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Molecular phylogenetics, vocalizations, and species limits in *Celeus* woodpeckers (Aves: Picidae)

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ABSTRACT

Species limits and the evolutionary mechanisms that have shaped diversification of woodpeckers and allies (Picidae) remain obscure, as inter and intraspecific phylogenetic relationships have yet to be comprehensively resolved for most genera. Herein, we analyzed 5020 base pairs of nucleotide sequence data from the mitochondrial and nuclear genomes to reconstruct the evolutionary history of *Celeus* woodpeckers. Broad geographic sampling was employed to assess species limits in phenotypically variable lineages and provide a first look at the evolution of song and plumage traits in this poorly known Neotropical genus. Our results strongly support the monophyly of *Celeus* and reveal several novel relationships across a shallow phylogenetic topology. We confirm the close sister relationship between *Celeus spectabilis* and the enigmatic *Celeus obrieni*, both of which form a clade with *Celeus flavus*. The Mesoamerican *Celeus castaneus* was placed as sister to a *Celeus undatus–grammicus* lineage, with the species status of the latter drawn into question given the lack of substantial genetic, morphological, and vocal variation in these taxa. We recovered paraphyly in *Celeus elegans*; however, this result appears to be the consequence of mitochondrial introgression from *Celeus lugubris* considering the monophyly of *elegans* at the ß-FIBI7 locus. A second instance of paraphyly was observed in *Celeus flavescens* with deep genetic splits and substantial phenotypic variation indicating the presence of two distinct species in this broadly distributed lineage. As such, we advocate elevation of *Celeus flavescens ochraceus* to species status. Our analysis of *Celeus* vocalizations and plumage characters demonstrates a pattern of lability consistent with a relatively recent origin of the genus and potentially rapid speciation history.

1. Introduction

Recent molecular phylogenetic investigation of woodpeckers and allies (Piciformes: Picidae) has brought significant advances in understanding higher-level relationships within this diverse near-global radiation, resolving the phylogenetic position of most picid genera and confirming polyphyly in five broadly distributed clades (Webb and Moore, 2005; Benz et al., 2006; Moore et al., 2006; Fuchs et al., 2007). By comparison, intrageneric relationships and the regional evolutionary histories for much of this diversity are little known, as few groups have been examined within a modern phylogenetic context at the species level. Moreover, several instances of plumage convergence evidenced throughout the family suggest traditional phenotype-based taxonomic arrangements may not accurately reflect phylogenetic relationships among some woodpecker lineages (Weibel and Moore, 2002; Benz et al., 2006; Moore et al., 2006).

The Neotropics support by far the highest picid diversity, encompassing ~102 of the 216 species currently recognized within the family, including three non-insular endemic genera *Piculus*, *Veniliornis*, and *Celeus* (Winkler and Christie, 2002). Among these, *Celeus* is the least known and most speciose, comprising 12 species restricted to Central and South America. Independent molecular data have recently confirmed that the Old World purported congeneric Rufous woodpecker (*Micropternus* [*Celeus*] *brachyurus*) is in fact nested within a southeast Asian clade and sister to *Meiglyptes* (Benz et al., 2006; Fuchs et al., 2007). The present center of *Celeus* diversity lies in the Amazon basin, where as many as five species may be sympatric through ecological partitioning and differing foraging strategies that specialize on a broad suite of ant and termite species. As of yet, conventional species-level phylogenetic analyses are lacking within *Celeus* woodpeckers, and taxonomic arrangements remain based principally on plumage characters and bill morphology, traits that exhibit a high degree of inter and intraspecific variability and are potentially homplaseous (Short, 1972, 1982). Consequently, *Celeus* represents a prime clade in need of detailed molecular phylogenetic investigation to clarify intrageneric relationships and resolve uncertainty surrounding the species status of several widely allopatric lineages.

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In his analysis of *Celeus* systematic relationships, Short (1972) examined approximately 800 specimens, comparing bill morphology and an extensive suite of plumage characters to arrive at the conclusion that although *Celeus castaneus* exhibits significant plumage differences from *Celeus elegans*, *Celeus lugubris*, and *Celeus flavescens*, the shared similarities in bill morphology indicated a common evolutionary history among these taxa, and thus recognized the four taxon '*elegans* superspecies' as a clade distinct from other members of the genus. Short's comparative analysis further identified two distinct groups within the six subspecies of *C. elegans*, long-crested '*elegans*' forms from the Guyana shield, and short-crested '*jumana*' forms throughout Amazonia; however, explicit phylogenetic hypotheses were not made given the potential hybridization between *C. elegans* '*jumana*' and the partially sympatric *C. lugubris* (Short, 1972). Subsequent phenotype-based taxonomic treatments of *Celeus* (Short, 1982; Winkler and Christie, 2002) have provided little additional insight on relationships among the remaining congenerics.

In the present investigation, we employ a ~5 kb molecular data set and model-based phylogenetic methods to test the position of *C. castaneus* within Short's hypothesized '*elegans* superspecies'. Through broad geographic taxon sampling in *C. elegans* and *C. flavescens*, we explore the relationships among the '*jumana*' and '*elegans*' forms, as well as the phylogenetic position of the distinctive *Celeus flavescens ochraceus*. The result is a well-supported phylogenetic framework of *Celeus* woodpeckers that addresses the status of the enigmatic *Celeus obrieni*, clarifies species limits within *C. flavescens*, and suggests recent mitochondrial introgression between *C. elegans* and *C. lugubris*. Lastly, we provide a first look at *Celeus* vocalizations and plumage characters within a phylogenetic context, and highlight the need for further avenues of research in this poorly known genus.

2. Methods

2.1. Taxonomic sampling

We sampled 43 ingroup specimens encompassing all currently recognized species within *Celeus* and at least two specimens per taxon, with the exception of *C. obrieni*, represented solely by the holotype (Table 1). Intraspecific genetic samples were selected from distinct geographic regions to examine genetic diversity across well-known biogeographic boundaries and evaluate species limits within broadly distributed and phenotypically variable lineages. Although limited by tissue availability, intraspecific sampling was focused within *C. elegans* and *C. flavescens*, both of which exhibit prominent geographic forms of questionable species status. Outgroup taxa were drawn from two related woodpecker genera, *Dryocopus lineatus* and *Piculus chrysochloros*, based on recent higher-level phylogenetic studies within the Picidae (Webb and Moore, 2005; Benz et al., 2006).

Table 1
Summary of specimens included in this study.

#	Species	Country of Origin	Source	Voucher #
1	*Celeus castaneus*	Mexico	UNAM	99–162
2	*Celeus castaneus*	Panama: Bocas del Toro	USNM	1977
3	*Celeus flavus*	Guyana	KUNHM	5840
4	*Celeus flavus*	Guyana	KUNHM	5738
5	*Celeus flavus*	Ecuador: Sucumbios	ANSP	2737
6	*Celeus flavus*	Brazil: Pará	USNM	6880
7	*Celeus flavus*	Peru: Loreto	KUNHM	1036
8	*Celeus flavus*	Brazil: Bahia	AMNH	242714[a]
9	*Celeus elegans*	Ecuador: Sucumbios	ANSP	3224
10	*Celeus elegans*	Brazil: Mato Grosso	LSUMNS	35528
11	*Celeus elegans*	Guyana	USNM	12777
12	*Celeus elegans*	Brazil: Rondônia	FMNH	389780
13	*Celeus elegans*	Guyana	USNM	10473
14	*Celeus elegans*	Bolivia: La Paz	FMNH	391072
15	*Celeus elegans*	Brazil: Roraima	FMNH	389194
16	*Celeus elegans*	Peru: Loreto	LSUMNS	4364
17	*Celeus elegans*	Guyana	KUNHM	5764
18	*Celeus flavescens*	Paraguay	KUNHM	304
19	*Celeus flavescens*	Brazil: Maranhão	FMNH	63975[a]
20	*Celeus flavescens*	Brazil: Pará	AMNH	278666[a]
21	*Celeus flavescens*	Brazil: Maranhão	AMNH	242703[a]
22	*Celeus flavescens*	Brazil: Minas Gerais	FMNH	191173[a]
23	*Celeus flavescens*	Brazil: São Paulo	FMNH	344388[a]
24	*Celeus flavescens*	Brazil: Espírito Santo	FMNH	208004[a]
25	*Celeus flavescens*	Brazil: Bahia	AMNH	242688[a]
26	*Celeus grammicus*	Ecuador: Napo	ANSP	3253
27	*Celeus grammicus*	Ecuador: Morona-Santiago	ANSP	2477
28	*Celeus grammicus*	Bolivia: Santa Cruz	LSUMNS	105252
29	*Celeus grammicus*	Brazil: Rondônia	FMNH	389782
30	*Celeus grammicus*	Peru: Loreto	LSUMNS	6892
31	*Celeus loricatus*	Panama: Colon	LSUMNS	28510
32	*Celeus loricatus*	Ecuador: Esmeraldas	LSUMNS	11832
33	*Celeus lugubris*	Argentina: Corrientes	USNM	5899
34	*Celeus lugubris*	Paraguay	KUNHM	3204
35	*Celeus lugubris*	Bolivia: Santa Cruz	LSUMNS	6534
36	*Celeus obrieni*	Brazil: Piauí	AMNH	242687[a]
37	*Celeus spectabilis*	Peru: Madre de Díos	LSUMNS	45460
38	*Celeus spectabilis*	Peru: Ucayalí	LSUMNS	10664
39	*Celeus torquatus*	Guyana	KUNHM	1305
40	*Celeus torquatus*	Brazil: Amazonas	LSUMNS	25574
41	*Celeus torquatus*	Bolivia: Pando	LSUMNS	9422
42	*Celeus undatus*	Guyana	KUNHM	5829
43	*Celeus undatus*	Guyana	KUNHM	5765
44	*Dryocopus lineatus*	Peru	KUNHM	799
45	*Piculus chrysochloros*	Paraguay	KUNHM	2966

Tissue sources: KUNHM, University of Kansas Natural History Museum and Biodiversity Research Center; LSUMNS, Louisiana State University Museum of Natural Science; UNAM, Museo de Zoologıa, Universidad Nacional Autónoma de México; USNM, United States National Museum of Natural History; FMNH, Field Museum of Natural History.

[a] Museum specimens sequenced from toepad samples.

2.2. Sequencing protocols

Whole genomic DNA was extracted from muscle tissue using proteinase K digestion under manufacturer's protocols (DNeasy tissue kit, Qiagen). Given the relatively shallow genetic divergences within the Picidae, we selected a suite of rapid evolving mtDNA genes (NADH dehydrogenase subunits 2 and 3 [ND2, 1041 bp; ND3, 351], ATP synthase subunits 6 and 8 [ATP6, 684 bp; ATP8, 168 bp], cytochrome c oxidase subunit 3 [COXIII, 192 bp], Control Region [CR, 957 bp]), as well as two nuclear loci (intron 7 of the ß-fibrinogen gene [ß-FIBI7, 911 bp], and a segment of the nonhistone chromosomal protein HMG-17 gene including exon 2 and adjacent mRNAs [HMGN2, 693 bp]), all of which were amplified via polymerase chain reaction (PCR) in 25 µl reactions using PureTaq RTG PCR beads (GE Healthcare). Primers used for this study are summarized in Table 2, and thermocycle parameters include an initial 3 min at 94 °C, followed by 35 cycles of 20 s at 94 °C, 15 s at 53 °C, and 60 s at 72 °C, followed by a 7 min final extension at 72 °C and 4 °C soak. This protocol was modified to incorporate an annealing touch down of eight cycles at 60 °C, eight cycles at 57 °C, and 25 cycles at 55 °C for CR reactions and both nuDNA markers. ND2, ND3, and HMGN2 were sequenced for both fresh and ancient DNA samples whereas the remaining markers were only sequenced for fresh samples.

All PCR products were visualized on a 1% agarose gel stained with ethidium bromide and amplicons were subsequently cleaned of unincorporated DNTPs and primers with ExoSaP-IT purification (USB Corp.) Purified PCR products were cycle sequenced with ABI Prism BigDye v3.1 terminator chemistry under manufacture's