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

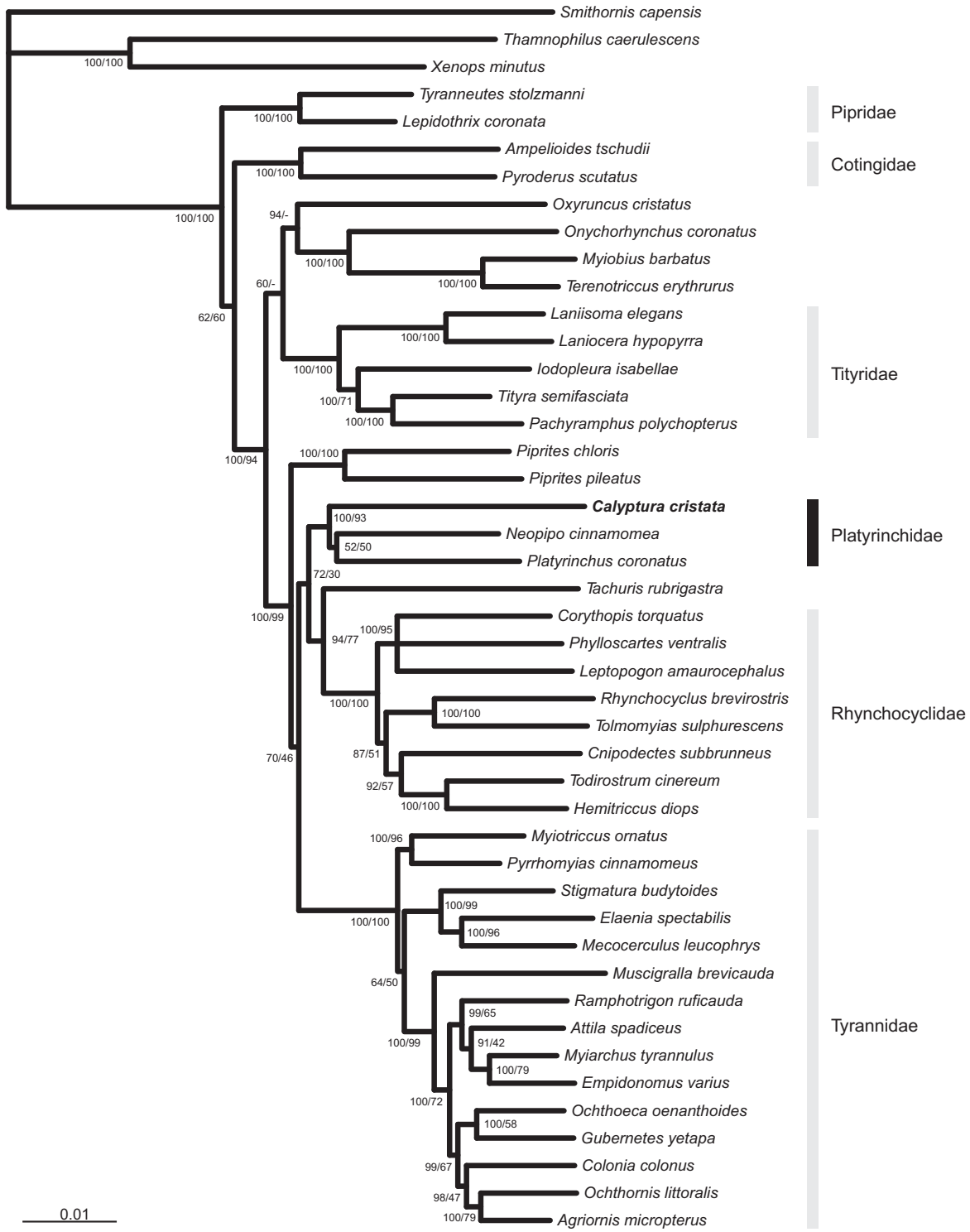


**Figure 1.** Majority rule (50%) consensus tree from the Bayesian analysis of the concatenated total DNA dataset (G3PDH intron 11, myoglobin intron 2, ODC introns 6–7 and RAG-1). Bayesian posterior probability values and ML bootstrap values are indicated next to each node before and after the slash,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