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Phylogenomic analysis confirms the relationships among toucans, toucan-barbets, and New World barbets but reveals paraphyly of *Selenidera* toucanets and evidence for mitonuclear discordance

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ABSTRACT

We reconstruct the species-level phylogenetic relationship among toucans, toucan-barbets, New World barbets using phylogenomic data to assess the monophyly and relationships at the family, generic, and specific levels. Our analyses confirmed (1) the monophyly of toucans (Aves: Ramphastidae), toucan-barbets (Aves: Semnornithidae), and New World barbets (Aves: Capitonidae) and that the toucan-barbets are sister to the toucans, an arrangement suggested, but poorly supported, in previously published phylogenies; (2) the paraphyly of lowland *Selenidera* toucanets with respect to *Andigena* mountain-toucans; and (3) evidence of some mitonuclear discordance, suggesting introgression or incomplete lineage sorting. For example, mitonuclear conflict in the phylogenetic placement of *Ramphastos vitellinus* subspecies suggests that Amazonian populations of *Ramphastos vitellinus ariel* may have introgressed mitogenomes derived from other Amazonian *vitellinus* taxa. To reconstruct the phylogenetic history of toucans, toucan-barbets, and New World barbets, we included all species-level taxa from the three families, with the addition of outgroups from the two major clades of Old World barbets (Megalaimidae and Lybiidae). We analyzed a combination of UCE sequences and whole mitochondrial genome sequences to reconstruct phylogenetic trees.

Keywords: barbets, mitonuclear discordance, phylogenomics, toucans, ultraconserved elements

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LAY SUMMARY

- We analyzed relationships between all toucans, toucan-barbets, and New World barbets using both nuclear and mitochondrial DNA sequence data.
- We found highly supported relationships that showed that toucan-barbets are most closely related to toucans to the exclusion of New World barbets.
- Our results were consistent with previous research indicating that some *Selenidera* toucanets may be more closely related to *Andigena* mountain-toucans than they are to other *Selenidera* species.
- We found biologically relevant discordance between phylogenetic reconstructions using nuclear and mitochondrial data that give insight into potential historical introgression within *Ramphastos*.

El análisis filogenómico confirma las relaciones entre los tucanes, los barbudos tucanes y los barbudos del Nuevo Mundo, pero revela la parafilia de las tucanetas de *Selenidera* y brinda evidencia de discordancia mitonuclear

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RESUMEN

Reconstruimos la relación filogenética a nivel de especie entre tucanes, barbudos tucanes y barbudos del Nuevo Mundo utilizando datos filogenómicos para evaluar la monofilia y las relaciones a nivel de familia, género y especie. Nuestros análisis confirmaron (1) la monofilia de los tucanes (Aves: Ramphastidae), los barbudos tucanes (Aves: Semnornithidae) y los barbudos del Nuevo Mundo (Aves: Capitonidae), y que los barbudos tucanes son hermanos de los tucanes, un arreglo sugerido pero pobremente apoyado en filogenias publicadas anteriormente; (2) la parafilia de las tucanetas de las tierras bajas del género *Selenidera* en relación a los tucanes de montaña del género *Andigena*; y (3) la evidencia de alguna discordancia mitonuclear, lo que sugiere introgresión o clasificación incompleta de linajes. Por ejemplo, el conflicto mitonuclear en la ubicación filogenética de las subespecies de *Ramphastos vitellinus* sugiere que las poblaciones amazónicas de *Ramphastos vitellinus ariel* pueden tener introgresión de mitógenos derivados de otros taxones *vitellinus* amazónicos. Para reconstruir la historia filogenética de los tucanes, los barbudos tucanes y los barbudos del Nuevo Mundo, hemos incluido todos los taxones a nivel de especie de las tres familias, con la adición de grupos externos de los dos clados principales de barbudos del Viejo Mundo (Megalaimidae y Lybiidae). Hemos analizado una combinación de secuencias de elementos ultraconservados y de secuencias del genoma mitocondrial completo para reconstruir árboles filogenéticos.

Palabras clave: barbudos, discordancia mitonuclear, elementos ultraconservados, filogenómica, tucanes

INTRODUCTION

Toucans, toucan-barbets, and New World barbets are iconic Neotropical piciform birds found throughout the Neotropics from Mexico south to Argentina. This expansive distribution has been leveraged to examine broad questions about biogeography, endemism, and the evolution of plumage coloration (Haffer 1974, Prum and Samuelson 2016). In particular, these species have often served as models for testing hypotheses about the effects of biogeographic barriers such as rivers, mountains, and other isolating geographic features on speciation in the tropics (Naka and Brumfield 2018, Bonaccorso and Guayasamin 2013, Haffer 1974).

Despite this group's clear utility for testing hypotheses about replicate biogeographic histories of co-distributed genera across the Neotropics, precise phylogenetic relationships among species, genera, and families of toucans, toucan-barbets, and New World barbets have yet to be resolved. Studies have either reconstructed higher-level relationships among genera and families (Lanyon and Zink 1987, Barker and Lanyon 2000, Moyle 2004), focused on species-level phylogenies within genera (Hackett and Lehn 1997, Weckstein 2005, Eberhard and Bermingham 2005, Puebla-Olivares et al. 2008, Patané et al. 2009, Bonaccorso et al. 2011, Patel et al. 2011, Lutz et al. 2013), or focused on one species complex (Armenta et al. 2005). All previous studies have been based on either morphological characters (Cracraft and Prum 1988, Prum 1988a, Prum 1988b), allozymes (Lanyon and Zink 1987), or Sanger DNA sequences from only a few mitochondrial or nuclear loci (Hackett and Lehn 1997, Barker and Lanyon 2000, Moyle 2004, Weckstein 2005, Eberhard and Bermingham 2005, Puebla-Olivares et al. 2008, Patané et al. 2009, Bonaccorso et al. 2011, Patel et al. 2011, Lutz et al. 2013). Consequently, many relationships among genera, families, and species are uncertain.

Higher-level and generic relationships within toucans, toucan-barbets, and New World barbets have not been confidently established (Moyle 2004, Barker and Lanyon 2000), which has important implications for reconstructing the evolutionary history of these taxa. For example, Haffer (1974) hypothesized that highland and lowland toucan genera were reciprocally monophyletic groups, implying that members of the genera *Aulacorhynchus* and *Andigena*, which live in montane regions, were sister clades to the exclusion of all other toucans. If montane toucan genera were monophyletic, it would indicate a single historical biogeographic transition between montane and lowland regions, whereas non-monophyly would suggest a more complex biogeographic history of multiple transitions between lowland and highland areas. Lanyon and Zink's (1987) analysis of allozyme data supported the sister relationship between *Aulacorhynchus* and *Andigena*, but did not report support values (Figure 1). Lanyon and Zink (1987) also found that New World barbets were sister to all toucans rather than African (Lybiidae) or Asian (Megalaimidae) barbets, but they did not include *Semnornis* in their study. Prum (1988b) included *Semnornis* in his morphological analysis of the toucans, toucan-barbets, and New World barbets and found that *Semnornis* was sister to toucans to the exclusion of the New World barbets, but once again did not include support values (Figure 1). Barker and Lanyon (2000) analyzed DNA sequences from the mitochondrial cytochrome *b* (cyt *b*) gene from representatives of all genera of barbets and toucans and found that *Semnornis* was sister to all toucans and New World barbets, but with low statistical support (Figure 1). Using both maximum likelihood and Bayesian methods to analyze a combination of mitochondrial cyt *b* and nuclear beta fibrinogen intron 7 DNA sequence data, Moyle (2004) was also unable to fully resolve the basal phylogenetic relationships among toucans, toucan-barbets, and New World barbets.

Several studies have focused on more detailed species-level phylogenetic reconstructions of one or two genera of toucans or New World barbets. The toucans (Ramphastidae) include colorful and charismatic large-bodied genera such as *Ramphastos* (toucans), *Aulacorhynchus* (emerald toucanets), *Pteroglossus* (araçaris), *Andigena* (mountain-toucans) and *Selenidera* (toucanets). Within toucans, *Ramphastos* is split into smooth-billed yelping (*Ramphastos tucanus*, *Ramphastos swainsonii*, and *Ramphastos ambiguus)* and channel-keel-billed croaking clades (all other *Ramphastos*), with the smooth-billed toucans arising from within the channel-keel-billed croaking clade (Weckstein 2005). *Ramphastos toco* was identified as sister to all other *Ramphastos* toucans (Weckstein 2005). Two lowland toucan genera, *Ramphastos* toucans and *Pteroglossus* araçaris, have relatively low genetic divergence (Hackett and Lehn 1997, Eberhard and Bermingham 2005, Patel et al. 2011, Weckstein 2005, Patané et al. 2009). Conversely, *Aulacorhynchus* harbors underappreciated genetic divergence and geographic variation. For example, morphological and genetic data have provided evidence that many subspecies within the *Aulacorhynchus prasinus* complex should be considered full species (Navarro et al. 2001, Puebla-Olivares et al. 2008, Bonaccorso et al. 2011, Bonaccorso and Guayasamin 2013). Many of these species breaks coincide with major biogeographic breaks between isolated highland regions (Navarro et al. 2001).

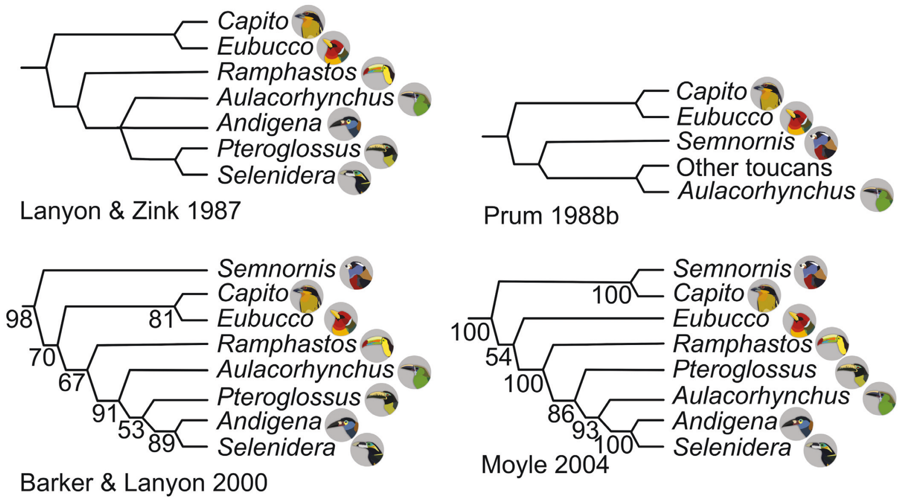

FIGURE 1. Previous phylogenetic reconstructions of toucan, toucan-barbet, and New World barbet generic relationships. Bootstrap support values from each paper are reported if they were published with the tree.

Previously reconstructed relationships between *Selenidera* and *Andigena* are complicated and not fully resolved. Lutz et al. (2013) found that *Selenidera* may be paraphyletic. Specifically, *Selenidera spectabilis* was sister to *Andigena*, and together, these taxa were sister to the rest of *Selenidera* (Lutz et al. 2013). However, this paraphyly had mixed support in the mitochondrial dataset and little resolution in the nuclear dataset (Lutz et al. 2013).

The smaller-bodied but equally colorful New World barbet family (Capitonidae: Genera *Capito* and *Eubucco*) and toucan-barbet family (Semnornithidae: Genus *Semnornis*) were thought to be closely related based on both groups having relatively small bills, but the limited genetic sampling in phylogenetic studies has not resolved these higher-level relationships (Barker and Lanyon 2000, Moyle 2004). Only two molecular phylogenetic studies have been published on New World barbets: One assessing the relationships in the Black-spotted Barbet complex (*Capito niger*) and one focusing on the description of *Capito fitzpatricki* (Armenta et al. 2005, Seeholzer et al. 2012).

In this study, we test previously published hypotheses about the relationships among families, genera, species, and many subspecies of toucan, toucan-barbets, and New World barbets, using a phylogenomic-scale dataset generated by next-generation sequencing of libraries enriched for ultraconserved elements (UCEs). Our study includes all species and most subspecies of toucans, toucan-barbets, and New World barbets in addition to a diverse sampling of Old World barbets to serve as outgroup taxa, which allows us to reassess the monophyly of the New World genera.

METHODS

Taxon Sampling

We sampled 102 individuals comprising 8 outgroup taxa and 94 ingroup individuals using museum-vouchered specimens (see Table 1 for details). The ingroup consisted of representatives of all 53 species and as many subspecies as available from the three ingroup families (Ramphastidae, Capitonidae, and Semnornithidae; Clements et al. 2022). We sequenced taxa from within Ramphastidae including *Pteroglossus* (17 of the 23 subspecies), *Ramphastos* (12 of the 17 subspecies), *Aulacorhynchus* (13 of the 25 subspecies), *Selenidera* (7 of the 7 subspecies), and *Andigena* (5 of the 7 subspecies). We sequenced taxa from within Capitonidae including *Capito* (12 of the 20 subspecies) and *Eubucco* (9 of the 14 subspecies) and both species in the family Semnornithidae, genus *Semnornis* (2 of the 3 subspecies). We also sequenced 8 outgroup taxa from multiple genera of Asian (Megalaimidae) and African (Lybiidae) barbet lineages to root the trees.

DNA Extraction, UCE Enrichment, and Sequencing

For each specimen, we extracted DNA from ~20 mg of pectoral muscle previously stored at −80°C. DNA was extracted from muscle using standard DNeasy protocols for tissues (Qiagen, Valencia, CA). For five taxa, *Aulacorhynchus huallagae*, *Capito hypoleucus hypoleucus*, *Ramphastos vitellinus citreolaemus*, *Selenidera reinwardtii reinwardtii*, and *Selenidera maculirostris*, no frozen tissue samples were available and therefore we used a clean scalpel blade to cut a toepad from a museum study skin for DNA extraction. These toepad samples were extracted in a lab where no frozen bird tissues have been extracted, following a procedure developed by Andrés Cuervo and colleagues (personal communication). To wash the external surface of these samples, absolute ethanol, and AE buffer from a QIAamp DNA Micro kit (Qiagen, Valencia, CA) were added to an extraction tube and used to wash each toepad prior to extraction. These washed toepad fragments were then placed in a new tube, minced, and incubated at 56°C for 48 hr in a mixture of buffer ATL, proteinase K, and 20 μL of 1M DTT. After incubation, we followed the Qiagen DNeasy Blood and Tissue protocol with the following exceptions: (1) we added 1 μL of carrier RNA during step 1, (2) we added cold absolute ethanol and incubated the mixture for 1 hr at 4°C during step 2, (3) we used spin columns from a QIAquick PCR purification kit during step 5, and (4) we concentrated the extract to 100 μL in a SpeedVac after elution.

We quantified all DNA concentrations using real-time qPCR fluorescent detection (Blotta et al. 2005) with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR). We then sent all extracted samples with 3–30 ng μL^{-1} of DNA to RAPiD Genomics (Gainesville, FL, USA) for library preparation, UCE

TABLE 1. Taxa included in this study, identified to subspecies where applicable, including tissue number and locality. Outgroup taxa are listed at the bottom.

Genus	Species	Subspecies	Tissue source	Tissue #[+]	Skin #[*]	Locality
Andigena	*cucullata*		LSUMNS	B1273	101870	Bolivia: La Paz
Andigena	*hypoglauca*	*hypoglauca*	LSUMNS	B32020	MHNSM	Peru: Cajamarca
Andigena	*hypoglauca*	*lateralis*	LSUMNS	B8309	128378	Peru: Pasco
Andigena	*laminirostris*		LSUMNS	B7777		Ecuador: Pichincha
Andigena	*nigrirostris*	*spilorhynchus*	LSUMNS	B32513	169750	Peru: Cajamarca
Aulacorhynchus	*albivitta*	*atrogularis*	LSUMNS	B21201		Peru: Madre de Dios
Aulacorhynchus	*albivitta*	*cyanolaemus*	LSUMNS	B33837	172078	Peru: Cajamarca
Aulacorhynchus	*albivitta*	*dimidiatus*	LSUMNS	B10742	156323	Peru: Ucayali
Aulacorhynchus	*coeruleicinctis*		LSUMNS	B39644	168852	Bolivia: Santa Cruz
Aulacorhynchus	*derbianus*	*derbianus*	LSUMNS	B22829	162605	Bolivia: La Paz
Aulacorhynchus	*derbianus*		LSUMNS	B33174	MHNSM	Peru: Cajamarca
Aulacorhynchus	*haematopygus*		LSUMNS	B7850	ANSP 177539	Ecuador: El Oro
Aulacorhynchus	*huallagae*		ANSP		108177	Peru: La Libertad
Aulacorhynchus	*prasinus*	*caeruleogularis*	LSUMNS	B26403	163529	Panama: Chiriquí
Aulacorhynchus	*prasinus*	*cognatus*	LSUMNS	B1373	104668	Panama: Darién
Aulacorhynchus	*prasinus*	*prasinus*	LSUMNS	B19279	UNAM 9769	Mexico
Aulacorhynchus	*sulcatus* (1)		KU	12020	111217	Venezuela: Sucre
Aulacorhynchus	*sulcatus* (2)		KU	12025	111218	Venezuela: Lara
Aulacorhynchus	*sulcatus* (3)		KU	12021	111219	Venezuela: Aragua
Aulacorhynchus	*whitelianus*	*duidae*	LSUMNS	B7589	FMNH 318901	Venezuela: Amazonas
Aulacorhynchus	*whitelianus*	*osgoodi*	USNM	B10573	621744	Guyana
Capito	*auratus*	*auratus*	LSUMNS	B4805		Peru: Loreto
Capito	*auratus*	*nitidior* (1)	LSUMNS	B6947	119450	Peru: Loreto
Capito	*auratus*	*nitidior* (2)	LSUMNS	B25572	184595	Brazil: Amazonas
Capito	*auratus*	*punctatus*	LSUMNS	B27553	161653	Peru: Loreto
Capito	*aurovirens*		LSUMNS	B7344	119446	Peru: Loreto
Capito	*brunneipectus*		LSUMNS	B25499	184593	Brazil: Pará
Capito	*dayi*		LSUMNS	B18305	153280	Bolivia: Santa Cruz
Capito	*hypoleucus*	*hypoleucus*	ANSP		162682	Colombia: Antioquia
Capito	*maculicoronatus*	*maculicoronatus*	LSUMNS	B28524	163498	Panama: Colón
Capito	*maculicoronatus*	*rubrilateralis*	LSUMNS	B2178	108765	Panama: Darién
Capito	*niger*	*niger*	USNM	B09373	UG A 251	Guyana: North West
Capito	*quinticolor*		LSUMNS	B29985		Ecuador: Esmeraldas
Capito	*squamatus*		LSUMNS	B7738	ANSP 178084	Ecuador: Bolivar
Capito	*fitzpatricki*		KU	18267	CORBIDI AV 2792	Peru: Ucayali
Capito	*wallacei*		LSUMNS	B27757		Peru: Loreto
Eubucco	*bourcierii*	*aequitorialis*	LSUMNS	B11755	ANSP 180266	Ecuador: Esmeraldas
Eubucco	*bourcierii*	*anomolus*	LSUMNS	B2108	108245	Panama: Darién
Eubucco	*bourcierii*	*orientalis*	LSUMNS	B6053	ANSP 176705	Ecuador: Morona-Santiago
Eubucco	*bourcierii*	*salvini*	LSUMNS	B16038	138683	Costa Rica: Heredia
Eubucco	*richardsoni*	*auranticollis*	LSUMNS	B5496	116711	Peru: San Martin
Eubucco	*richardsoni*	*nigriceps*	LSUMNS	B2617	109524	Peru: Loreto
Eubucco	*tucinkae*		LSUMNS	B10659	156319	Peru: Ucayali
Eubucco	*versicolor*	*steerii*	LSUMNS	B27728	161657	Peru: Loreto
Eubucco	*versicolor*	*versicolor*	LSUMNS	B22873		Bolivia: La Paz
Pteroglossus	*aracari*	*aracari*	LSUMNS	B35547	MPEG 55313	Brazil: Pará
Pteroglossus	*aracari*	*atricollis*	USNM	B09755	KU 88945	Guyana: North West
Pteroglossus	*azara*	*azara*	FMNH	JAP-839	456643	Brazil: Amazonas
Pteroglossus	*azara*	*flavirostris*	FMNH	JAP-474	456647	Brazil: Amazonas
Pteroglossus	*azara*	*mariae* (1)	LSUMNS	B40614		Peru: Loreto
Pteroglossus	*azara*	*mariae* (2)	LSUMNS	B8991	132260	Bolivia: Pando
Pteroglossus	*bailloni*		LSUMNS	B25891	ANSP	Paraguay
Pteroglossus	*beauharnaesii*		LSUMNS	B35532	MPEG 54566	Brazil: Mato Grosso
Pteroglossus	*bitorquatus*	*reichenowi*	FMNH	PPBIO-127	456656	Brazil: Pará
Pteroglossus	*bitorquatus*	*sturmii*	LSUMNS	B35533	MPEG 54572	Brazil: Mato Grosso

Table 1. Continued

Genus	Species	Subspecies	Tissue source	Tissue #[+]	Skin #[*]	Locality
Pteroglossus	*castanotis*	*australis* (1)	LSUMNS	B35266	MPEG 54536	Brazil: Mato Grosso
Pteroglossus	*castanotis*	*australis* (2)	LSUMNS	B37611		Bolivia: Santa Cruz
Pteroglossus	*castanotis*	*castanotis* (1)	FMNH	JAP-855	456651	Brazil: Amazonas
Pteroglossus	*castanotis*	*castanotis* (2)	LSUMNS	B27624		Peru: Loreto
Pteroglossus	*frantzii*		LSUMNS	B16075	138690	Costa Rica: Puntarenas
Pteroglossus	*inscriptus*	*humboldti* (1)	LSUMNS	B8819	132278	Bolivia: Pando
Pteroglossus	*inscriptus*	*humboldti* (2)	FMNH	JAP-574	456652	Brazil: Amazonas
Pteroglossus	*inscriptus*		LSUMNS	B35444	MPEG 54573	Brazil: Mato Grosso
Pteroglossus	*pluricinctus*		LSUMNS	B7112	119461	Peru: Loreto
Pteroglossus	*torquatus*		LSUMNS	B28584	164089	Panama: Colón
Pteroglossus	*torquatus*	*erythropygius*	LSUMNS	B100021	139983	Zoo/captive
Pteroglossus	*torquatus*	*sanguineus*	LSUMNS	B11783	ANSP 180273	Ecuador: Esmeraldas
Pteroglossus	*viridis*		LSUMNS	B20231	165700	Brazil: Amazonas
Ramphastos	*ambiguus*	*swainsonii*	LSUMNS	B2309		Panama: Darién
Ramphastos	*ambiguus*		FMNH	P10-236	473978	Peru
Ramphastos	*brevis*		LSUMNS	B12175	ANSP 180277	Ecuador: Pichincha
Ramphastos	*dicolorus*		KU	B282		Paraguay: Caazapá
Ramphastos	*sulfuratus*	*brevicarinatus*	LSUMNS	B28577	164099	Panama: Colón
Ramphastos	*sulfuratus*	*sulfuratus*	KU	B2007		Mexico: Campeche
Ramphastos	*toco*	*albogularis*	LSUMNS	B1477	127162	Zoo/captive
Ramphastos	*tucanus*	*cuvieri* (1)	LSUMNS	B27691	161634	Peru: Loreto
Ramphastos	*tucanus*	*cuvieri* (2)	LSUMNS	B9392	132309	Bolivia: Pando
Ramphastos	*tucanus*	*tucanus* (1)	KU	B1356		Guyana
Ramphastos	*tucanus*	*tucanus* (2)	LSUMNS	B35550	MPEG 54563	Brazil: Pará
Ramphastos	*vitellinus*	*ariel* (Amazonia)	LSUMNS	B35667	MPEG 55175	Brazil: Pará
Ramphastos	*vitellinus*	*ariel* (Atlantic Forest)	LSUMNS	B35555	MPEG 55148	Brazil: São Paulo
Ramphastos	*vitellinus*	*citreolaemus*	ANSP		161487	Colombia: Bolivar
Ramphastos	*vitellinus*	*culminatus*	LSUMNS	B2860	109542	Peru: Loreto
Ramphastos	*vitellinus*	*vitellinus*	KU	B1237		Guyana
Selenidera	*gouldii* (1)		LSUMNS	B35413	MPEG 54568	Brazil: Mato Grosso
Selenidera	*gouldii* (2)		FMNH	389772	389772	Brazil: Rondônia
Selenidera	*maculirostris*		ANSP		169989	Brazil: São Paulo
Selenidera	*nattereri*		LSUMNS	B25399	165746	Brazil: Amazonas
Selenidera	*piperivora*		LSUMNS	B35643	MPEG 55302	Brazil: Pará
Selenidera	*reinwardtii*	*langsdorfii*	LSUMNS	B27756		Peru: Loreto
Selenidera	*reinwardtii*	*reinwardtii*	ANSP		187040	Ecuador: Sucumbíos
Selenidera	*spectabilis*		LSUMNS	B2120	108768	Panama: Darién
Semnornis	*frantzii*		LSUMNS	B16019	138685	Costa Rica: Heredia
Semnornis	*ramphastinus*	*ramphastinus*	LSUMNS	B7771	ANSP 178078	Ecuador: Pichincha
Buccanodon	*duchaillui*		ANSP	23632	190270	Equatorial Guinea: Centro Sur
Caloramphus	*fuliginosus*		LSUMNS	B20474	148751	Zoo/captive
Gymnobucco	*bonapartei*	*bonapartei*	ANSP	23793	190269	Equatorial Guinea: Centro Sur
Lybius	*torquatus*		LSUMNS	B5904	122625	Zoo/captive
Megalaima	*annamensis*		ANSP	29992	122925	Vietnam: Kon Tum
Pogoniulus	*scolopaceus*	*flavisquamatus*	ANSP	23844	190271	Equatorial Guinea: Centro Sur
Trachyphonus	*vaillantii*		LSUMNS	B5900	122171	Zoo/captive
Tricholaema	*hirsuta*		ANSP	23600	100501	Equatorial Guinea: Centro Sur

[*] All skins are housed at the tissue source institution unless noted by a museum acronym ahead of the skin number.

[+] All samples without tissue numbers are sequenced from tissue cut from toepads of specimens.

enrichment, and sequencing (Faircloth et al. 2012, McCormack et al. 2013). We used a standard 2.5k probe set (Faircloth et al. 2012) with additional avian-specific UCE probes (McCormack et al. 2013). Libraries were pooled and were divided between three lanes on an Illumina HiSeq 2000 and paired-end 100 base pair (bp) or 150 bp reads were generated on each of these lanes.

Mitochondrial Quality Control and Assembly

All raw read files were trimmed for adapters and low-quality regions with the FASTX-Toolkit from the Hannon lab (http://hannonlab.cshl.edu/fastx_toolkit/) using a custom script. Mitochondrial genomes were assembled using Geneious 8.1.8 (Kearse et al. 2012) by mapping reads for a given specimen onto the *Pteroglossus azara flavirostris* mitochondrial genome reference sequence deposited in NCBI (NC_008549.1, Gibb et al. 2007). We used the *map to reference* function with a medium–low sensitivity setting iterated up to five times with no trimming. We generated a consensus sequence for each individual with a strict 50% threshold.

For quality control and annotation, we submitted individual sequences to the MITOS2 (http://mitos2.bioinf.uni-leipzig.de/index.py, Bernt et al. 2013) online portal and then downloaded the resultant sequences along with .gff annotation files. Both sequence and annotation files were imported into Geneious and all annotations were compared by eye to the *Pteroglossus azara flavirostris* mitochondrial genome (NC_008549) by ENO. We then aligned all high-quality mitochondrial sequences in Geneious and input this alignment into Gblocks 0.91b for further quality control (Castresana 2000, Talavera and Castresana 2007). We allowed for gap positions with half of the sequences due to some sequences missing data and some informative indels in the mitochondrial genome. All other parameters were set to the defaults. Some individuals (*Buccanodon duchaillui*, *Calorhamphus fuliginosus*, *Capito aurovirens*, *Capito auratus auratus*, *Capito auratus punctatus*, *Capito dayi*, *Capito fitzpatricki*, *Capito hypoleucus hypoleucus*, *Eubucco bourcierii salvini*, *Eubucco richardsoni nigriceps*, *Eubucco veriscolor veriscolor*, *Lybius torquatus*, *Selenidera gouldii* [FMNH 389772], *Selenidera reinwardtii langsdorfii*, and *Trachyphonus vaillantii*) were removed for this step because they had long gaps in their sequences, which would have caused Gblocks to erroneously remove sequence data from the overall alignment. The individuals with missing data listed previously were added back into the alignment after this step and sections of the mitochondrial genome that Gblocks annotated out of the partial alignment were removed from the sequences. Only non-overlapping coding regions were used as input for mitochondrial phylogenetic analyses.

UCE Processing

UCEs were processed using the Phyluce 1.5.0 (Faircloth 2016) pipeline. Initial sequences were preprocessed for quality control with Illumiprocessor (Faircloth 2013), which uses Trimmomatic (Bolger et al. 2014). Read assembly was completed with Phyluce (Faircloth 2016) using the Trinity 2.0.6 (Grabherr et al. 2011) assembler. All samples were aligned using MAFFT (Katoh et al. 2013) and trimmed with gblocks following the default Phyluce settings. Only UCE loci that were found in ≥ 75% of the taxa were used for analyses.

Model Testing

We used PartitionFinder2 (Lanfear et al. 2017) on the CIPRES science gateway 3.3 (Miller et al. 2010) to analyze both UCE and mitochondrial alignments to test for the best partitioning scheme and substitution models for each partition. These partitions and models were calculated starting from the finest partitioning scheme available: every UCE and every codon position for each mitochondrial gene. We chose the best partitions and models using the corrected Akaike Information Criterion.

The UCE and mitochondrial alignments were handled differently due to the size of the alignments. For the UCE alignment, an initial RAxML 8.2.10 (Stamatakis 2014) tree was generated from unpartitioned UCE data and a GTR model was used as a starting tree for PartitionFinder2 analysis. The rcluster search scheme was used due to the quantity of UCEs in the alignment. For the mitochondrial alignment, no starting tree was provided and a greedy search scheme was used.

Phylogenetic Analyses

All maximum likelihood trees were generated using RAxML on the CIPRES science gateway. Two trees were generated using RAxML: a UCE tree and a mitochondrial tree. Unlinked partitions from PartitionFinder2 were used for these analyses. For both alignments, PartitionFinder2 indicated that GTR + I + G was the best model for most of the partitions; however, we used GTR + G to avoid over-parameterization (Stamatakis 2014). We used the program ExaBayes 1.5 to conduct a Bayesian analysis of the mitochondrial data. We ran four chains for 10 million generations and sampled these chains every 1,000 generations with a 10% burn-in. We used the *consense* tool to summarize the results. We also completed a quartet-based analysis using the UCE data. We used the alignment with UCEs present in ≥ 75% of the samples as an input for the program SVDQuartets 5.5.9 (Chifman and Kubatko 2015). We sampled 100,000 random quartets and generated 100 bootstraps.

Single Nucleotide Polymorphism Analyses (SNPs)

We identified single nucleotide polymorphisms (SNPs) by phasing all individuals using the program Phyluce. In short, we did this by exploding UCE alignments, using the phyluce wrapper to do multiple alignments with BWA 0.7.0-r313 (Li and Durban 2009), and phased SNPs. We then called SNPs from these phased data, using only one SNP per UCE to create a dataset of putatively unlinked SNPs. We input these SNPs into the program SNAPP (Bryant et al. 2012) to visualize the relationships among *Andigena* and *Selenidera* and assess whether there was support for conflicting topologies in the nuclear data. We also analyzed SNPs from the *Ramphastos vitellinus* complex using the program DFOIL 2017-011-25 (Pease and Hahn 2015) after filtering for missing data and heterozygous bases using custom scripts (https://github.com/emilyostrow/UCEtoDFOIL). For SNP analyses within *Ramphastos vitellinus*, we did not include *Ramphastos vitellinus citreolaemus*, as these sequences came from a toepad DNA extract and introduced too much missing data.

RESULTS

Sequence Alignment

The 75% UCE alignment used in this study for all 102 taxa (listed in Table 1) included 2,112 UCE loci with an alignment length of 0.93 Mbp. The mean length of individual UCEs was 566.02 bp with a range of 46–3,949 bp. Due to missing data, there was an average of 82 taxa included in each gene tree. In general, we recovered fewer UCEs from toepad samples compared to frozen tissue samples. In particular, *Ramphastos vitellinus citreolaemus* was missing sequence data for 90.05% of sites in the UCE alignment, which likely explains the long branch leading to this taxon in Figure 3.

The protein-coding mitochondrial gene alignment (excluding areas such as the control region and tRNAs) generated for this study was 11,340 bp long. Most sequences were complete except for a few individuals, as noted in the methods section, missing large amounts of data. There are

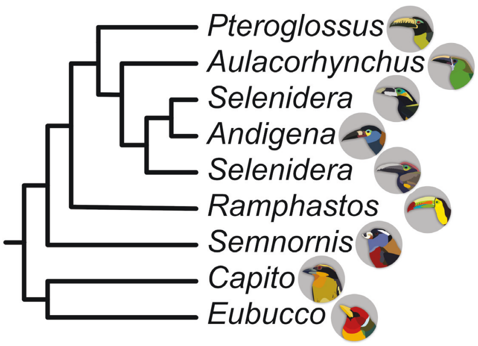

FIGURE 2. Phylogenetic tree of the relationships of toucan, toucan-barbet, and New World barbet genera. All nodes in these trees have full RAxML bootstrap support in analyses of UCEs and mitochondrial data.

two 3 bp indels in the mitochondrial alignment unique to the two toucan genera *Ramphastos* and *Aulacorhynchus*. These indels are at the beginning of NADH Dehydrogenase Subunit-1 and are 3 bp apart.

Higher-Level and Generic Relationships

All families were recovered as monophyletic with strong Bayesian inference support (1.00) and maximum likelihood bootstrap support (100%). Toucan-barbets are strongly supported as sister to toucans, and New World barbets are strongly supported as sister to toucans + toucan-barbets in all analyses using both mitochondrial and UCE data (Figures 2–4).

All genera within each of the focal families are monophyletic, except for *Selenidera*. The relationships between the species in *Selenidera* and their close relatives in *Andigena* are complicated. In every phylogenetic reconstruction, regardless of method or dataset analyzed, we found that *Selenidera* is paraphyletic with respect to *Andigena*; however, relationships within the *Selenidera* + *Andigena* clade differ depending on the data type analyzed (e.g., UCE versus mitochondrial data). In the UCE tree, *Selenidera spectabilis* is sister to the rest of *Selenidera* and *Andigena* (Figure 3), whereas in the mitochondrial tree, both *Selenidera spectabilis* and *Selenidera piperivora* are sister to *Andigena* and this clade is sister to the rest of *Selenidera* (Figure 4). The quartet-based tree (Supplementary Material Figure S1) is in agreement with the UCE tree. The SNAPP analysis produced a reconciled tree with both genera as monophyletic, but indicates that there is some support for *Selenidera spectabilis* as sister to *Andigena* (Figure 5). The *Selenidera* + *Andigena* clade is sister to *Aulacorhynchus*. *Pteroglossus* is sister to the *Aulacorhynchus* + *Selenidera* + *A ndigena* clade and *Ramphastos* is sister to all other toucans.

Species- and Population-Level Relationships

The UCE maximum likelihood tree (Figure 3) and quartet-based tree (Supplementary Material Figure S1) were concordant at highly supported nodes. The quartet-based tree had fewer nodes with high support compared with the maximum likelihood tree. The RAxML mitochondrial (Figure 4) and UCE trees are generally concordant but differ slightly. For example, our maximum likelihood analysis of UCE data places *Pteroglossus bailloni* as sister to all other *Pteroglossus*, whereas the maximum likelihood analysis of the mitochondrial data places *Pteroglossus bailloni* as sister to the *Pteroglossus viridis* + *Pteroglossus inscriptus* clade. The *Pteroglossus viridis* + *Pteroglossus inscriptus* clade is sister to the remaining *Pteroglossus*. The remaining *Pteroglossus* can be divided into three subclades that are consistent in both analyses. The first contains *Pteroglossus torquatus* + *Pteroglossus frantzii*, the second contains *Pteroglossus aracari* + *Pteroglossus pluricinctus* + *Pteroglossus castanotis*, and the last contains *Pteroglossus azara*, *Pteroglossus bitorquatus*, and *Pteroglossus beauharnaesii*. However, how these subclades are related varies with analyses. In the UCE phylogeny *Pteroglossus torquatus* + *Pteroglossus frantzii* are sister to *Pteroglossus aracari* + *Pteroglossus pluricinctus* + *Pteroglossus castanotis* but in the mitochondrial tree *Pteroglossus torquatus* + *Pteroglossus frantzii* is sister to all remaining *Pteroglossus*. Mitochondrial and UCE subspecific topologies within these species differ throughout *Pteroglossus*.

For *Aulacorhynchus*, the trees generated from mitochondrial and nuclear data only differ in their placement of *Aulacorhynchus prasinus prasinus*. In the UCE tree, *Aulacorhynchus prasinus prasinus* is sister to the rest of the *Aulacorhynchus prasinus* + *Aulacorhynchus albivitta* clade, whereas in the mitochondrial tree, *Aulacorhynchus prasinus prasinus* is embedded within the clade as sister to *Aulacorhynchus prasinus caeruleogularis*.

All of the polytypic *Capito* species with subspecific sampling in our study are monophyletic. Overall, for *Capito* barbets, the UCE and mitochondrial trees are concordant with few exceptions. The only case of mitonuclear discordance was within the *Capito auratus* species. *Capito auratus punctatus* is placed within *Capito auratus nitidor* in the mitochondrial tree, but is sister to both *Capito auratus nitidor* individuals in the UCE tree. Most polytypic *Eubucco* species sampled with multiple subspecies are monophyletic with the exception of *Eubucco bourcierii*. *Eubucco bourcierii orientalis* is sister to *Eubucco versicolor* in the UCE tree and sister to all other *Eubucco bourcierii* in the mitochondrial tree. However, *Eubucco bourcierii* may be monophyletic, because the placement of *Eubucco bourcierii orientalis* is not strongly supported in either the UCE or mitochondrial phylogenetic trees.

At the subspecific scale, relationships among taxa within the *Ramphastos vitellinus* clade are in conflict in the mitochondrial and UCE trees. In the UCE tree, the Atlantic Forest *Ramphastos vitellinus ariel* and the Amazonian *Ramphastos vitellinus ariel* are sisters. However, in the mitochondrial tree, the Atlantic Forest *Ramphastos vitellinus ariel* is sister to all Amazonian *Ramphastos vitellinus* taxa and the Amazonian *Ramphastos vitellinus ariel* falls within a clade including all Amazonian *Ramphastos vitellinus* taxa. An analysis of SNP data for the *Ramphastos vitellinus* complex using DFOIL indicates that there was ancestral introgression from *Ramphastos vitellinus culminatus* and *Ramphastos vitellinus vitellinus* into the Amazonian *Ramphastos vitellinus ariel*.

DISCUSSION

Toucans, toucan-barbets, and New World barbets serve as model taxa for numerous studies, including the potential adaptive value of large toucan bills (Tattersall et al. 2009), reconstructions of Neotropical biogeographical history, endemism (Prum 1988a, Naka and Brumfield 2018, Bonaccorso and Guayasamin 2013, Haffer 1974), phylogeographic

FIGURE 3. RAxML phylogenetic reconstruction using UCE data and the GTR + G model. All nodes have full support except those noted.

history (Armenta et al. 2005), and even the evolution of mimicry in plumage coloration (Prum and Samuelson 2016). However, no study to date has sufficient taxonomic sampling to provide a complete species-level phylogeny for this group and published studies have either focused on species-level phylogenies within particular families or genera (e.g., Hackett and Lehn 1997, Weckstein 2005, Eberhard and Bermingham 2005, Puebla-Olivares et al. 2008, Patané et al.

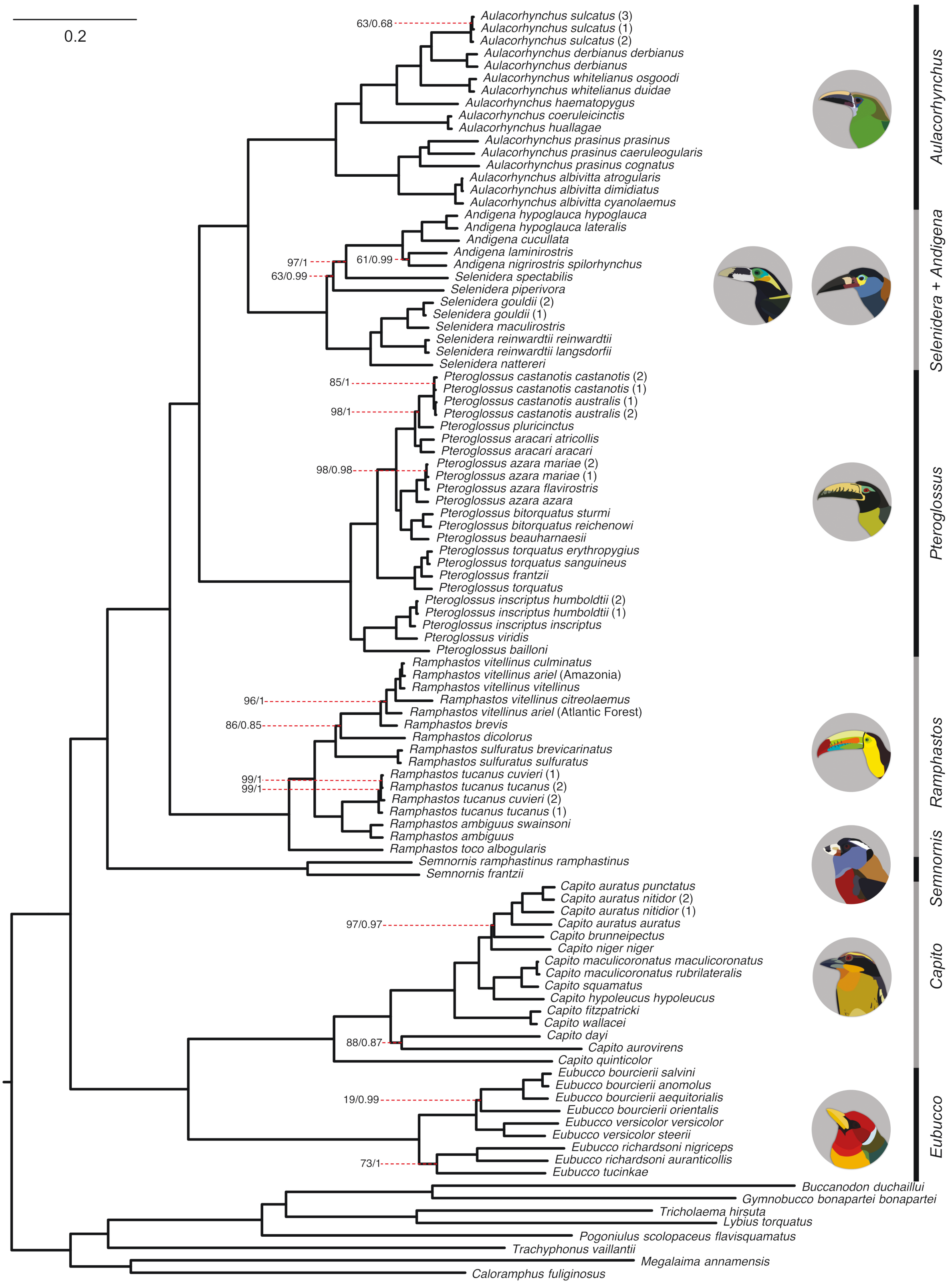

FIGURE 4. RAxML phylogenetic reconstruction using mitochondrial data and the GTR + G model. All nodes have full support in both maximum likelihood (bootstrap values) and Bayesian analyses (posterior probability values) except those noted. Support values are reported as maximum likelihood bootstrap support/Bayesian inference support.

2009, Bonaccorso et al. 2011, Patel et al. 2011, Lutz et al. 2013) or have included only representatives of each genus to reconstruct higher-level relationships (Lanyon and Zink 1987, Barker and Lanyon 2000, Moyle 2004). All published studies to date have also been limited in their genetic sampling, with the most recently published studies relying mostly

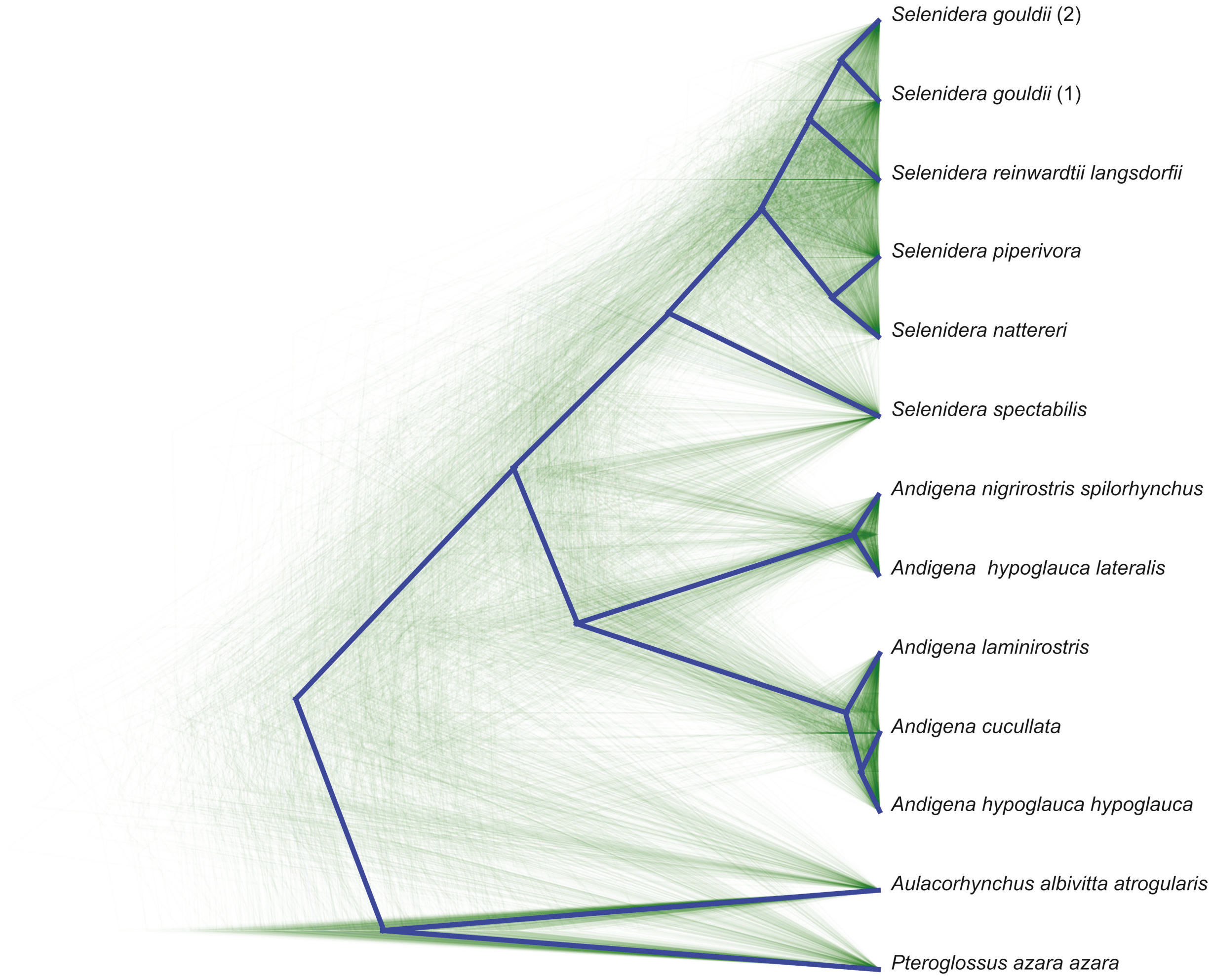

FIGURE 5. SNAPP tree showing the *Selenidera* + *Andigena* clade. The thick blue tree is the consensus tree.

on mitochondrial DNA sequence data collected with Sanger sequencing approaches (e.g., Barker and Lanyon 2000, Moyle 2004). Therefore, we focused on reconstructing a complete and robust species-level phylogeny based on phylogenomic data, including as much subspecific variation as possible, to test hypotheses about phylogenetic relationships at the level of family, genus, and species. Without a robust phylogeny for this broadly interesting group of birds we cannot properly assess the taxonomic limits within these families, genera, and species or conduct comparative phylogenetic and biogeographic analysis.

Higher-level and Generic Relationships

Aptly named, toucan-barbets have a mix of characters from both toucans and New World barbets. For example, toucan-barbets are more similar to New World barbets in body size, bill size and diet, but their calls are perhaps more similar to toucans than New World barbets (Winkler et al. 2020, e.g., xeno-canto *Semnornis frantzii* XC274431). However, Toucan-barbets also have unique morphological features in comparison to toucans and New World barbets, such as fewer and finer rictal bristles than most barbets, and the tip of their upper mandible fits into a notch in the lower mandible, making their phylogenetic affinities confusing.

In fact, previous phylogenetic studies have recovered all three of the possible topologies among these three families. For example, Prum (1988b) analyzed morphological data and found that toucan-barbets were sister to toucans. However, based on an analysis of mitochondrial DNA, Barker and Lanyon (2000) found that toucan-barbets were sister to a clade of toucans and New World barbets. Moyle (2004) had somewhat equivocal results depending on the analytical method used. However, all three of these studies (Moyle 2004, Barker and Lanyon 2000, Prum 1988b) found weak support for these basal relationships and were therefore unable to determine the phylogenetic relationships of toucan-barbets with respect to the toucans and New World barbets with certainty, which has bearing on understanding the evolution of characteristics such as body size, bill size and shape, and vocalizations. We found that toucan-barbets are sister to toucans and that New World barbets are sister to toucans + toucan-barbets. Given that the placement of these families was unclear in previous studies, our highly supported phylogenomic results help to clarify the evolutionary history of traits such as vocal characters and bill size. For example, our topology would support the hypothesis that large toucan bills evolved from small-billed barbet ancestors.

The generic relationships in this study most closely match Moyle (2004), who found that the New World barbet genera *Eubucco* and *Capito* form reciprocally monophyletic clades. Within toucans, *Ramphastos* is sister to all other toucan genera, *Pteroglossus* is sister to *Aulacorhynchus*, and *Andigena* + *Selenidera*, and *Aulacorhynchus* is sister to *Andigena* + *Selenidera* (Figure 1). The mitochondrial tree generated by Barker and Lanyon (2000) was also similar to our tree. However, our study found that *Pteroglossus* was sister to *Aulacorhynchus* and a clade containing *Selenidera* + *Andigena,* whereas Barker and Lanyon (2000) found *Aulacorhynchus* was sister to *Pteroglossus* and the *Selenidera* + *Andigena* clade. This result has bearing on understanding the evolutionary and biogeographic history of the montane genera *Aulacorhynchus* and *Andigena*. Haffer (1974) hypothesized that lowland and highland toucan taxa form reciprocally monophyletic groups, implying that the 2 montane genera *Aulacorhynchus* and *Andigena* were sister clades to the exclusion of all other toucans. If montane toucan genera were monophyletic then it would suggest a single historical biogeographic transition between montane and lowland regions, whereas non-monophyly would indicate a more complex biogeographic history of multiple transitions between lowland and highland areas. In this case, given that our data shows that *Andigena* is more closely related to *Selenidera* it suggests a more complex biogeographic history than a single transition between highland and lowland areas.

The Saffron Toucanet (*Pteroglossus bailloni*), an endemic of the Atlantic Forest, was historically considered a monotypic genus (*Baillonius bailloni*) allied with *Andigena* due to the unique morphological characteristics of this species, notably its unique yellow plumage compared to the overall green coloration of other taxa in the genus *Pteroglossus* (Short and Kirwin 2020b). Several studies, which were based on mitochondrial or allozyme data, found that *Baillonius* was either sister to all other *Pteroglossus* (Hackett and Lehn 1997) or found within *Pteroglossus* (Barker and Lanyon 2000, Kimura et al. 2004, Eberhard and Bermingham 2005, Periera and Wajntal 2008, and Patel et al. 2011). Kimura et al. (2004) argued that *Baillonius bailloni* should be subsumed into *Pteroglossus*. Our mitochondrial tree most closely resembled the previous studies using mitochondrial DNA, whereas analysis of UCEs placed *Pteroglossus bailloni* as sister to the rest of *Pteroglossus*.

Previous studies have indicated that *Selenidera* might be paraphyletic with respect to *Andigena* (Kimura et al. 2004, Lutz et al. 2013). Neither study found strong support for the paraphyly of *Selenidera* and Lutz et al. (2013) suggested that this problem needed to be addressed with additional data. Lutz et al. (2013) based their phylogenetic reconstructions on three mitochondrial genes and one nuclear intron, thus their tree topology was heavily influenced by the mitochondrial data and matched that of our mitochondrial tree (Figure 4) with *Selenidera spectabilis* sister to all *Andigena* and *Selenidera piperivora* sister to *Selenidera spectabilis* + *Andigena*. This entire clade is then sister to the rest of *Selenidera*. Kimura et al. (2004) had a similar topology but had less complete taxon sampling.

Selenidera piperivora and *Selenidera spectabilis* are unique in many ways but have characteristics of both *Selenidera* and *Andigena*. Although *Selenidera piperivora* and *Selenidera spectabilis* generally look like *Selenidera* they are vocally more similar to *Andigena* (Short and Horne 2001). In fact, female *Selenidera piperivora* have calls so similar to *Andigena*, that they have been observed responding to *Andigena laminirostris* songs (e.g., xeno-canto XC450798 for *Selenidera piperivora* and XC776750 for *Andigena laminirostris*, Short and Horne 2001). Also, *Selenidera piperivora* females have a gray belly that is similar in coloration to the blue–gray belly of the *Andigena* mountain-toucans (Short and Kirwin 2020a). In comparison to *Selenidera piperivora*, *Selenidera spectabilis* has fewer characteristics that are similar to *Andigena*, but both of these *Selenidera* species are sometimes grouped into a superspecies (Short and Kirwin 2020a). Based on our analysis of mitochondrial data, *Selenidera* is likely paraphyletic as found by Lutz et al. (2013). The UCE data suggest that *Selenidera piperivora* is sister to all *Selenidera* and *Andigena* together. Therefore, given that both UCE and mitochondrial trees indicate that *Selenidera* is paraphyletic with respect to *Andigena* we suggest that all taxa in this clade should be classified as *Selenidera*, which has historical taxonomic priority over *Andigena* (Gould 1837; Gould 1851). This would resolve the problem of paraphyly found in this study and by Lutz et al. (2013).

Species- and Population-Level Relationships and Mitonuclear Conflict

For all toucan, toucan-barbet and New World barbet genera excluding *Andigena* and *Selenidera*, most of the species-level relationships that we recovered through separate analyses of UCEs and mitochondrial sequences are similar. The SNAPP tree generated by analyzing UCE data indicates that there may be a signature of incomplete lineage sorting or gene flow between *Selenidera spectabilis* and *Andigena*. Where there are differences, the mitochondrial tree generally matches trees published in the literature with similar taxon sampling, (e.g., *Pteroglossus* and *Ramphastos*; Patané et al. 2009, Patel et al. 2011). This is not surprising because the majority of genes used in the analyses presented in previous studies are mitochondrial genes (e.g., Patané et al. 2009, Patel et al. 2011). Therefore, we believe it is important to discuss the conflict discovered among our mitochondrial and UCE datasets.

Ramphastos vitellinus subspecies have complicated dynamics including broad hybrid zones between Amazonian taxa. *Ramphastos vitellinus ariel* also has disjunct populations in southeast Amazonia and the Atlantic Forest (Haffer 1974). The *Ramphastos vitellinus* complex shows some mitonuclear discordance, a finding that is not unexpected given its biogeographical history. In the UCE trees, the two *Ramphastos vitellinus ariel* individuals are sisters, but in the mitochondrial tree southeast Amazonian *Ramphastos vitellinus ariel* is more closely related to other Amazonian *Ramphastos vitellinus* than it is to the Atlantic Forest *Ramphastos vitellinus ariel*. Haffer (1974) suggested that there might have been multiple bouts of interbreeding between Amazonian toucan populations during interglacial periods. Rivers may have acted as important barriers between *Ramphastos vitellinus* populations that maintained divergences between populations (Naka and Brumfield 2018). Our data provide support for historical introgression, as the DFOIL results show ancestral introgression from *Ramphastos vitellinus culminatus* and *Ramphastos vitellinus vitellinus* into southeast Amazonian *Ramphastos vitellinus ariel* (Table S1). Furthermore, the close mitochondrial phylogenetic affinities (with low mitochondrial divergence) among Amazonian *Ramphastos vitellinus ariel* and other Amazonian *Ramphastos vitellinus* taxa compared to the high mitochondrial divergence and non-sister relationship of Atlantic Forest *Ramphastos vitellinus ariel* are also consistent with mitochondrial capture due to introgression.

This is the first study to include nearly complete taxon sampling for all toucans, toucan-barbets, and New World barbets. We analyzed ~2,500 UCEs and all mitochondrial protein-coding genes to reconstruct phylogenies. The higher-level relationships within these trees were strongly supported and indicated that nearly all genera, except for *Selenidera*, were monophyletic. Our data suggest a complicated history for the genus *Selenidera* that may be more appropriately represented by models including reticulate evolution. We found that overall, our mitochondrial results tend to concur with previous molecular studies, but that the nuclear UCE data differ from the mitochondrial data and shed light on toucan, toucan-barbet, and New World barbet evolutionary history, such as revealing signatures of historical or ongoing gene flow.

Supplementary Material

Supplementary material is available at *Ornithology* online.

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Ethics statement

We declare no competing interests with this research.

Author contributions

J.D.W., J.M.B., A.A., and E.N.O. conceived of and designed the experiment. T.A.C. did lab work and collected data. E.N.O. and T.A.C. analyzed the data. E.N.O., T.A.C., and J.D.W. wrote and substantially edited the paper. J.D.W. and J.M.B. contributed funding.

Data availability

All data are available at NCBI https://www.ncbi.nlm.nih.gov/genbank/. The bioproject accession number is PRJNA894463.

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