



Nuclear DNA from a 180-year-old study skin reveals the phylogenetic position of the Kinglet *Calyptura calyptura cristata* (Passeriformes: Tyrannides)

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The Kinglet *Calyptura calyptura cristata* is one of the most enigmatic bird species in South America, known only from specimens collected in the 19th century and a few recent observations. Knowledge of its biology is scanty and its systematic position is obscure. Traditionally, *Calyptura* was placed in the Cotingidae, but associated with genera that are now known to fall outside the Cotingidae. In an attempt to clarify its phylogenetic position, sequence data from four nuclear markers were obtained from a 180-year-old museum study skin of *Calyptura*, and incorporated into a comprehensive dataset of tyrant flycatchers, cotingas, manakins and allies. Our analyses demonstrate that *Calyptura* is most closely related to *Platyrinchus* and *Neopipo* and that these three genera constitute a deep branch in the clade containing the Rhynchocyclidae (tody-tyrants and flatbills) and Tyrannidae (typical tyrant flycatchers). The *Calyptura* specimen is one of the oldest avian museum specimens from which a substantial amount of nuclear DNA sequence data have been obtained, and highlights the immense value of museum collections for DNA-based phylogenetic studies.

Keywords: Platyrinchidae, Cotingidae, suboscines, nuclear DNA, museum specimen.

The rare and enigmatic Kinglet *Calyptura calyptura cristata* is one of the few members of Tyrannides (tyrant flycatchers, cotingas, manakins and allies) whose relationships are still unknown. Due to the almost complete lack of behavioural and anatomical data, its taxonomic history has been rather uneventful, although speculative. It was placed in Cotingidae by Sclater (1888), based solely on its pycnospidean tarsus, and this has remained its position until recently, when it was given an *incerta sedis* position in some recent systematic revisions (e.g. Remsen *et al.* 2012).

All specimens of *Calyptura* were collected in the 19th century, but for most of the *c.* 55 specimens, precise locality data are either missing, or are vague and potentially misleading. The only specimens with reasonably precise and traceable locality data are from the Rosário area, northeast

of Rio de Janeiro (Collar *et al.* 1992, Lambert & Kirwan 2010). The species was rediscovered in 1996 when a pair was observed near Teresópolis, northeast of Rio de Janeiro (Pacheco & Fonseca 2001). Subsequently, claims have been made that the species was observed on three occasions near Ubatuba in the coastal part of São Paulo state, but these have been questioned (Lambert & Kirwan 2010).

There are no anatomical specimens (skeletons or whole birds stored in alcohol) and information on its ecology and behaviour is scanty and anecdotal. P. W. Lund, who collected three specimens (of which one was used for our molecular study) near Rosário in 1827–28, stated that they hopped around in trees or shrubbery, one uttering a sparrow-like chirp, and that stomach contents included small insects and probably also seeds (Krabbe 2007). Other information, summarized by Lambert and Kirwan (2010), indicates that the species inhabits the higher and wilder places in

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montane forest, sometimes at forest edge and in secondary growth, where it moves through both canopy and lower shrubbery at edges, exploring vine tangles and epiphytes in search of small berries and insects, and that it has a surprisingly loud voice. Berries of a species of Solanacean scrub are mentioned as a potential food source and it has also been suggested that the species may have a predilection for mistletoe fruit (Snow 2004, Lambert & Kirwan 2010).

Habitat loss is likely to have caused a serious decline in the population of *Calyptura* (Collar *et al.* 1992, Lambert & Kirwan 2010). However, there may be additional explanations for the virtually complete absence of records from the entire 20th century. Lambert and Kirwan (2010) consider several possible explanations pertaining to its ecology, such as nomadism or altitudinal migration, microhabitat specialization or a highly cryptic behaviour. They also suggest that part of the problem may be purely logistic: large portions of the remaining potential habitat are never visited and it is generally difficult to obtain good views of the forest canopy at many places that receive regular visits from ornithologists and birdwatchers.

The original reason for placing *Calyptura* in Cotingidae was its type of tarsal scutellation (Sclater 1888) but molecular systematic studies have demonstrated that tarsal scutellation is of little phylogenetic value in Tyrannides. In his review of Cotingidae systematics, Snow (1973) grouped *Calyptura* with the three species of purpletuff (*Iodopleura*), although he noted that the two were not obviously related. In light of the molecular systematics of Tyrannides (Johansson *et al.* 2002, Chesser 2004, Ericson *et al.* 2006, Ohlson *et al.* 2008, Tello *et al.* 2009), there is little support for a close relationship between *Calyptura* and Cotingidae. *Iodopleura* has now been transferred to the Tityridae (Ericson *et al.* 2006, Ohlson *et al.* 2008, Tello *et al.* 2009) and *Calyptura* is now often treated as *incerta sedis* (Remsen *et al.* 2012). With the recent advances in laboratory procedures for working with old museum study skins (Irestedt *et al.* 2006, Kirchman *et al.* 2010), it is now feasible to obtain high-quality DNA sequence data from specimens well over 100 years of age. Here we present an attempt to assess the systematic position of *Calyptura cristata*, using DNA sequence data from four different nuclear markers. The DNA was extracted from a tissue sample of one of the specimens collected by P. W. Lund in 1827–28 and the

sequence data were incorporated into a comprehensive dataset of Tyrannides, mostly consisting of data downloaded from GenBank.

METHODS

DNA was extracted from a *c.* 2 × 2 × 1-mm piece of tissue taken from the inside of an open study skin of *Calyptura cristata*. Extraction procedures largely followed those used in Irestedt *et al.* (2006), using the Qiagen DNeasy Tissue Micro Kit, with some modification to their recommended protocol; 20 µL of DTT (dithiothreitol) was added to break disulfide bonds; and an additional 10–20 µL of Proteinase K was added roughly half-way through the lysis process, as Proteinase K may become inactive or depleted during long lysis periods (in this case *c.* 24 h). In the elution stage, elution buffer volume was decreased to yield a higher DNA concentration and the sample was incubated for 5 min. The extraction from the *Calyptura* specimen was carried out in separate facilities dedicated to extracting old DNA. All equipment and surfaces were sterilized before the extraction, by irradiation with ultraviolet light or sanitized with 10% bleach.

PCR-amplification and sequencing of fragmented DNA from old study skins require careful primer design, as target regions need to be broken down into short overlapping fragments. By using primer combinations that amplify short DNA fragments, the risk of amplifying contaminations is minimized, as study skins generally contain large numbers of short fragments, whereas longer fragments are absent or only exist in small numbers (Irestedt *et al.* 2006). For our *Calyptura* specimen, we used primers that amplified fragments of 200–250 bp, as this fragment length has been found to work for the majority of museum study skins (Irestedt *et al.* 2006, and personal experience). However, for a few fragments we were able to amplify almost 350 bp from our *Calyptura* specimen. A list of primers used in this study can be found in the supporting information (Table S1). Thermocycling programmes varied depending on estimated annealing temperatures of the primers, but typically involved a 5-min denaturation at 95 °C followed by three phases of denaturation (95 °C) for 40 s, annealing (typically 55–65 °C) for 1 min and extension (72 °C) for 1 min, with a decrease in annealing temperature of 2–3 °C between each phase. The first two phases were

run for four cycles and the third for 32–36 cycles. A final extension phase at 72 °C was run for 8 min.

All the PCR products were loaded on an agarose gel for electrophoresis and the target bands were excised and cleaned from gel remains using the Sigma GelClean Kit. Strong bands were sequenced directly, whereas weaker bands were reamplified using the same settings and primer combinations as in the initial PCR. Sequencing was carried out on an ABI 3130xl capillary array, using the same primers that had been used during PCR-amplification. Each segment of the target sequences was checked separately against the corresponding sequence segments in a number of other Tyrannides species to control for the uniqueness of the *Calyptura* sequences, and the overlapping portions between segments were checked for mismatches. A number of fragments were PCR-amplified and sequenced a second time for confirmation.

Sequence data were aligned in MEGALIGN and regions with gaps corrected by eye. All gaps were treated as missing data in the analyses. Phylogenetic relationships were assessed with maximum likelihood (ML) and Bayesian inference (BI). Substitution models for each gene were selected under the Akaike information criterion (AIC; Akaike 1973) through MRMODELTEST 2.2 (Nylander 2004) in conjunction with PAUP* (Swofford 2002). Both the ML and the BI analyses of the concatenated datasets were performed as partitioned analyses, treating each locus as a separate partition and allowing parameters of the respective substitution models to vary independently of each other.

The ML analyses were conducted using RAXML v7.2.6 (Stamatakis 2006), as implemented in RAXMLGUI v0.93 (Silvestro & Michalak 2010). We performed three ML analyses with rapid bootstrapping (1000 replicates) and a thorough ML search. BI analyses were run using the program MRBAYES 3.1.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) on the freely available University of Oslo Bioportal (www.bioportal.uio.no). Three sets of analyses were performed: (1) on each gene separately; (2) on the concatenated nuclear intron dataset; and (3) on the complete dataset. Several preliminary analyses were performed to explore the effect of chain temperature on the mixing behaviour of the chains. We found that lowering of the temperature to 0.05 resulted in better mixing of the chains than the default

temperature of 0.2. All BI analyses were performed with four chains, one cold chain and three incrementally heated chains and were run for 25 million generations, with trees sampled every 1000 generations. Trees saved before the target distribution had been reached (the burnin phase) were discarded and the final phylogenetic tree was estimated from 15 000 trees from each run.

RESULTS

Sequence characteristics

We obtained sequence data from RAG-1, myoglobin intron 2, ODC introns 6–7 and G3PDH intron 11 for *Calyptura cristata*. These loci were incorporated into a dataset with several representatives from all of the major lineages in Tyrannides, with two representatives of Furnariidae and one representative of Eurylaimidae as outgroups, yielding a final dataset of 45 terminal taxa (Table 1). Despite the old collection date of the sample of *Calyptura cristata*, we succeeded in sequencing the entire length of all four target regions. No mismatches between overlapping segments were found in any of the target sequences and none of the *Calyptura cristata* sequence segments turned out to be identical to any other corresponding segment in any other species checked. The other complementary samples for the study also yielded full-length sequence data for all target regions. GenBank numbers for newly obtained sequences for this study are given in Table 1. Total alignment length, sequence length variation, proportion of variable and parsimony-informative sites, base composition and substitution patterns are shown in Table 2.

Phylogenetic results

The topology of the trees from the combined dataset for the ML and BI analyses was similar, with the exception of a few nodes where support was low in both analyses (Fig. 1). The deeper nodes in Tyrannides conform to the results of Ericson *et al.* (2006) and Ohlson *et al.* (2008), with a strongly supported node uniting Tityridae, Rhynchocyclidae (*sensu* Tello *et al.* 2009), Tyrannidae and an array of species-poor, deep lineages (*Oxyruncus*, the Onychorhynchine clade, *Piprites*, *Platyrrinchus*, *Neopipo* and *Tachuris*; altogether *c.* 25 species; for convenience hereafter referred to as the oddball

Table 1. List of taxa used in this study, with sample identification and GenBank numbers for all sequence data included. Sample identification pertains to all myoglobin, G3PDH and ODC sequences, and to the RAG-1 sequences of *Piprites pileatus* and *Calyptura cristata*. The remainder of the RAG-1 sequence data were downloaded from GenBank and were generated from other individuals. Institutional acronyms: NRM: Swedish Museum of Natural History, USNM: National Museum of Natural History, Smithsonian Institution, ZMUC: Zoological Museum, University of Copenhagen.

Scientific name	Sample id	Collection locality	Myoglobin	G3PDH	ODC	RAG-1
<i>Smithornis capensis</i>	ZMUC125518	Iringa, Tanzania	DQ786004	DQ785929	DQ785971	DQ320608
<i>Thamnophilus caerulescens</i>	NRM967007	Concepción, Paraguay	AY065783	AY336587	DQ435504	FJ461176
<i>Xenops minutus</i>	ZMUC125002	El Oro, Ecuador	AY590060	AY590082	EF212127	FJ461153
<i>Ampelioides tschudii</i>	ZMUC127031	Guayas, Ecuador	DQ470543	DQ470516	EU231841	FJ501597
<i>Pyroderus scutatus</i>	NRM967030	Concepción, Paraguay	AY065786	AY336582	DQ435498	FJ501734
<i>Tyrannetes stolzmanni</i>	ZMUC126866	Pastaza, Ecuador	EU231743	EU231645	EU231844	FJ501760
<i>Lepidothrix coronata</i>	ZMUC126073	Napo, Ecuador	EU231745	EU231647	EU231846	FJ501655
<i>Laniisoma elegans</i>	ZMUC127782	Morona-Santiago, Ecuador	EU231747	EU231649	EU231848	FJ501651
<i>Laniocera hypopyrra</i>	ZMUC125879	Mato Grosso, Brazil	DQ470554	DQ470527	EU231849	FJ501652
<i>Iodopleura isabellae</i>	ZMUC125762	Napo, Ecuador	DQ435519	DQ435467	DQ435485	FJ501648
<i>Tityra semifasciata</i>	NRM976667	Amambay, Paraguay	JF970155	JF970144	JF970166	FJ501754
<i>Pachyramphus polychopterus</i>	NRM967032	Concepción, Paraguay	AY338747	AY336573	DQ435493	FJ501699
<i>Oxyruncus cristatus</i>	NRM967078	Amambay, Paraguay	AY338745	AY336572	DQ435492	FJ501698
<i>Onychorhynchus coronatus</i>	ZMUC126915	Guayas, Ecuador	EU231751	EU231653	EU231853	FJ501696
<i>Myiobius barbatus</i>	ZMUC137122	Sao Paulo, Brazil	JF970156	JF970145	JF970167	FJ501675
<i>Terentotriccus erythrurus</i>	ZMUC126692	Napo, Ecuador	EU231753	EU231655	EU231855	FJ501753
<i>Hemipipo chloris</i>	ZMUC127972	Morona-Santiago, Ecuador	EU231754	EU231656	EU231856	FJ501717
<i>Piprites pileatus</i>	ZMUC128817	Southeastern Brazil	DQ435524	DQ435472	DQ435496	JF970177
<i>Calyptura cristata</i>	ZMUC379	Rio de Janeiro, Brazil	JF970157	JF970146	JF970168	JF970178
<i>Tachuris rubrigastra</i>	ZMUC135914	Junín, Peru	EU231755	EU231657	EU231857	FJ501751
<i>Neopipo cinnamomea</i>	USNMB11797	Guyana	EU231756	EU231658	EU231858	FJ501690
<i>Platyrinchus coronatus</i>	ZMUC126491	Napo, Ecuador	JF970158	JF970147	JF970169	FJ501720
<i>Cnipodectes subbrunneus</i>	ZMUC125226	Guayas, Ecuador	EU231761	EU231663	EU231863	FJ501616
<i>Leptopogon amaurocephalus</i>	NRM937317	Caazapa, Paraguay	DQ435520	DQ435468	DQ435487	FJ501657
<i>Phylloscartes ventralis</i>	ZMUC126247	Chuquisaca, Bolivia	EU231757	EU231659	EU231859	FJ501711
<i>Corythopis torquatus</i>	ZMUC126377	Sucumbíos, Ecuador	JF970159	JF970148	JF970170	FJ501622
<i>Rhynchocyclus brevirostris</i>	ZMUC127248	Costa Rica	JF970160	JF970149	JF970171	FJ501738
<i>Tolmomyias sulphurescens</i>	ZMUC130316	Alto Paraguay, Paraguay	JF970161	JF970150	JF970172	FJ501757
<i>Todirostrum cinereum</i>	NRM947036	Alto Paraguay, Paraguay	AY338740	AY336575	DQ435506	FJ501755
<i>Hemitriccus diops</i>	NRM956601	Itapua, Paraguay	EU231766	EU231668	EU231868	FJ501638
<i>Myiopiccus ornatus</i>	ZMUC125759	Napo, Ecuador	EU231789	EU231691	EU231892	FJ501686
<i>Hirundinea ferruginea</i>	ZMUC125257	Cochabamba, Bolivia	EU231790	EU231692	EU231893	FJ501643
<i>Stigmatura budytoides</i>	NRM966804	Boquerón, Paraguay	DQ435528	DQ435476	DQ435503	FJ501748
<i>Elaenia spectabilis</i>	NRM986766	Misiones, Paraguay	JF970162	JF970151	JF970173	FJ501628
<i>Mecocerculus leucophrys</i>	ZMUC125277	Ancash, Peru	EU231774	EU231676	EU231877	FJ501667
<i>Muscigralla brevicauda</i>	ZMUC125316	Lambayeque/Piura, Peru	EU231810	EU231712	EU231913	FJ501671
<i>Attila spadiceus</i>	ZMUC125869	Mato Grosso, Brazil	EU231795	EU231697	EU231898	FJ501603
<i>Ramphotrigon ruficauda</i>	ZMUC125895	Mato Grosso, Brazil	EU231799	EU231701	EU231902	FJ501737
<i>Myiarchus tyrannulus</i>	NRM937173	Pres. Hayes, Paraguay	DQ435521	DQ435469	DQ435489	FJ501674
<i>Empidonomus varius</i>	NRM956628	Caazapa, Paraguay	EU231809	EU231711	EU231912	FJ501630
<i>Ochthoeca oenanthoides</i>	ZMUC126270	Potosí, Bolivia	JF970163	JF970152	JF970174	FJ501693
<i>Colonia colonus</i>	NRM976648	Amambay, Paraguay	EU231817	EU231719	EU231920	FJ501617
<i>Gubernetes yetapa</i>	NRM976700	Concepción, Paraguay	AY338739	AY336578	DQ435483	FJ501635
<i>Ochthornis littoralis</i>	USNMB11416	Guyana	JF970164	JF970153	JF970175	FJ501694
<i>Agriornis micropterus</i>	ZMUC126599	La Paz, Bolivia	JF970165	JF970154	JF970176	FJ501594

taxa). This topology is also found in the nuclear intron, myoglobin and ODC trees, although the support is weak (posterior probability = 85%) in the myoglobin tree. The G3PDH tree is practically unresolved with respect to deep nodes and the

RAG-1 tree conforms to the results of Tello *et al.* (2009) in placing Tityridae, *Oxyruncus* and the Onychorhynchine clade with Cotingidae (see Supporting Information Fig. S1 for the individual gene trees).

Table 2. Data characteristics and Bayesian estimates of parameters for the four studied genetic markers. A unique 628-bp insertion in the ODC sequence of *Piprites chloris* has been excluded in the calculations. Substitution rates are calculated with the G-T rate set to 1.

	G3PDH	Myoglobin	ODC	RAG-1
Number of sites in alignment	383	775	784	2872
Sequence length variation	318–349	689–750	559–747	2857–2872
Number of variable sites	250 (65.3%)	340 (43.9%)	379 (48.3%)	833 (29.0%)
Number of parsimony-informative sites	134 (35.0%)	131 (16.9%)	197 (25.1%)	369 (12.8%)
Selected substitution model	GTR+G	GTR+G	GTR+G	GTR+G+I
Base frequencies (%)				
A	23.8	29.8	32.2	32.3
C	19.1	21.2	18.8	19.5
G	29.2	21.9	20.2	23.2
T	27.9	27.1	28.8	25
Substitution rate				
r(AC)	1.03	1.04	0.72	1.52
r(AG)	4.83	5.2	3.48	5.69
r(AT)	0.89	0.53	0.55	0.92
r(CG)	1.81	1.45	0.87	1.2
r(CT)	6.02	5.86	2.44	7.54
r(GT)	1	1	1	1
pInv	–	–	–	0.4
Gamma shape parameter (alpha)	1.36	0.83	1.23	1.35

All gene trees identify a clade formed by Tyrannidae and Rhynchocyclidae together with *Platyrinchus*, *Neopipo*, *Tachuris* and *Piprites* (hereafter referred to as the Tyrant Flycatcher clade). This clade is recovered in all trees although both ML and BI support values are often low in individual gene trees. Apart from the monophyly of Tyrannidae and Rhynchocyclidae, internal relationships among deeper lineages are not concordant and likewise often receive low support values in individual gene trees.

Our results give clear evidence that *Calyptura* does not belong in the Cotingidae. In all gene trees, it is placed among the species-poor and deep lineages within the Tyrant Flycatcher clade. The exact position differs among the individual trees, but in the tree from the combined dataset it is placed with strong support (ML 93 and BI 100) in an unresolved clade with *Platyrinchus* and *Neopipo* (Fig. 1). This relationship is also discovered with strong support in the myoglobin and ODC trees, as well as in the nuclear intron tree, whereas the G3PDH and RAG-1 trees yield different positions (Fig. S1).

DISCUSSION

Recently, Kirchman *et al.* (2010) assessed the systematic position of *Heliangelus zusii*, based on

mitochondrial DNA data from the > 100-year-old unique type specimen. Here we report results of a study that incorporates sequence data from four nuclear markers (one protein-coding gene and three introns) from a 180-year-old museum specimen. This represents one of the oldest avian museum specimens from which such a substantial amount of nuclear sequence data have been obtained with conventional methods, demonstrating that issues concerning relationships of extinct or extremely rare birds can now be successfully resolved using tiny amounts of tissue that cause hardly any visible damage to the old specimens.

Recent studies of the systematics of Tyrannides (Ericson *et al.* 2006, Ohlson *et al.* 2008, Tello *et al.* 2009) have been in congruence and demonstrated that the majority of Tyrannides can be divided into five principal clades (Cotingidae, Pipridae, Tityridae, Rhynchocyclidae and Tyrannidae). However, deeper relationships, involving the relationships of the oddball taxa, differ among the studies. Tello *et al.* (2009) placed *Oxyruncus* and the Onychorhynchine clade together with Tityridae and Cotingidae in a clade they named Cotingoidea, whereas Ericson *et al.* (2006) and Ohlson *et al.* (2008) obtained support for a different topology, with all of the oddball taxa together with Tityridae, Rhynchocyclidae and Tyrannidae forming a monophyletic clade separate from the

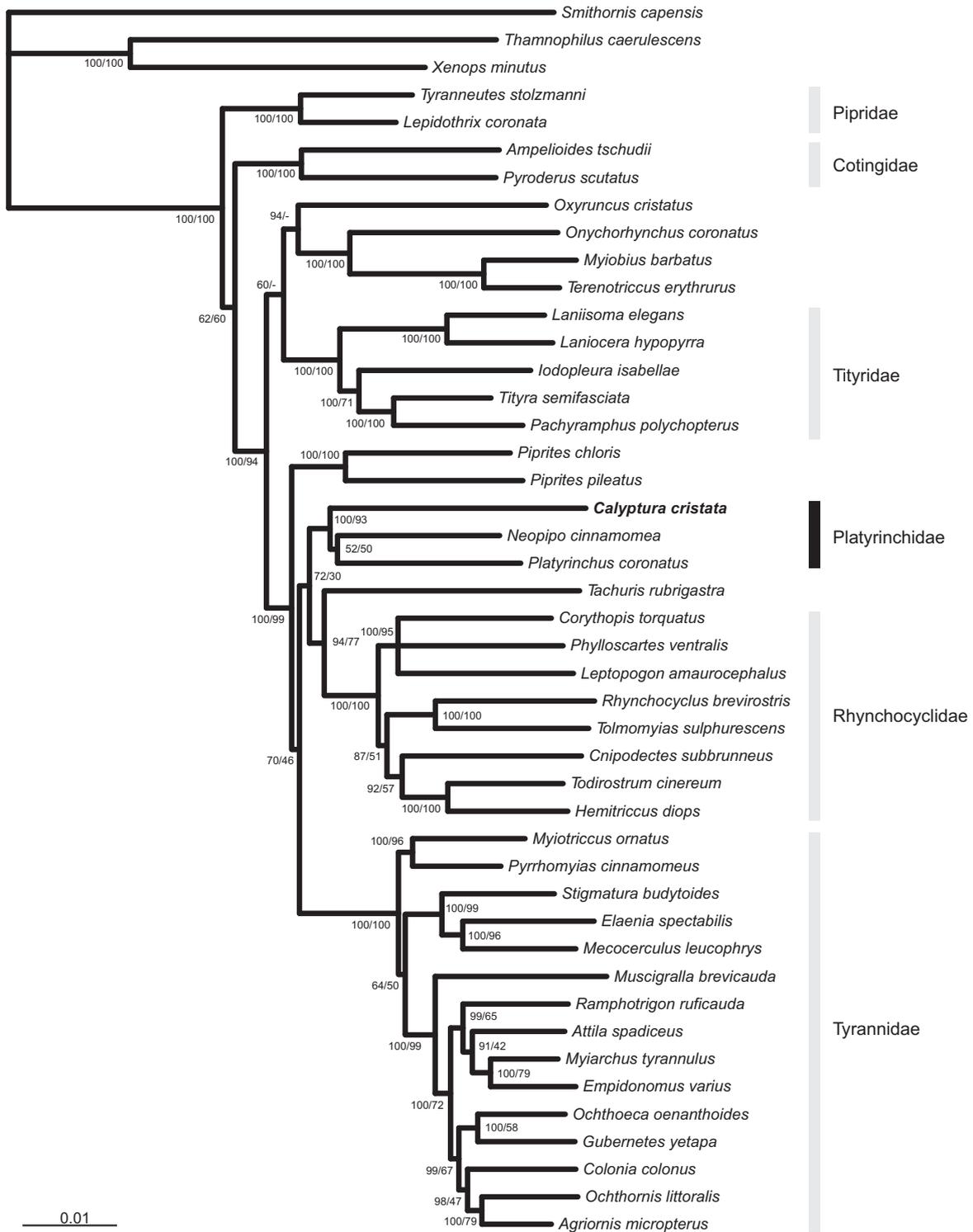


Figure 1. Majority rule (50%) consensus tree from the Bayesian analysis of the concatenated total DNA dataset (G3PDH intron 11, myoglobin intron 2, ODC introns 6–7 and RAG-1). Bayesian posterior probability values and ML bootstrap values are indicated next to each node before and after the slash, respectively. Major clades in Tyrannides are marked with grey bars at the right-hand side of the tree, the proposed family Platyrrinchidae (see discussion) is highlighted by a black bar.

Cotingidae and Pipridae. The dataset used in this study, with three nuclear introns and RAG-1, yielded a topology essentially similar to that found by Ericson *et al.* (2006) and Ohlson *et al.* (2008).

Both the studies of Ohlson *et al.* (2008) and Tello *et al.* (2009) demonstrate that *Platyrinchus*, *Tachuris*, *Neopipo* and *Piprites* are deep lineages that cluster at the base of the Tyrant Flycatcher clade. Our results show that we can add *Calyptura cristata* to this array of morphologically diverse taxa. This part of the Tyrannides tree is characterized by short branch-lengths, poor resolution and partly conflicting topologies in different studies. The initial divergences in the Tyrant Flycatcher clade have consistently been difficult to resolve. This situation remains after combining data from the two main studies (Ohlson *et al.* 2008, Tello *et al.* 2009) and it is likely that it reflects a bout of rapid diversification early in the evolutionary history of this clade. Rhynchocyclidae and Tyrannidae are always unambiguously supported, but the relationships of *Piprites*, *Neopipo*, *Platyrinchus* and *Tachuris* to these families and to each other are not clear. These relationships are not unambiguously resolved by this study either. However, we receive good support for *Calyptura cristata* forming a clade with *Neopipo cinnamomea* and *Platyrinchus*. Myoglobin and ODC yield strong support for this relationship, whereas RAG-1 and G3PDH place them close together, but not as a monophyletic clade (Fig. S1). A sister relationship between *Neopipo* and *Platyrinchus* was found by Ohlson *et al.* (2008) and, with different molecular markers, by Rheindt *et al.* (2008). In Tello *et al.* (2009), *Neopipo* and *Platyrinchus* were both placed in unresolved positions at the base of the Tyrant Flycatcher clade. Denser taxon sampling and inclusion of sequence data from additional markers are needed to establish the relationships among these three genera.

This novel hypothesis is quite unexpected, as *Calyptura cristata* appears to have little in common in morphology or ecology with these two genera. *Platyrinchus* species have exceptionally wide and flat bills surrounded by prominent rictal bristles, an adaptation to their specialized upward-striking foraging technique (Fitzpatrick 1985). *Neopipo* also has a broad-based and rather flat bill adapted for a similar foraging technique, although much less extreme than that of *Platyrinchus* species and with much less developed rictal bristles. *Calyptura* is set apart from these two genera,

having a sturdy bill with a distinctly arched culmen and very deep gonyx of the lower mandible. On the whole, it is more reminiscent of *Piprites* species, which mainly feed by perch-gleaning and also eat berries to a large extent. The biology of *Calyptura cristata* is virtually unknown but bill morphology and scanty evidence on behaviour reporting it to feed in the canopy and edge of forest, partly on fruit (summarized in Lambert & Kirwan 2010), point to ecological characteristics markedly different from its closest relatives, which are insectivores of the understorey of closed humid forest. Taken together with ecomorphological characteristics of the other deep clades in the Tyrant Flycatcher clade, this indicates that a great morphological and behavioural diversity was already present in the initial stages of its history. Only two of its subclades (Rhynchocyclidae and Tyrannidae) contain any significant species-level diversity today, raising interesting issues about the underlying factors responsible for the differences in evolutionary success within this clade. Closer comparative studies of these odd birds will be challenging, but we hope that new searches in the mountain ranges of southeastern Brazil will eventually lead to a documented rediscovery of *Calyptura cristata* and that this will produce more data that may shed light upon the adaptive changes in this small group.

To reflect better the results from recent molecular phylogenetic studies, tyrant flycatcher classification will have to be extensively modified. A major step forward was taken by Tello *et al.* (2009), who proposed several changes to the traditional classification, among them dividing the traditional broad Tyrannidae into two families: Rhynchocyclidae and Tyrannidae. The remaining members of their Tyrannoidea are a small number of taxa for which the precise relationships have still not been resolved. In this study we show that three of these genera (*Platyrinchus*, *Neopipo* and *Calyptura*) form a well-supported deep lineage in Tyrannoidea and propose that they should be recognized as a family-level taxon, for which the name Platyrinchidae (Bonaparte 1854) is available (see Tello *et al.* 2009):

Family Platyrinchidae (Bonaparte 1854) (type genus: *Platyrinchus* Desmarest 1805).

Diagnosis: The most inclusive crown clade that includes *Platyrinchus coronatus*, *Neopipo cinnamomea* and *Calyptura cristata*, but not *Todirostrum*

cinereum, *Hirundinea ferruginea*, *Myiarchus tyrannulus*, *Tityra semifasciata*, *Lepidothrix coronata* or *Ampelioides tschudii*. There are no known morphological synapomorphies but the clade has been diagnosed as monophyletic based on good support (ML 93 and BI 100) from molecular DNA sequence data.

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REFERENCES

- Akaike, H.** 1973. Information theory as an extension of the maximum likelihood principle. In Petrov, B.N. & Csaki, C. (eds) *Second International Symposium in Information Theory*: 267–281. Budapest: Akademiai Kiado.
- Chesser, R.T.** 2004. Molecular systematics of the New World suboscine birds. *Mol. Phylogenet. Evol.* **32**: 11–24.
- Collar, N.J., Gonzaga, I.P., Krabbe, N., Madroño Nieto, A., Naranjo, L.G., Parker, T.A.III & Wege, D.C.** 1992. *Threatened Birds of the Americas. The ICBP/IUCN Red Data Book*, 3rd edn. Cambridge, UK: ICBP.
- Ericson, P.G.P., Zuccon, D., Ohlson, J.I., Johansson, U.S., Alvarenga, H. & Prum, R.O.** 2006. Higher level phylogeny and morphological evolution of tyrant flycatchers, cotingas, manakins and their allies (Aves: Tyrannida). *Mol. Phylogenet. Evol.* **40**: 471–483.
- Fitzpatrick, J.W.** 1985. Form, foraging behavior and adaptive radiation in the Tyrannidae. In Buckley, P.A., Foster, M.S., Morton, E.S., Ridgely, R.S. & Buckley, F.G. (eds) *Neotropical Ornithology*: 447–470. Ornithological Monographs No. 36. Washington, DC: The American Ornithologists' Union.
- Huelsenbeck, J.P. & Ronquist, F.** 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.
- 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). *Zool. Scr.* **35**: 567–580.
- Johansson, U.S., Irestedt, M., Parsons, T.J. & Ericson, P.G.P.** 2002. Basal phylogeny of the Tyrannoidea based on comparisons of cytochrome b and exons of nuclear c-myc and RAG-1 genes. *Auk* **119**: 984–995.
- Kirchman, J.J., Witt, C.C., McGuire, J.A. & Graves, G.R.** 2010. DNA from a 100-year-old holotype confirms the validity of a potentially extinct hummingbird species. *Biol. Lett.* **6**: 112–115.
- Krabbe, N.** 2007. Birds collected by P. W. Lund and J. T. Reinhardt in south-eastern Brazil between 1825 and 1855, with notes on P. W. Lund's travels in Rio de Janeiro. *Rev. Bras. Ornitol.* **15**: 331–357.
- Lambert, F. & Kirwan, G.M.** 2010. The twice-vanishing 'pardalote': what future for the Kinglet Calyptura? *Neotrop. Birding* **6**: 4–17.
- Nylander, J.A.A.** 2004. MRMODELTEST2 version 2.2. <http://www.abc.se/~nylander/>.
- Ohlson, J.I., Fjeldså, J. & Ericson, P.G.P.** 2008. Tyrant flycatchers coming out in the open: phylogeny and ecological radiation in Tyrannidae (Aves: Passeriformes). *Zool. Scr.* **37**: 315–335.
- Pacheco, J.F. & Moreira da Fonseca, P.S.** 2001. The remarkable rediscovery of the Kinglet Calyptura *Calyptura cristata*. *Cotinga* **16**: 44–47.
- Remsen, J.V. Jr., Cadena, C.D., Jaramillo, A., Nores, M., Pacheco, J.F., Pérez-Emán, J., Robbins, M.B., Stiles, F.G., Stotz, D.F. & Zimmer, K.J.** 2012. Version 2 April 2012. *A Classification of the Bird Species of South America*. American Ornithologists' Union. <http://www.museum.lsu.edu/~Remsen/SACCBaseline.html>
- Rheindt, F.E., Norman, J.A. & Christidis, L.** 2008. Phylogenetic relationships of tyrant-flycatchers (Aves: Tyrannidae), with an emphasis on the elaeiniine assemblage. *Mol. Phylogenet. Evol.* **46**: 88–101.
- Ronquist, F. & Huelsenbeck, J.P.** 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
- Slater, P.L.** 1888. *Catalogue of the Birds in the British Museum*, vol. 14. London: Trustees of the British Museum.
- Silvestro, D. & Michalak, I.** 2010. RAXMLGUI: a graphical front-end for RAXML. Available at <http://sourceforge.net/projects/raxmlgui/>.
- Snow, D.W.** 1973. The classification of the Cotingidae (Aves). *Breviora* **409**: 1–27.
- Snow, D.W.** 2004. Family Cotingidae (Cotingas). In del Hoyo, J., Elliot, A. & Christie, D.A. (eds) *Handbook of the Birds of the World*, Vol. 9. Barcelona: Lynx Ediciones.
- Stamatakis, A.** 2006. RAXML-VI-HPC: Maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.
- Swofford, D.L.** 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods)*, version 4.0. Sunderland, MA: Sinauer Associates.
- Tello, J.G., Moyle, R.G., Marchese, D.J. & Cracraft, J.** 2009. Phylogeny and phylogenetic classification of the tyrant flycatchers, cotingas, manakins and their allies (Aves: Tyrannidae). *Cladistics* **25**: 429–467.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used specifically to obtain sequence data from the study skin of *Calyptura cristata*. The primers were used for both PCR-amplification and sequencing.

Figure S1. The majority rule (50%) consensus trees from the Bayesian analyses of the G3PDH intron 11 (A), the myoglobin intron 2 (B), the ODC introns 6–7 (C), the concatenated nuclear

intron dataset (D) and the RAG-1 dataset (E). Support values as in Figure 1.

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