin region due to the locations of these additional primers. For the fresh material all fragments were amplified in one piece, but we were unable to sequence the ODC intron 6 from *Pitta kochi*.

Sequence lengths and alignments

Taking into account the fact that a few short fragments for some taxa are missing for the myoglobin intron 2 and the ODC regions, and that we were unable to sequence intron 6 in ODC for *P. kochi*, the sequence lengths obtained from the individual genes vary as follows. The sequences for myoglobin intron 2 ranged between 691 bp (*Furnarius cristatus*) and 720 bp (*Tyrannus savanna* and *Pipra fasciicauda*) for all taxa, except for *Pitta anerythra* where an autapomorphic insertion of 149 bp made this sequence 820 bp. The G3PDH intron 11 varied in length between 268 bp (*Passer montanus*) and 343 bp (*Pyroderus scutatus*). The sequenced ODC region (including exon 6) varies between 569 bp (*Pitta venusta*) and 638 bp (*Tityra cayana*), except for *F. cristatus* (497 bp) and *Rhinocrypta lanceolata* (403 bp), which contain considerable deletions compared to the other taxa.

Most indels observed in the introns were autapomorphic and were mainly found in particularly variable and repetitive regions. In these variable regions some indels varied in length between taxa, particularly in the relatively more indel-rich G3PDH intron 11 and ODC intron 7, which makes it difficult to know if these indels are homologous or represent independent evolutionary events. However, several apparently synapomorphic indels were observed when mapping the data onto the tree topology obtained from the Bayesian analyses of the combined data set. A few indels were also found to be

incongruent with the phylogenetic tree obtained from the analysis of the combined data set. These were generally found in the most variable regions and some of the single base pair insertions actually consist of different bases. For more details of indel length and positions see the alignments of the individual gene regions deposited at EMBL, accession numbers ALIGN_001037 (myoglobin), ALIGN_001038 (ODC), ALIGN_001039 (G3PDH).

Models for nucleotide substitutions and phylogenetic relationships

The a priori selection of nucleotide substitution models suggested that the GTR + Γ model had the best fit for all three gene regions. Although the same substitute models were selected for all three regions, we still applied a partitioned analysis of the combined data set, as the nucleotide state frequencies, as well as the gamma distribution, differed between the three gene regions. For Bayes estimates of parameters and substitution rates see Table 3. After discarding the burn-in phase the inference for the myoglobin, ODC, and G3PDH regions was based on a total of 97 500 samples from the posterior, while that for the combined data set was based on a total of 97 000 samples. For the phylogenetic inference, the mode of the posterior distribution of topologies was presented as a majority-rule consensus tree from each analysis (Figs 2, 3).

The trees obtained from the Bayesian analyses of the individual gene partitions (Fig. 2), as well as the tree obtained from the combined data set (Fig. 3), are topologically very similar and are, overall, well resolved. The only strongly supported conflicts observed concern: (1) the position of one group of pittas (*Pitta erythrogaster*, *P. kochi*, *P. arcuata*, *P. granatina* and *P. venusta*) in the ODC tree, which is in conflict with its position in the trees based on the G3PDH, myoglobin and the combined data sets, and (2) the position of *Sapayoa*

aenigma in the myoglobin tree that differs from that found in the G3PDH, ODC and combined trees. The few other relationships that are in conflict are all present at short nodes with posterior probabilities below 95%. In fact, most nodes are congruently present and supported in the trees obtained from all three gene regions as well as in the tree obtained from the combined data set.

Among the well-supported relationships suggested by the combined data set (and in most cases also by all three gene trees) are the monophyletic origins of the pittas, as well as of a broadbill–asities–*Sapayoa* clade. There is also strong support for dividing the pittas into three major clades and the broadbills and allies into two major radiations. The precise phylogenetic position of *S. aenigma* is not well supported, but two gene trees and the combined tree suggest that it is a basal member of a clade of broadbills that includes the Asian genus *Calyptomena* and the African genus *Smithornis*. Within the other main radiation of broadbills and allies, the asities are found to form the first branch, the African genus *Pseudocalyptomena* the second, followed by a group consisting of all Asian broadbill genera except *Calyptomena*.

The tree topology obtained from the parsimony jackknifing analysis of the combined data set is almost entirely congruent with the tree obtained from the Bayesian analysis. Only one topological conflict was observed, with a sister relationship between the outgroup taxa *Pipra fasciicauda* and *Tyrannus savana* suggested by the parsimony jackknifing tree. One node resolved in the combined Bayesian tree (a sister relationship between *Pitta cyanea* and the *P. elliotii* and *P. gurneyi* clade) is also unresolved in the parsimony jackknifing tree. The parsimony jackknife support values are shown in Fig. 3.

Discussion

The potential of avian study skins as a source for nuclear DNA in molecular phylogenetics

DNA is often damaged when exposed to conditions such as heat, light, acid, and alkylating or oxidizing agents. Today, tissue and blood collections for molecular research are stored in as good condition as possible. However, as the function of the DNA molecule and its potential in various areas of science have only have been known for slightly more than 50 years, older museum specimens have not been maintained in such a way as to prevent damage to the DNA. Consequently, the DNA obtained from older museum collections is always damaged to various degrees. This can cause problems for PCR amplification and sequencing.

An additional and crucial problem is the increased risk of contamination, with small amounts of intact foreign DNA out-competing the target DNA. It is a fact that several published sequences based on ancient DNA have later proven to be erroneous (see review by Willerslev & Cooper 2005). It has also been shown that damage to both mitochondrial and

Table 3 Mean Bayes estimates of parameters for the three studied genetic markers. Substitution rate parameters are calculated with the rate for G \leftrightarrow T set to 1. The models for nucleotide substitutions for the individual genes were used also in the analysis of the concatenated data set.

	Myoglobin	ODC	G3Pdh
No. of sites (aligned sequence)	886	735	409
No. of variable sites (%)	381 (43%)	381 (52%)	266 (65%)
No. of informative sites (%)	238 (27%)	218 (30%)	170 (42%)
ML model selected	GTR + G	GTR + G	GTR + G
r(AC)	1,14	1,38	0,99
r(AG)	5,58	4,74	3,83
r(AT)	0,75	0,76	0,7
r(CG)	1,72	1,42	1,88
r(CT)	5,44	3,44	3,86
r(GT)	1	1	1
Shape	1,45	1,42	3,65

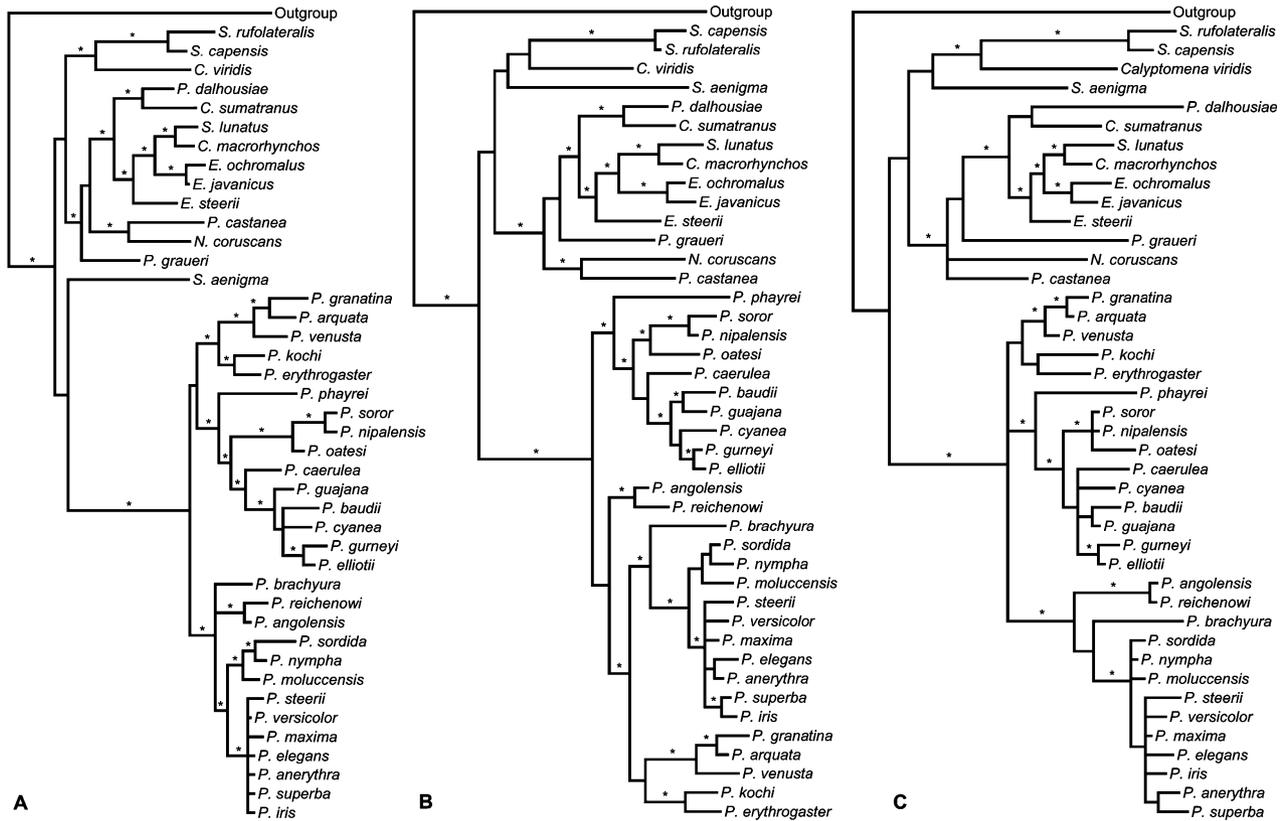


Fig. 2 A–C. The majority rule consensus trees obtained from the Bayesian analyses of the individual genes. —A. Tree obtained from the analyses of the myoglobin intron 2 data set. —B. Tree obtained from the Bayesian analyses of the ODC (ornithine decarboxylase) intron 6 and 7 data set. —C. Tree obtained from the analyses of G3PDH (glyceraldehydes-3-phosphodehydrogenase) intron 11 data set. Posterior probability values 95% and above are indicated with an asterisk at the branch.

nuclear ancient DNA could lead to artifactual sequences (Binladen *et al.* 2005). However, the specimens used in these studies are much older, and it might be assumed that they contain smaller amounts of DNA that is more damaged (and therefore likely to be much more sensitive to contamination), than that extracted from museum study skins that are typically 25–100 years old.

If some preventive measures are taken, such as keeping the DNA extracted from the footpads of the study skins separately from fresh avian DNA samples, never amplifying DNA from fresh and old samples together, and generally handling old DNA material carefully (e.g. using sterilized filter tips), our results suggest that the risk of contamination should be rather low for avian study skins. However, the fragment size that is targeted by a given primer combination is probably also an important factor when attempting to minimize contamination. Most samples extracted from footpads produce distinct bands for fragments of around 200 bp, while the strength of the bands is generally weaker or sometimes non-existent for fragments of around 350 bp. This indicates that

most samples contain a fairly good amount of DNA (when measuring the concentration of a few samples found to contain between 2 and 8 µg/µl DNA) but that longer fragments only exist in small numbers.

Thus, primer combinations for fragments that are too long most likely increase the risk of a poor ratio between the desired fragment and contaminants (if present). Not only is the length of the target fragment as defined by the primers vital, but how well a given primer pair act together is also critical to obtaining good results, possibly reducing the risk of contamination. In Fig. 4 the amplification results produced by the three primer combinations are compared. Even though the primers have a good fit to the templates and amplify virtually the same region (with only minor differences in fragment size), the results differ considerably. One of the combinations also forms a lot of primer dimers and the desired fragment is clearly visible only for the fresh DNA extract. In the second combination fewer primer dimers are visible and some of the study skin extracts produce visible bands. In the last combination, which produces almost no

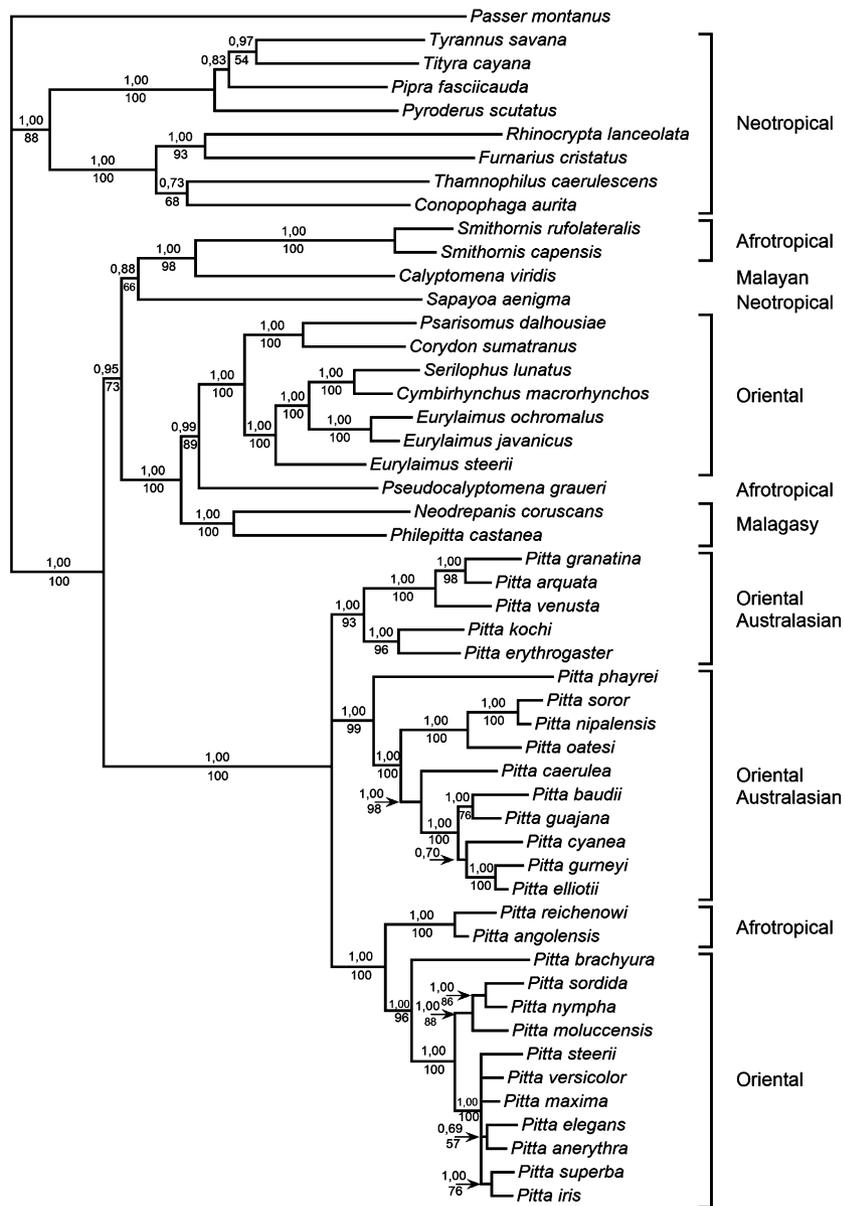


Fig. 3 The 50% majority rule consensus tree obtained from the analyses of the combined data set (myoglobin intron 2, ODC intron 6 and 7, and G3PDH intron 11). Posterior probability values and jackknife support values above 50% are indicated at the nodes.

visible primer dimers, the desired fragment is clearly visible for all study skins. This result clearly indicates the importance of using primer combinations with good properties when the desired template fragment exists in low concentration, as most of the primers otherwise might form primer dimers instead of building up the required fragment.

We have found no reason to suspect that any of the sequences obtained from the museum study skins examined in this study are erroneous. It should be noted, however, that a few extractions that produced noisy sequence chromatograms were discarded and replaced by others. Unique sequences

were obtained from all three gene regions from all taxa represented by museum study skin. Furthermore, their phylogenetic positions in the individual gene trees were congruent, which makes sense both with respect to what is known from non-molecular characters such as plumage patterns and from comparisons with the DNA from fresh material of related species. For example, the skin sequences from *Eurylaimus ochromalus* grouped with the sequences from fresh material of *E. javanicus*; *Pitta arcuata* and *P. granatina* grouped with *P. venusta*; *P. reichenowi* grouped with *P. angolensis*; and *P. gurneyi*, *P. cyanea* and *P. guajana* formed a clade with *P. elliotii* and

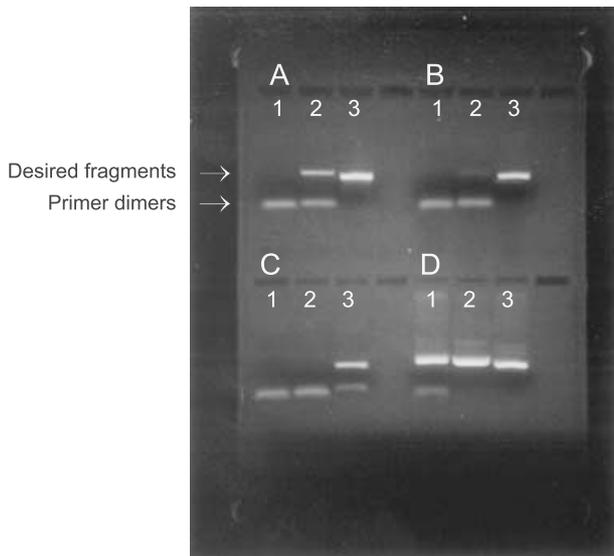


Fig. 4 A–D. Amplification results between the three primer combinations are compared for extracts from study skins —A–C. (*E. steerii*, *P. phayrei*, *P. gurneyi*) and from a fresh DNA extract —D. (*P. soror*). Even though all primers have a good fit to the templates and amplify intron 6 in ODC (with only some minor differences in fragment size), the results differ considerably between primer combinations. In the first combination of primers (OD6 and ODintR2) a lot of primer dimers are visible for all extracts, but only the fresh material produce a visible band of the desired fragment. In the second combination (OD6 and ODintR3) fewer primer dimers are visible and some of the study skin extracts produce visible bands. The last combination (ODintF2 and ODintR3) produces almost no visible primer dimers and the desired fragment is clearly visible for all study skins. These results clearly indicate that it is very important to use primer combinations that perform well together when working with study skin extracts. Our interpretation of the results is that when study skin extracts with low concentrations of the desired DNA fragment and a primer combination with a strong tendency to form primer dimers are combined in a PCR, the ratio between produced primer dimers and the desired fragment will be so unfavourable that it is unlikely to produce a visible band of the desired DNA fragment.

P. baudi. For a few species (*P. nipalensis* and *P. venusta*) fresh tissue samples became available late during this study and highly similar sequences were obtained from both skins and tissue (only a few base pairs differ in the combined data set). When adding both kinds of sequences to the analyses they always grouped together.

There is no doubt that museum study skins are an invaluable source of nuclear (as well as mitochondrial) DNA and that they can complement fresh samples in avian systematics. Although the age of the specimens is of general importance for the quality of the DNA, there is also considerable variation due to other factors. The reasons for this are often difficult to

elucidate, but are most likely due to the collectors' use of chemicals for preservation or the climatic conditions the skins have been exposed to. As shown herein, fragment size and the primers' properties are important contributory factors to obtaining good results and minimizing the risk of contamination. If properly designed protocols and primers are used, nuclear fragments of *c.* 350 bp (and possibly longer) could be amplified for a large proportion of the study skins present in museums worldwide. This will considerably improve our ability to target rarely collected and/or endangered species in molecular systematics. New enzyme cocktails with repairing enzymes will probably further improve the ability to obtain good DNA sequences from avian study skins. For further discussion of the use of museum material as a source for DNA and the potential of new enzyme cocktails, see e.g. Hajibabaei *et al.* (2005).

Phylogenetic relationships: molecular and morphological evidence

We believe that the overall good congruence between the trees obtained from the individual gene partitions justifies the assumption that the tree derived from the combined data set represents a well supported hypothesis of the evolutionary history of the Old World suboscines. Some nodes are obviously less firmly established, i.e. those supported by modest posterior probability support values (less than 0.95), or those for which a strongly supported, alternative topology is observed in any of the gene trees. However, most nodes are congruently supported by all three gene partitions (Fig. 2) lending strong support to the combined tree (Fig. 3).

The relationships among broadbills and asities in the combined tree are in overall good agreement with a previous phylogenetic hypothesis for the group based predominantly on syringeal characters (Prum 1993). They disagree, however, with those obtained in a phylogeny based mainly on myological characters in the hindlimb (Raikow 1987). That the variation in syrinx morphology in passerines is generally consistent with molecular data is obvious when comparing the extensive morphological study by Ames (1971) with recent molecular findings (e.g. Barker *et al.* 2002, 2004; Ericson *et al.* 2002a; Irestedt *et al.* 2002). Hindlimb myology, on the other hand, has been found to be at odds with molecular data in several studies (McCracken *et al.* 1999; Irestedt *et al.* 2004b, 2006). This has led to suggestions that hindlimb characters, as directly involved in locomotion and foraging, may be particularly susceptible to convergence and thus less useful in analyses of deeper passerine relationships than syringeal morphology (Irestedt *et al.* 2004b). However, Prum (1993) noted extensive internal congruence regarding the relationships among broadbills and asities between the syringeal and hindlimb trees, respectively, when rooting the latter tree with *Smithornis*.

The topological conflicts that do exist between the combined tree and the interpretations by Prum (1993) concern the relative position of some basal broadbills. While Prum (1993) found *Smithornis* to be the sister taxon (with *Calyptomena* as the next branch up) to all other broadbills and the asities, our phylogeny suggests that *Calyptomena* and *Smithornis* (and possibly *Sapayoa aenigma*) form their own clade that is sister to the other broadbills and the asities. It is noticeable that this discrepancy is supported not by syrinx morphology but by two skeletal characters. This disagreement could thus possibly be explained by these skeletal characters being less consistent than previously assumed. The conflict between the combined tree and the syrinx data regarding the position of *Pseudocalyptomena graueri* is more difficult to explain, but could possibly be due to a local rooting problem. Of the morphological characters re-evaluated by Olson (1971), a vinculum between the deep plantar tendons (a character used to set broadbills apart from other passerine birds in the early classifications) was the only distinctive morphological character of the family not shared with one or more other group of suboscines. Later, Raikow (1987) found a vinculum also in asities, in agreement with the nested position of the asities within the broadbills suggested herein. The observation of a weak and sometimes absent vinculum in *Calyptomena* and *Smithornis* agrees with the major division of broadbills and asities suggested by our data. Our phylogeny is also consistent with the suggestion by Dekker & Dickinson (2000) that the broadbills in the Philippines (Mindanao wattled broadbill and Visayan wattled broadbill) are better placed in a genus of their own, *Sarcophanops*, than in the genus *Eurylaimus* in which they are placed in most classifications.

Pittas have been poorly represented in analyses of passerine systematics employing modern techniques and phylogenetic methods. The morphological analysis by Raikow (1987) and the DNA–DNA hybridization study of Sibley & Ahlquist (1990) both included only four species of pittas each. Present knowledge of the evolutionary relationships among pittas thus virtually rests on interpretations based on external similarities (e.g. Whitehead 1893; Erritzoe & Erritzoe 1998). Nevertheless, the phylogenetic relationships suggested herein based on molecular data in many respects agree well with these phenetic studies.

The tree obtained from the combined data set supports the radiations of three major groups of pittas. The first is formed by *Pitta arcuata*, *P. granatina*, *P. venusta*, *P. erythrogaster* and *P. kochi*. These pittas have previously been suggested as being closely related based on external morphology (e.g. Whitehead 1893; Wolters 1982; Lambert & Woodcock 1996), with the extensive crimson/red coloration of the underparts the most distinctive character. All these species also have greenish or bluish backs, short tails, and most are small-sized. In line with previous opinion our phylogeny also suggests that this

group can be further subdivided into two evolutionary groups, the first formed by *P. kochi* and *P. erythrogaster*, the second by *P. arcuata*, *P. granatina* and *P. venusta*. Although our phylogeny includes all the pitta species that belong to this major radiation according to Erritzoe (2003), the group needs further investigation, as both *P. erythrogaster* and *P. granatina* comprise several subspecies/populations with disjunct distributions. It is worth noting that *P. kochi*, *P. arcuata*, and *P. venusta* have sympatric distribution with *P. erythrogaster* and *P. granatina*, respectively, although their altitude preferences differ somewhat (Lambert & Woodcock 1996; Erritzoe & Erritzoe 1998). It may be that *P. kochi*, *P. arcuata*, and *P. venusta* are autapomorphic, mountain radiations derived from ancestral stocks of *P. erythrogaster* and *P. granatina*, thus rendering the latter two taxa nonmonophyletic.

The second, major radiation of pittas consists of a rather heterogeneous assemblage of species (*Pitta elliotii*, *P. gurneyi*, *P. cyanea*, *P. baudi*, *P. guajana*, *P. caerulea*, *P. nipalensis*, *P. soror*, *P. oatesi*, and *P. phayrei*) that could be subdivided into three clades. Of these, *P. phayrei* forms the most basal clade, *P. nipalensis*, *P. soror* and *P. oatesi* a second clade. The last clade consists of the remaining species. Such a division agrees rather well with morphology and with earlier studies (Whitehead 1893; Wolters 1982), although *P. caerulea* (probably together with *P. schneideri*, not included here) and *P. baudi* are often kept separate from the other species in the third clade. However, *P. cyanea* has, based on its plumage with a blue back and a banded belly, sometimes been suggested as an ‘evolutionary link’ that unites *P. caerulea* and *P. schneideri* with *P. elliotii*, *P. gurneyi*, and *P. guajana* (Erritzoe 2003). Plumage similarities have also been used as an indication that *P. caerulea* and *P. schneideri* should be grouped together with *P. nipalensis*, *P. soror* and *P. oatesi* (Lambert & Woodcock 1996). *P. phayrei*, the taxon suggested to form the most basal branch within this radiation, has always been considered a very aberrant species of pitta, based on its autapomorphic cryptic coloration, slender decurved bill, and rather short legs. In some classifications it is placed in its own genus, *Anthocinclia*, and it has also been proposed as being the most ‘primitive’ pitta — the sister to all other pittas (Erritzoe 2003). However, the systematic position of *P. phayrei* within the second major radiation of pittas is supported by its possession of sexual dimorphism. Sexual dimorphism occurs in all other species within this radiation, but is absent in all other pitta species. Another synapomorphy for the clade is possibly the possession of cryptic juvenile plumage. This condition is present in all species for which it has been studied (Erritzoe 2003).

While the first two clades have their distributions concentrated to South-east Asia and the Sunda Islands (*Pitta erythrogaster* is an exception being distributed from the Philippines to Australia, with many distinct subspecies inhabiting the different islands), the third major radiation of pittas has a

much wider distribution, with members being found all the way from Africa to Australia. Most species within this radiation have rather uniform plumages, i.e. *P. angolensis*, *P. brachyura*, *P. moluccensis*, *P. nympha*, *P. versicolor*, *P. elegans*, *P. anerythra* and *P. megarhyncha* (not studied herein), with green upperparts with a blue wing-patch, and cinnamon-buff underparts with (in most species) a red vent. All species also have dark heads, often with paler supercilium, and many species also show a white wing-patch in flight. However, *P. reichenowi*, *P. sordida*, *P. steerii*, *P. iris*, *P. superba* and *P. maxima* more or less differ from the general plumage pattern described above. The plumages of *P. reichenowi*, *P. sordida*, *P. steerii* and *P. iris* differ from the above in the colouration of the underparts. *P. superba* and *P. maxima*, on the other hand, have also a different colour on their backs. These differences are probably the reason why the affinities of the latter two species have been difficult to untangle (Erritzoe 2003). Nevertheless, both species have retained several plumage features that are typical for this group, such as a blue wing-patch and a red vent.

Noticeably, several species within this third clade of pittas are migratory, and the distributions of migratory and sedentary species, respectively, in the combined tree suggest that a migratory habit is plesiomorphic within this radiation. Our results thus support the idea of a migratory ancestor giving rise to the species found in remote areas like Halmahera, Manus, the Solomon Islands, and Australia (Lambert & Woodcock 1996). It is also worth noting that species such as *Pitta angolensis*, *P. brachyura*, *P. moluccensis*, *P. nympha*, *P. versicolor* and *P. sordida* (and possibly also some of the subspecies of *P. elegans*) that do perform migratory movements (Lambert & Woodcock 1996), generally have retained the typical plesiomorphic plumage pattern of the group (*P. sordida* differs in having green underparts). In contrast, sedentary species on islands or remote landmasses have much more derived plumages. This suggests that the shift in habit, from being migratory to becoming sedentary on small islands or on remote landmasses would have promoted plumage diversification within this radiation of pittas. It remains to be clarified whether plumage diversification is constrained by the migratory habit (migrating birds may need the camouflage provided by a duller plumage, or that the energy-cost for migration may have reduced the ability for plumage diversification). A different explanation could be that genetic drift in small, sedentary populations has promoted plumage diversification.

The phylogeny of the Old World suboscines, and especially the inclusion of the neotropical *Sapayoa aenigma* in the clade, raises some biogeographical questions. There are data that suggest that the earliest evolution of the passerines took place in Gondwana during the late Cretaceous (Barker *et al.* 2002, 2004; Ericson *et al.* 2002a). After the break-up of Gondwana the Old World suboscines appear to have evolved on the Indian tectonic plate (Ericson *et al.* 2003). It has been

hypothesized that the ancestor of *S. aenigma* colonized South America from Antarctica in a 'second wave', after the phylogenetic lineages leading to the New World and Old World suboscines, respectively, had split (Fjeldså *et al.* 2003). Alternatively the *S. aenigma* ancestor could have spread from the Old World to the New World either eastwards via Beringia or westwards via Greenland. Both routes at times allowed dispersal of tropical organisms in the Tertiary. The present distribution of the members of the basal lineages of Old World suboscines gives little information about the geographical origin of the group as such — all major tropical areas are equally likely. An exception is the larger clade of broadbills where the asities and *Pseudocalyptomena* form the basal branches implying a Madagascan or African origin. Members of this group subsequently dispersed to Asia.

Classification

Our phylogenetic hypothesis firmly places *Sapayoa* among the asities and broadbills. There is also weak support for placing *Sapayoa* as sister to *Calyptomena* and *Smithornis*, and these as the sister group to Philepittidae and the remaining Eurylaimidae. This is not compatible with the retention of the families Eurylaimidae and Philepittidae in the traditional sense (e.g. del Hoyo *et al.* 2003; Dickinson 2003), as this would make Eurylaimidae paraphyletic. One way of reconciling this would be to treat all these as one family (Eurylaimidae). We believe that this is a less attractive solution, as *Sapayoa* undoubtedly is a very isolated taxon both morphologically and biogeographically, and we advocate its treatment in a family of its own. The molecular data leave no doubt as to the inclusion of *Sapayoa* among the Old World suboscines, although this taxon differs from all other members of this group in having intrinsic syringeal musculature (Prum 1990). Although the family name Sapayoidae has already appeared in print (Dickinson *et al.* 2003), it is not available because, when originally proposed, it did not meet the requirements of the Code (ICZN 1999), viz. Articles 13.1 (statement of differences from other similar taxa), 16.1 (statement of being a new name) and 16.2 (designation of the type genus). We thus propose the new family name Sapayoidae (type genus *Sapayoa* Hartert, 1903) for the monotypic *Sapayoa aenigma*. We regard the spelling proposed here as more euphonious than Sapayoidae.

The genera *Smithornis* and *Calyptomena* must be removed from Eurylaimidae and placed in a separate family. We thus propose resurrecting the family name Calyptomenidae Bonaparte, 1850, which takes precedence over Smithornithidae Bonaparte, 1853, for these two genera. Our results also show that the genus *Eurylaimus*, as currently defined (e.g. del Hoyo *et al.* 2003), is not monophyletic. Consequently we propose restricting the name *Eurylaimus* Horsfield, 1821 to the species *E. javanicus* and *E. ochromalus*, and to resurrect the genus

name *Sarcophanops* Sharpe, 1877 for the species *steeri* and *samarensis* (the latter taxon has not been included in our study, but it shares a similar morphology and biogeographical distribution with *steeri*). Our results show strong support for a sister clade relationship between the asities and the remaining broadbills. Although the inclusion of Philepittidae in a restricted Eurylaimidae, as proposed by Prum (1993), is consistent with our data, we prefer to keep them as separate families to highlight their morphological and behavioural distinctiveness. Therefore we restrict the family Eurylaimidae to include the genera *Pseudocalyptomena*, *Psarisomus*, *Corydon*, *Sarcophanops*, *Serilophus*, *Cymbirhynchus* and *Eurylaimus*, and propose to maintain the family Philepittidae for the genera *Philepitta* and *Neodrepanis*.

Pittidae is a very well defined and superficially homogeneous family. In most modern classifications all species have been included in a single genus, *Pitta*, although a resurrection of several genera used in the older literature was preferred by Wolters (1982). We feel that the division of Pittidae into three reciprocally well-supported and morphologically coherent clades should be reflected in the classification. Therefore, we propose that the name *Pitta* Vieillot, 1816 should be restricted to the third clade (*reichenowi* through *iris*), that the first clade (*granatina* through *erythrogaster*) should be separated as *Erythropitta* Bonaparte, 1854 and that the second clade (*phayrei* through *elliottii*) should be separated as *Hydrornis* Blyth, 1843. The latter clade is the most heterogeneous in morphology, although all members are united in their exhibition of sexual plumage dimorphism and possibly also by the presence of a distinctive spotted juvenile plumage.

Acknowledgements

Tissue and/or footpad samples were obtained from the following: Museum Victoria, Melbourne (Les Christidis), Zoological Museum, University of Copenhagen (Jon Fjeldså and Jan Bolding Kristensen), Muséum National d'Histoire Naturelle, Paris (Eric Pasquet), Senckenberg Forschungsinstitut and Naturmuseum (Gerald Mayr), Academy of Natural Science, Philadelphia (Leo Joseph), Field Museum of Natural History (David E. Willard), National Museum of Natural History, Leiden (Réne Dekker), National Museum of Natural History, Smithsonian Institution (James Dean, Storrs L. Olson), University of Washington, Burke Museum (Sharon Birks), Yale University, Peabody Museum (Richard O. Prum). Jon Fjeldså and two anonymous reviewers are thanked for comments on the manuscript, and Pia Eldenäs and Keyvan Mirbakhsh are thanked for practical support in the laboratory. Fieldwork in Vietnam was supported by a grant within the Swedish Research Links programme to Nguyen Cu and Per Ericson. The Swedish Research Council (grant no. 621-2004-2913 to P.E.) funded the laboratory work.

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