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## Phylogenetic relationships of the Mangrove Hummingbird, “*Amazilia*” *boucardi* (Apodiformes: Trochilidae) of Costa Rica

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### ABSTRACT

**Introduction:** A recent revision of the generic classification of the Trochilidae based on DNA sequences revealed many inconsistencies with the current generic classification, largely based on plumage characters subject to homoplasy, especially in the Trochilini, the largest tribe. A thorough generic reorganization brought the classification into accord with the phylogeny, but due to lack of genetic data, two species remained unclassified. One of these was the Mangrove Hummingbird, “*Amazilia*” *boucardi*, endemic to Costa Rica and included in the IUCN red list of threatened species.

**Objective:** To obtain molecular evidence to clarify the generic relationships of “*A.*” *boucardi*.

**Methods:** We isolated DNA from tissues of this species and amplified 4 nuclear and 4 mitochondrial fragments and compared these with homologous fragments from 56 species in the Trochilini, constructing phylogenetic trees with maximum likelihood and Bayesian methods.

**Results:** Our phylogenetic analyses confirmed the placement of *boucardi* in the Trochilini and definitely excluded it from *Amazilia* but placed it with high confidence in the genus *Chrysuronia* Bonaparte, 1850, within which its closest relative is *C. coeruleogularis*, which also inhabits mangroves.

**Conclusions:** Our genetic data based on nuclear and mitochondrial regions clearly indicate the relationship of *A. boucardi* and *L. coeruleogularis*. Moreover, it is also supported by their habitat distribution in the mangroves of the Pacific coast of Costa Rica and Western Panama. Therefore, we suggested to exclude *A. boucardi* as “*incertae sedis*”.

**Key words:** endemic hummingbird; mangroves; nuclear and mitochondrial genes; phylogeny; taxonomy.

### RESUMEN

#### Relaciones filogenéticas del Colibrí manglero “*Amazilia*” *boucardi* (Apodiformes: Trochilidae) de Costa Rica

**Introducción:** Una revisión reciente de la clasificación de la familia Trochilidae con base en secuencias de ADN demostró muchas incongruencias con la clasificación genérica previa, que había sido hecho con base en caracteres del plumaje muy sujetos a homoplasia, especialmente en la tribu más grande, Trochilini. Una reorganización de los géneros logró llevar su clasificación genérica a la concordancia con la filogenia, pero debido a la ausencia de datos genéticos, dos especies permanecieron sin clasificar. Una de estas fue el colibrí de manglar *Amazilia boucardi*, una especie endémica de Costa Rica, considerada como amenazada en la lista roja de la UICN.



**Objetivo:** Obtener evidencia molecular para esclarecer las relaciones genéricas de *A. boucardi*.

**Métodos:** Se aisló ADN de tejidos de esta especie y se amplificaron 4 fragmentos de ADN del núcleo y 5 de la mitocondria, y se compararon con fragmentos homólogos de 56 especies en la tribu Trochilini, generando árboles filogenéticos con métodos de máxima verosimilitud y bayesiano.

**Resultados:** Los análisis filogenéticos obtenidos confirmaron la ubicación de *boucardi* en Trochilini y definitivamente la excluyó del género *Amazilia*, pero la ubicó con un alto grado de confianza en el género *Chrysuronia* Bonaparte, 1850, dentro los cuales su pariente más cercano es *C. coeruleogularis*, que también habita manglares.

**Conclusiones:** Nuestros datos genéticos basados en regiones nucleares y mitocondriales indican claramente la relación entre *A. boucardi* and *L. coeruleogularis*. Es más, lo anterior se sustenta por su distribución en los manglares de la costa Pacífica de Costa Rica y oeste de Panamá. Por lo tanto, sugerimos excluir a *A. boucardi* como “*incertae sedis*”.

**Palabras clave:** colibrí endémico; filogenia; genes nucleares y mitocondriales; manglares; taxonomía.

## INTRODUCTION

The Mangrove Hummingbird *Amazilia boucardi* is an endemic species of Costa Rica, and has been considered Near Threatened (IUCN, 2021). Males are bronze-green above, the throat and breast are bright bluish green, the abdomen and undertail coverts white; the tail is blackish and shallowly forked with the outer two rectrices notably narrow. The female is mostly white below, spotted with bronze-green laterally, the tail is blackish, the outer two rectrices narrowly tipped grayish-white (Stiles & Skutch 1989). Its habitat is mangrove swamps, mainly those where the piñuela mangrove *Pelliciera rhizophorae* abounds, though it sometimes ventures into the adjoining forest edge or secondary growth. It feeds on nectar from *Pelliciera* flowers and insects, as well as visiting flowers of *Lonchocarpus* trees and epiphytes. In Costa Rica it is found along the Pacific coast and is now rare around the Golfo de Nicoya but more common southward, especially around the Golfo Dulce.

The species *boucardi* was described in the monotypic genus *Arena* by Mulsant and Verreaux (1877) but the genus name was changed to *Arinia* and finally *Arenella* because the first two names were preoccupied. Ridgway (1911) and Cory (1918) placed *boucardi* in the genus *Lepidopyga* because of similarity of its tail and plumage pattern to those of this genus, but Peters (1945) subsumed it into the large genus

*Amazilia* Lesson, 1843. The study of McGuire et al. (2014) derived a phylogeny of the Trochilidae based upon DNA sequencing of ca. 284 species representing nearly all the genera in the family and indicated that numerous reorganizations of the current generic classification (Schuchmann, 1999) were necessary. Over half of the incongruences between the previous generic taxonomy and the phylogeny occurred in the largest tribe, the Trochilini or Emeralds. A high proportion of the Emerald species show very limited morphological variation, with many genera having been based on homoplastic characters of the plumage. Following an extensive review of the many problems of nomenclature (Stiles et al., 2017a), Stiles et al. (2017b) proposed a new generic classification of the Emeralds; one of their major changes was the dismemberment of the genus *Amazilia*, species of which appeared in nine different genera based on molecular data. However, due to the lack of genetic material (DNA), the generic placement of two species including *boucardi* could not be determined and given the widespread homoplasy in plumage characters, Stiles et al. (2017b) were obliged to leave “*Amazilia*” *boucardi* as “*incertae sedis*” (uncertain place). Therefore, the present study aims to provide the first molecular data based on DNA segments from the nucleus and mitochondria to determine the generic relationships “*A. boucardi* within the Trochilini.

## MATERIALS AND METHODS

### **Tissue material, DNA extraction, PCR amplification and sequencing, DNA sequence data:**

A total of 3 tissue samples (heart, liver, and breast muscle) were included in the analyses. Because “*A. boucardi*” is an endemic species and it has been included in the red list of UICN, only one immature male specimen was collected by GA in Sierpe, Puntarenas Province, Costa Rica (8°52’06” N & 83°29’29” W). The specimen was identified by GA and FGS and deposited in the ornithology collection of the Museo Nacional de Costa Rica (voucher MN29285). Total DNA was isolated from each tissue using a modified CTAB 2 % protocol (Doyle & Doyle, 1990). DNA sequences for amplification were 4 nuclear fragments (intron 7 of beta fibrinogen “FGB”, intron 5 of adenylate kinase 1 “AK1”; a fragment of the ornithine decarboxylase gene from end exon 6 and beginning exon 8 “ODC”; a fragment of Z-linked muscle skeletal receptor tyrosine “MUSK”) and 4 mitochondrial segments (complete gene ND2, partial ND4, complete *tRNA*-His and *tRNA*-Ser). PCR amplifications and sequencing were generated using the primers described by McGuire et al. (2007). PCR reactions (25 µl) containing 80 ng (1.5 µl) were performed according to standard protocols. PCR products were purified using the kit NucleoSpin Gel and PCR clean up (Macherey-Nagel, USA). Sequencing reactions were performed in both directions using Big Dye Termination Cycle Sequencing kit ver. 3.1 (Applied Biosystems, USA) following the manufacturer’s protocol. Cycle sequencing products were visualized on an ABI 3730 automated sequencer (Applied Biosystem, USA). Sequences from 6 loci have been deposited in GenBank NCBI for FGB (accession number MT332711), AK1 (MT332712), ODC (MT332708), MUSK (MT332707), ND2 (MT332709) and ND4/*tRNA*-His/*tRNA*-Ser (MT332710). DNA sequences from 6 loci were retrieved directly from GenBank.

**Data analysis:** All sequences were manually edited using Geneious Prime version

2021.0.3 (BIOMATTERS Ltd, New Zealand) (Kearse et al., 2012) applying the alignment method MUSCLE ver. 3.8.425 using default settings, and then adjusted manually.

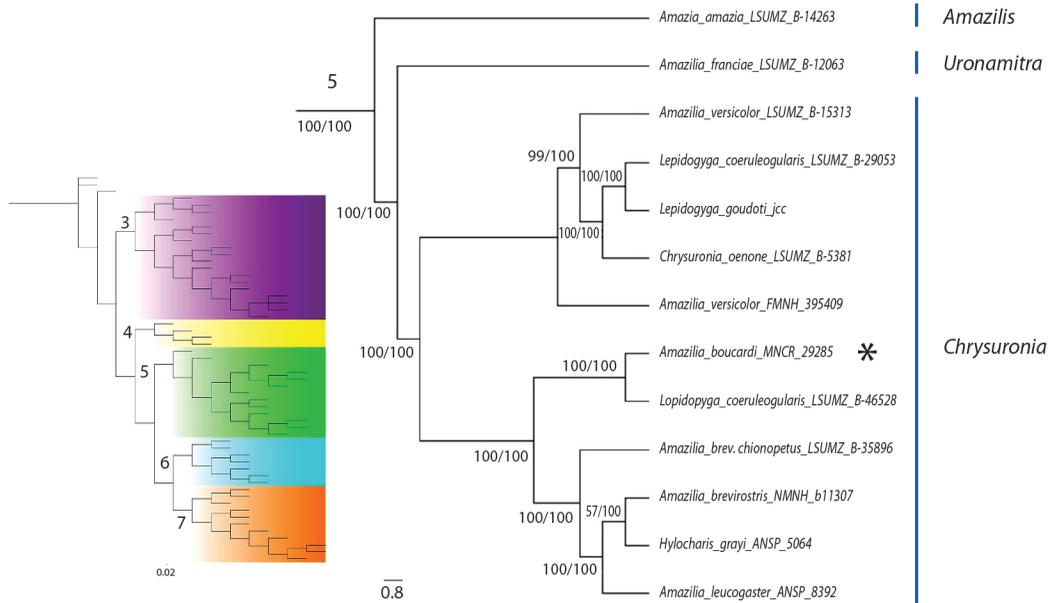
**Phylogenetic analysis:** Data partition and nucleotide substitution models were identical to those mentioned by McGuire et al. (2007) and McGuire et al. (2014). The selection of the best-fitting model for each region was: FGB GTR+I+G, AK1 GTR+G, ODC GTR+I+G, MUSK GTR+I+G, ND2 GTR+I+G, ND4 GTR+I+G, and *tRNAs* HKY+I. For the RAxML ver. 8.2.11 (Stamatakis, 2014) analyses, the GTRGAMMA model was used for both tree inference and bootstrapping, with 1 000 non-parametric bootstrap pseudoreplicates. Bayesian analyses applied to the concatenated matrix were conducted with the program MrBayes ver. 3.2.6 (Ronquist et al., 2011), 100 000 000 generations were iterated, and sampled every 1 000 generations, for two runs each with four chains; the first 25 % trees were discarded as burn-in. The final trees were visualized using FigTree ver. 1.4.4 (Rambault, 2018).

## RESULTS

### **PCR amplification, sequencing, and alignment:**

DNA amplification from three different tissues was successful and the sequences were the same, therefore we performed all analyses with DNA from the liver. The PCR products of the 6 loci, FGB (979 bp), AK1 (529 bp), ODC (603 bp), MUSK (596 bp), ND2 (945 bp), ND4/*tRNA*-His/*tRNA*-Ser/*tRNA*-Leu partial (885 bp), and BLAST analysis confirmed the identity. The same DNA fragments were obtained from GenBank for 56 Emerald species (Trochilini) according to McGuire et al. (2014) (SMT1). Alignments with MUSCLE of the concatenated data (5 009 positions) did not reveal any significant topological differences by visual comparison.

**Phylogenetic analysis:** The concatenated alignment of 6 loci for 56 species was used to generate a rooted Bayesian and RAxML tree



**Fig. 1.** Bayesian consensus tree inferred from a dataset of emerald hummingbirds group using six loci. Letters represent subgroup classifications according to Stiles et al. (2017b) and are highlighted in different colors. The numbers above the nodes correspond to percent of posterior probabilities values and maximum likelihood bootstrap. *Amazilia boucardi* is denoted by an asterisk.

(Fig. 1). The robustly resolved phylogeny of the tribe Trochilini achieved in our study subdivided the tribe Trochilini into four groups in accordance with the classification proposed by Stiles et al. (2017b). Analyses of the complete concatenated molecular data produced congruent phylogenies with two methodological approaches (Fig. 1). Our analysis confirmed the placement of *boucardii* in group D with strong support. This group has the greatest number of Emerald species, including those formerly in *Amazilia* but now separated in seven different genera. The analysis placed *boucardi* closest to “*Lepidopyga*” *coeruleogularis* (Gould, 1851), with both now included within the genus *Chrysuronia* Bonaparte 1850, as circumscribed by Stiles et al. (2017b), also with strong support. The genus *Lepidopyga* Reichenbach, 1855 is now considered a synonym of *Chrysuronia*. We therefore place *boucardi* in the genus *Chrysuronia*.

## DISCUSSION

Our conclusion that the closest relative of *C. boucardi* is *coeruleogularis* (possibly they are sister species) is interesting, because both are habitants of mangrove swamps along the Pacific coast of Middle America, *boucardi* in Costa Rica and *coeruleogularis* in Western Panama. In Colombia, the distribution of *C. coeruleogularis* then follows the Caribbean slope in mangroves and adjacent habitats, while its place in the Pacific mangroves southward from extreme Eastern Panama is occupied by *C. humboldtii* (deLatre & Bourcier, 1846). Our results also agree with the classification of *boucardi* in *Lepidopyga* by Ridgway (1911) and Cory (1918): both species are now included in *Chrysuronia*. This leaves only the Honduran Emerald “*Amazilia*” *luciae* (Lawrence, 1867) still unclassified due to lack of a genetic sample. Although Schuchmann (1999) considered this species

and *boucardi* to be possibly sisters (perhaps because of their geographical proximity), there are differences in their plumages that leave room for uncertainty, hence we prefer to leave *luciae* as “*incertae sedis*” until a genetic sample can be analyzed.

**Ethical statement:** the authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgments section. A signed document has been filed in the journal archives.

See supplementary material  
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