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Systematics of *Lepidothrix* manakins (Aves: Passeriformes: Pipridae) using RADcap markers

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ABSTRACT

Although recent molecular phylogenetic analyses of *Lepidothrix* manakins (family Pipridae) have helped clarify their evolutionary relationships, the placement of several lineages remains in question because of low or conflicting branch support. In particular, the relationship of *L. coronata* to other members of the genus and relationships within the *L. nattereri* + *L. vilasboasi* + *L. iris* clade have been difficult to resolve. We used RADcap to collect restriction site-associated DNA sequence data and estimate the first subspecies-level phylogeny of the genus *Lepidothrix* (17 of 18 currently recognized subspecies), and we included extensive geographic representation of the widespread and phenotypically variable *L. coronata*. We found strong support for the phylogenetic position and monophyly of *L. coronata*, and we resolved two clades separated by the Andes that, along with previous divergence time estimates and our assessment of morphological and vocal evidence, suggest the presence of two biological species: Velvety Manakin (*L. velutina*) west of the Andes and Blue-capped Manakin (*L. coronata*) east of the Andes. Species-level relationships within the *L. nattereri* + *L. vilasboasi* + *L. iris* clade remained poorly resolved in concatenated and coalescent-based analyses, with SNAPP analyses suggesting that the lack of reciprocal monophyly is due to extensive allele sharing among these taxa. Finally, we confirmed a previously documented hybrid between *L. coronata* and *L. suavissima* as an F1 individual, consistent with the view that hybridization between these two species is a rare event and that postmating reproductive barriers prevent successful backcrossing.

1. Introduction

The genus *Lepidothrix* Bonaparte, 1854 comprises 8 species and 18 subspecies (Dickinson and Christidis, 2014; Remsen et al., 2021; Snow, 2004) distributed across the Amazon Basin, east slope of the Andes, tepuis and lowlands of the Guiana Shield, Pacific lowlands of western Colombia and Ecuador, and southern Central America (Kirwan and Green, 2011; Snow, 2004). Species within this genus are largely allopatric, but several come into contact in areas that include headwater regions (Weir et al., 2015), east slope foothills of the Andes (Ridgely and Tudor, 1994), and the Guiana Shield (Stotz, 1993). Hybridization has been recorded between multiple species in these contact areas, particularly within the clade that includes *L. nattereri*, *L. vilasboasi*, and *L. iris* (Barrera-Guzmán et al., 2018; Dias et al., 2018; Weir et al., 2015) as well as a single record of a *L. coronata* × *L. suavissima* hybrid from northern Brazil (Stotz, 1993). At the intraspecific level, a contact zone between black-plumaged *L. coronata coronata* (to the north) and green-plumaged *L. c. caelestipileata* (to the south) extends for over 1500 km from Ucayali, Peru, northeast through Acre and Amazonas, Brazil, to the Madeira River (Haffer, 1970), with series of putative hybrids documented in eastern Peru along the lower Urubamba River (Moncrieff et al., 2020) and in Brazil along the BR-319 highway west of the lower Madeira River (de Abreu et al., 2018).

After Prum (1992) resurrected the genus *Lepidothrix*, molecular phylogenetic studies confirmed monophyly of the group and its sister relationship to *Cryptopipo* (Ohlson et al., 2013; Rêgo et al., 2007; Tello et al., 2009). Subsequent phylogenetic studies with species-level sampling for *Lepidothrix* have generally produced concordant trees, although the placement of *L. coronata* remains a point of uncertainty (Harvey et al., 2020; Leite et al., 2021). Relationships within the *L. nattereri* + *L. vilasboasi* + *L. iris* clade have also been difficult to resolve, with recent work proposing *L. vilasboasi* as a hybrid species (Barrera-Guzmán et al., 2018) and reporting ongoing introgression between *L. nattereri* and *L. iris* (Dias et al., 2018; Weir et al., 2015). Phylogeographic studies of *L. coronata* have shown several well-differentiated clades (Cheviron et al., 2005; Reis et al., 2020), but these studies have also highlighted the

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need for denser geographic sampling of and genome-scale data from *L. coronata* to better assess the relationship of these intraspecific clades to each other and to other taxa within the genus. An improved phylogenetic hypothesis for the genus will help address these lingering uncertainties

Further phylogenetic work will also facilitate studies of trait evolution in *Lepidothrix*. Plumage traits, in particular, vary strikingly across the genus, with males showing different combinations of bright plumage patches on the crown, rump, and belly (Igic et al., 2016), as well as presence or absence of "super black" plumage (McCoy and Prum, 2019) that may serve to emphasize bright plumage patches during lek displays for plain greenish females (Durães, 2009; Kirwan and Green, 2011; Snow, 2004). The evolutionary significance of these plumage traits is highlighted by recent work showing that rapid changes in crown color via hybridization and sexual selection may have promoted the speciation of *L. vilasboasi* (Barrera-Guzmán et al., 2018). These findings suggest that studying the evolution of bright plumage patches, aided by a robust phylogenetic framework, will be important for understanding speciation across the genus.

Here, we estimate the phylogeny of the genus *Lepidothrix* using samples collected from 17 of the 18 subspecies in the group (Fig. 1). We did not sample the subspecies *L. iris iris* of Brazilian Amazonia east of the Xingú River (Kirwan and Green, 2011). The phylogeny we infer is based on genetic data collected using RADcap (Hoffberg et al., 2016), a variant of restriction-site associated DNA sequencing (RAD-seq; Baird et al., 2008) that combines the benefits of sequence capture (e.g., consistent recovery of target loci, especially useful with historical samples) with those of RAD-seq (e.g., lower cost and efficient library preparation). In addition to inferring a phylogenetic hypothesis for the group, we demonstrate the utility of RADcap for phylogenetic studies, compare tree-building methods and data filtering schemes, and discuss the taxonomic implications of our results for the *L. coronata* species group. Finally, we use the data we collected to test the ancestry of a putative manakin hybrid, *L. coronata* × *L. suavissima*, identified by its intermediate plumage (Stotz 1993) and not included in prior genetic analyses.

2. Materials and methods

2.1. Study design and sample collection

This study included 78 tissue and four toepad samples of *Lepidothrix* manakins, chosen to represent the biogeographic and taxonomic diversity within the genus, as well as two tissues of *Cryptopipo holochlora*, which we used as an outgroup (Fig. 1; Table S1). We obtained samples

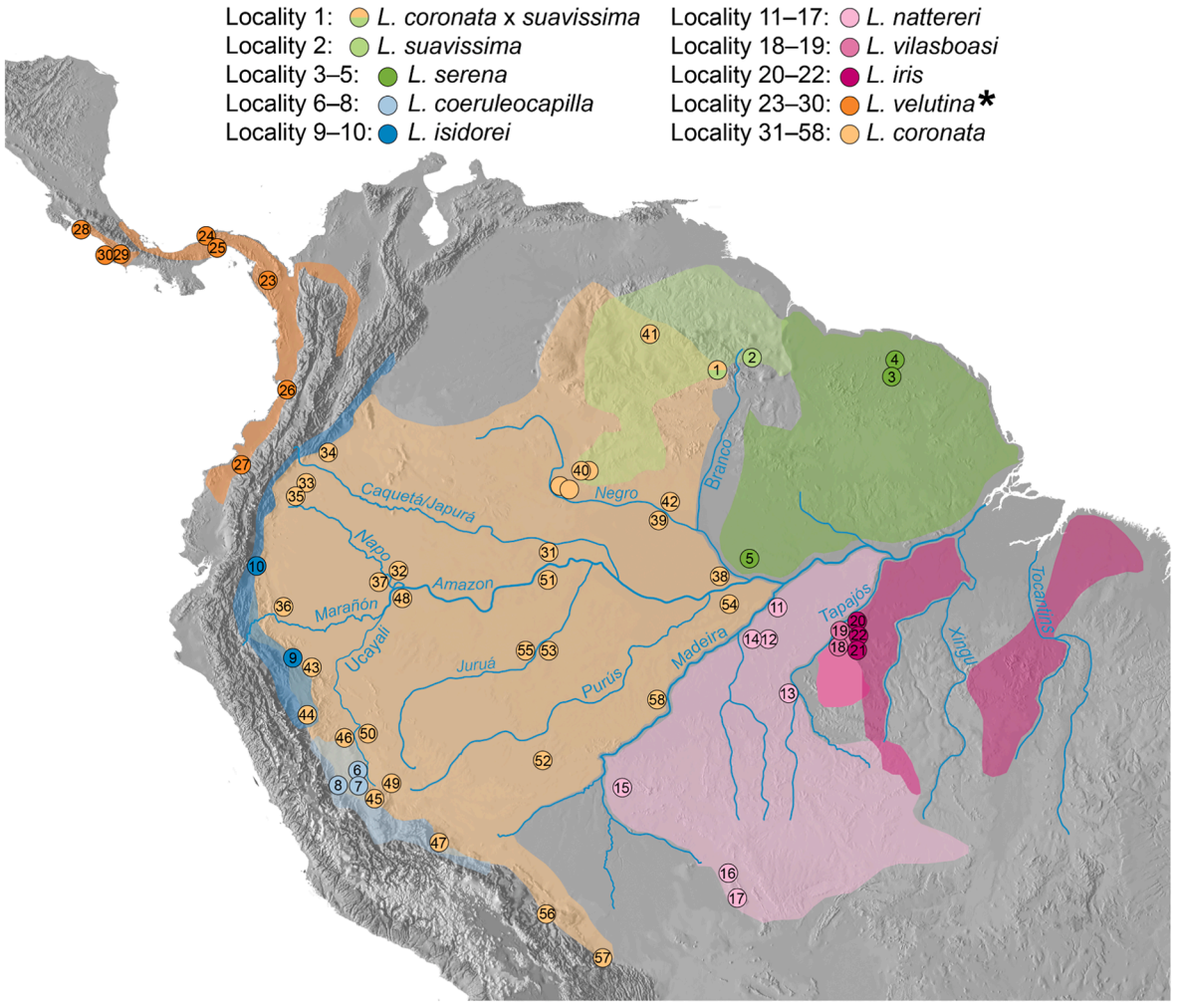

Fig. 1. Map of 58 sampling localities of *Lepidothrix* manakins used in phylogenetic analyses. Locality numbers correspond to numerals at the end of tip labels in Fig. 2. Samples of *L. coronata* from an additional three localities (unnumbered circles centered around locality 40) were used to test the ancestry of a *L. coronata* × *L. suavissima* hybrid. Geographic distributions of each species illustrated in consultation with published sources (Dickinson and Christidis, 2014; Hilty, 2021; Kirwan and Green, 2011; Schulenberg et al., 2010), citizen science records (eBird) with photographs, and museum specimens (LSUMNS and Marco Rego pers. comm.). The asterisk highlights our use of the new name we recommend (*L. velutina*) for populations of *L. coronata* (s.l.) from west of the Andes (see Section 4.5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from museum collections and from AEM's fieldwork in Peru during 2015–2019 (Moncrieff et al., 2020; see Acknowledgements for permit information). Tissues consisted of pectoral muscle preserved in ~ 95% ethanol or flash-frozen in liquid nitrogen.

2.2. DNA extraction, library preparation, enrichments, and sequencing

We extracted total DNA from tissues using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol, and we extracted total DNA from toepads using a phenol–chloroform protocol (Tsai et al., 2020). We quantified DNA extracts using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA) and ran all samples on agarose gels to check fragment length distributions. DNA fragments were shorter than expected in two tissues (ANSP 17140 and 20018), suggesting DNA degradation.

To initiate the RADcap workflow (Hoffberg et al., 2016), we designed and synthesized custom baits targeting 2495 putatively non-coding loci that were separated by > 75 kb across the *Lepidothrix coronata* genome (Supplemental Methods). We then prepared dual-digest RADseq (3RAD; Bayona-Vásquez et al., 2019) libraries using DNA extracted from all tissue samples except for the two extracts of low quality. After ligating unique inner indexes to identify each sample, we combined libraries into pools of 8 samples (with 5 samples in the final pool) that we amplified with a single cycle of PCR and a random octamer iTru5-8N primer to facilitate detection of PCR duplicates (Hoffberg et al., 2016). We then amplified pools with an additional 10–12 cycles of PCR, adding a unique, outer iTru7 index to identify each pool of libraries. Because the DNA extracts from toepads and the two degraded tissue samples were too short for restriction enzyme digestions, we prepared dual-indexed genomic libraries from these extracts using a KAPA HyperPrep library preparation kit (F. Hoffmann-La Roche AG, Basel, Switzerland) and iTru indexes (Glenn et al., 2019). We amplified each library with 11–14 PCR cycles before combining them into pools of 8 samples.

We then performed targeted enrichment of RADcap loci from each group of pooled samples using the custom baits we synthesized and following the manufacturer's manual v4.01 (myBaits Custom Kit; Daicel Arbor Biosciences, Ann Arbor, Michigan, USA). We substituted half the volume of Block C from the myBaits Kit with Chicken Hybloc™ DNA (Applied Genetics Laboratories, Melbourne, Florida, USA) to reduce the amount of non-specific DNA captured. After enrichment, we amplified pools with 10–13 cycles of PCR to recover targeted loci, and we cleaned PCR reactions using a GeneRead Size Selection Kit (Qiagen, Hilden, Germany). We ran 5 ng amplified, post-enrichment pools on a Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) to check fragment length distributions and ensure absence of adapter dimers, and we used a KAPA Library Quantification Kit (F. Hoffmann-La Roche AG) and a QuantStudio-6 (Applied Biosystems, Foster City, California, USA) to perform quantitative real-time PCR on enriched pools. We then combined enriched pools at equimolar ratios prior to sequencing, except that we included slightly more product (25%) for pools enriched from degraded sources of DNA. We collected sequence data from pooled samples, including samples for other projects, across three lanes of paired-end 150 bp sequencing on an Illumina HiSeq X (Novogene Corporation Inc., Sacramento, CA, USA), targeting 1.5 million read pairs for each RADseq library and 2 million read pairs for each genomic library. Libraries in each sequencing lane had non-overlapping index sequences.

2.3. Initial sequence data processing

We received FASTQ files from the sequencing center and demultiplexed the data according to library type. For RADseq libraries, we initially demultiplexed pools of libraries based on the outer iTru7 index using BBMap *demuxbyname.sh* (Bushnell, 2014) and subsequently demultiplexed individual libraries within each pool by inputting inner indexes to Stacks *process_radtags* (Catchen et al., 2013). We then ran Stacks *clone_filter* to remove PCR duplicates. For genomic libraries, we demultiplexed individual libraries with BBMap *demuxbyname.sh*, and we trimmed raw reads with Trimmomatic (Bolger et al., 2014). For all samples, we then produced BAM alignments to the reference *Lepidothrix coronata*-1.0 genome (NCBI RefSeq assembly accession GCF_001604755.1) using BWA *mem* (Li and Durbin, 2009) and SAMtools *view* (Li et al., 2009). For genomic libraries, we removed duplicates from BAM files using GATK 4.1.9 *MarkDuplicatesSpark* (Van der Auwera and O'Connor, 2020). Using SAMtools *view*, we divided the number of unique reads overlapping target capture regions specified by a BED file (-L -c -F 2432) by the number of unique reads (-c -F 2432) to calculate the percentage of on-target reads for toepad and tissue libraries.

Next, we performed one round of base quality score recalibration (BQSR), processing samples by the sequencing lane in which they were run. To identify "high-quality" variants for BQSR, we called sites in a population of libraries that included all individuals that were part of this project (n = 84) as well as 747 additional manakins that were part of different research projects. Specifically, we used GATK *HaplotypeCaller*, *GenomicsDBImport*, and *GenotypeGVCFs* to call variants, and we identified "high-quality" variants for BQSR using VCFtools 0.1.16 (Danecek et al., 2011) where we defined "high-quality" as having a minimum depth of 30X (–minDP 30), a minimum site quality > 30 (–minQ 30), a minimum genotype quality of 30 (–minGQ), a maximum of two alleles (–max-alleles 2), no indels (–remove-indels), and representation in at least 50% of all individuals (–max-missing 0.5). We recalibrated BAM files for all libraries by running GATK *BaseRecalibrator* and *ApplyBQSR* with the "high-quality" sites, and then we performed joint calling of variant sites across the population of libraries with GATK *HaplotypeCaller*, *GenomicsDBImport*, and *GenotypeGVCFs*. We used VCFtools (Danecek et al., 2011) to remove sites outside of the 2495 genomic intervals targeted by our custom RADcap baits, individuals with mean on-target depth < 10X, low quality sites (–minQ 30), indels (–remove-indels), and sites that were not biallelic (–min-alleles 2 –max-alleles 2).

2.4. Datasets for analysis

We applied additional filters to the resulting cleaned VCF file to produce nine subsets of individuals for phylogenetic analyses (total 67 individuals; Tables S2–S3) and one subset of individuals for analyses of the hybrid (total 24 individuals). The total number of unique individuals across all datasets was 84 (Table S1).

For estimation of the genus-level phylogeny, we were interested in examining the effects of site occupancy and differences among data subsets that included or excluded sites in linkage disequilibrium. So, we first extracted a set of individuals spanning the genus: 65 *Lepidothrix* manakins and two *Cryptopipo holochlora*, which served as an outgroup. We ran a second round of filtering to remove sites in this subset of individuals having a depth < 15X (–minDP 15) and a minor allele count < 3 (–mac 3; Linck and Battey, 2019). We then produced two data sets having a site occupancy of ≥ 75% (–max-missing 0.75) or ≥ 95% (–max-missing 0.95) which we refer to as "linked" VCF datasets because multiple SNPs were allowed within a single target RADcap locus (120 bp). We also prepared "unlinked" versions of these 75% and 95% complete datasets by using VCFtools to thin sites < 75 kb apart (–thin 75000; equivalent to keeping one SNP per RADcap locus). These treatments resulted in a total of four VCF files (Tables S2–S3).

We were interested in further investigating relationships among members of the *L. nattereri* + *L. vilasboasi* + *L. iris* clade because these relationships have previously been difficult to resolve. To maximize the number of clade-specific SNPs shared among members of this group, we prepared four additional VCF files (Tables S2–S3) modeled on the filtering scheme above, except that these files included only a 15-individual subset of taxa: 7 *L. nattereri*, 3 *L. vilasboasi*, 3 *L. iris*, and 2 *L. coeruleocapilla* as an outgroup (Table S1). Because we wanted to infer relationships and visualize estimates of allele sharing among members of this group using SNAPP (Bryant et al., 2012) and because SNAPP expects

complete matrices of SNPs that are not in linkage disequilibrium, we modified the clade-specific unlinked data set by dropping *L. coeruleocapilla* and ensuring there were no missing data (–max-missing 1).

For analyses to test the ancestry of the *Lepidothrix coronata* × *L. suavissima* hybrid from Roraima, Brazil (Stotz, 1993), we filtered the cleaned VCF file to include only the hybrid and 23 individuals representing the surrounding, parental populations: 18 *L. coronata carbonata* from northwestern Brazil and Venezuela and five *L. suavissima* from Guyana (Table S1). We then implemented the "unlinked" filtering approach described above to produce a VCF file with a site occupancy of ≥ 75% (–max-missing 0.75). We included an additional 17 individuals for the hybrid analyses that were not among the 67 used in phylogenetic analyses because these individuals were helpful for identifying fixed SNPs between *L. coronata* and *L. suavissima* populations (procedure described in Section 2.6) while they duplicated localities already represented in our phylogenetic analyses.

After preparing the VCF files for phylogenetic analyses, we produced consensus alignments in PHYLIP format using vcf2phylip v2.6 (Ortiz, 2019) for concatenated analyses with IQ-TREE 2.1.3 (Minh et al., 2020), and we removed ambiguously constant sites (see Minh et al., 2021) with variants coded by IUPAC ambiguity codes (Martin, 2018) because these sites must be removed to apply the IQ-TREE ascertainment bias correction model. We also used vcf2phylip to produce consensus alignments in NEXUS format for analyses with SVDquartets (Chifman and Kubatko, 2014) and SNAPP (Bryant et al., 2012).

2.5. Phylogenetic analyses

We used IQ-TREE 2.1.3 to select the best-fit substitution model (Kalyaanamoorthy et al., 2017) and infer maximum likelihood trees for each of eight PHYLIP-formatted alignments (Table S2) using ascertainment bias correction (-m MFP + ASC; Lewis, 2001), and we inferred branch support for each tree with 500 standard nonparametric bootstrap replicates (Felsenstein, 1985). We also inferred a coalescent-based phylogeny from the four, unlinked NEXUS files (Table S3) using SVDQuartets (Chifman and Kubatko, 2014), an efficient coalescent method designed for unlinked SNP data implemented in PAUP* 4.0a168 (Swofford, 2003). In SVDQuartets, we evaluated all quartets and performed 500 replicates of standard bootstrapping to obtain branch support. For all trees from concatenated and coalescent-based approaches, we collapsed branches with bootstrap support < 70 (Hillis and Bull, 1993) using ape v5.5 (Paradis and Schliep, 2019).

To infer relationships among members of the *L. nattereri* + *L. vilasboasi* + *L. iris* clade, we estimated an additional species tree with the coalescent method SNAPP v1.5.2 (Bryant et al., 2012) implemented in Beast v2.6.6 (Bouckaert et al., 2019). To infer the tree, we input the NEXUS file described above to BEAUTi v.2.6.6, assigned individuals to populations (based on subspecies), designated the presence of polymorphic-only sites, and set the MCMC chain length to 2,000,000 iterations with 10% burn-in. We left remaining settings at their defaults and exported an XML file, which we used to run six independent MCMC chains in SNAPP. We combined the resulting log files with LogCombiner v2.6.6 (Bouckaert et al., 2019) and checked for the convergence of parameters (effective sample size values > 1000) in Tracer v1.7.2 (Rambaut et al., 2018). After checking for convergence, we combined the trees files with LogCombiner, obtained a maximum clade credibility tree with TreeAnnotator v.2.6.6 (Drummond and Rambaut, 2007), and visualized the posterior distribution of trees with DensiTree v.2.2.7 (Bouckaert and Heled, 2014).

2.6. Testing ancestry of a rare hybrid

To examine the ancestry of the male *Lepidothrix coronata* × *L. suavissima* hybrid collected by Stotz (1993), we used SNMF 2.0 (Frichot et al., 2014) to estimate admixture coefficients for the hybrid and individuals from the surrounding, parental populations of *L. coronata carbonata* and *L. suavissima*. We then used a custom Python script to filter the VCF file input to SNMF and retain SNPs that were fixed between the two parental species. We used PGDSpider (Lischer and Excoffier, 2012) to convert this file of fixed SNPs to STRUCTURE format, and we used INTROGRESS (Gompert and Buerkle, 2010) to plot SNMF admixture coefficients relative to interspecific heterozygosity for all individuals at the fixed SNPs.

3. Results

3.1. Sequence data

Illumina sequencing resulted in an average of 1.7 million read pairs/sample (range = 333,258–7,344,948) for the 80 tissue samples and 7.2 million read pairs/sample (range = 6,118,112–8,225,412) for the 4 toepad samples (Table S1). After removal of PCR duplicates, we retained an average of 1.4 million read pairs/sample (range = 297,655–3,211,514; 85% reads retained) for the 80 tissue samples and 2.2 million read pairs/sample (range = 1,299,567–3,580,516; 30% reads retained) for the 4 toepad samples (Table S1). The percentage of unique, on-target reads was 51% for tissues and 11% for toepads.

3.2. Phylogenetics

Genus-wide phylogenetic trees inferred by IQ-TREE (Fig. 2; Fig. S1–S4) and SVDquartets (Fig. S5–S6) based on 1546–6815 SNPs (Tables S2–S3) showed consistent support for high-level relationships reported in previous studies (Harvey et al., 2020; Leite et al., 2021; Ohlson et al., 2013), including *L. serena* + *L. suavissima*, *L. coeruleocapilla* + *L. isidorei*, and *L. nattereri* + *L. vilasboasi* + *L. iris* clades. We found that the *L. serena* + *L. suavissima* clade was the first to diverge within the genus across all trees (Fig. S1–S6). The *L. coeruleocapilla* + *L. isidorei* clade was consistently recovered as sister to the *L. nattereri* + *L. vilasboasi* + *L. iris* clade, although this relationship was collapsed to a polytomy in the tree inferred by IQ-TREE based on the "unlinked" dataset with 95% site completeness (Fig. S4). These two clades in turn were recovered as sister to the *L. coronata* clade in all genus-wide trees except, again, for the tree inferred by IQ-TREE based on the "unlinked" dataset with 95% site completeness (Fig. S4) in which the relationships among the three clades were represented by a polytomy.

Species-level relationships, except for those in the *L. nattereri* + *L. vilasboasi* + *L. iris* clade, were also generally well-resolved and consistent among phylogenetic approaches. Neither IQ-TREE nor SVDquartets were able to consistently recover reciprocally monophyletic species within the *L. nattereri* + *L. vilasboasi* + *L. iris* clade across filtering schemes in the genus-wide (Fig. S1–S6) or clade-specific datasets (Fig. S7–S12). There was also low support for relationships in the SNAPP maximum clade credibility tree (Fig. 3A) based on 990 SNPs (Table S3). The posterior distribution of SNAPP trees suggested that extensive allele sharing among taxa provides one explanation for the lack of resolution within this clade (Fig. 3B). The relationship between *L. serena* and *L. suavissima* was also poorly resolved in the IQ-TREE "unlinked" and SVDquartets trees (Fig. S3–S6), although reciprocal monophyly was strongly supported in the IQ-TREE "linked" trees (Fig. S1–S2), which included more sites by allowing multiple SNPs on the same RADcap locus.

At the intraspecific level, we observed a similar pattern of greater resolution in the IQ-TREE "linked" trees (Fig. 2; Fig. S1–S2) compared to the IQ-TREE or SVDquartets "unlinked" trees (Fig. S3–S6). Across all data sets, we recovered *L. coronata* as monophyletic with strong support. We also observed a split between west-of-Andes (*L. coronata velutina* and *L. c. minuscula*) and Amazonian populations (*L. c. caquetae*, *L. c. carbonata*, *L. c. coronata*, *L. exquisita*, *L. c. caelestipileata*, *L. c. regalis*) that was strongly supported by all trees, except the 95% complete "unlinked" IQ-

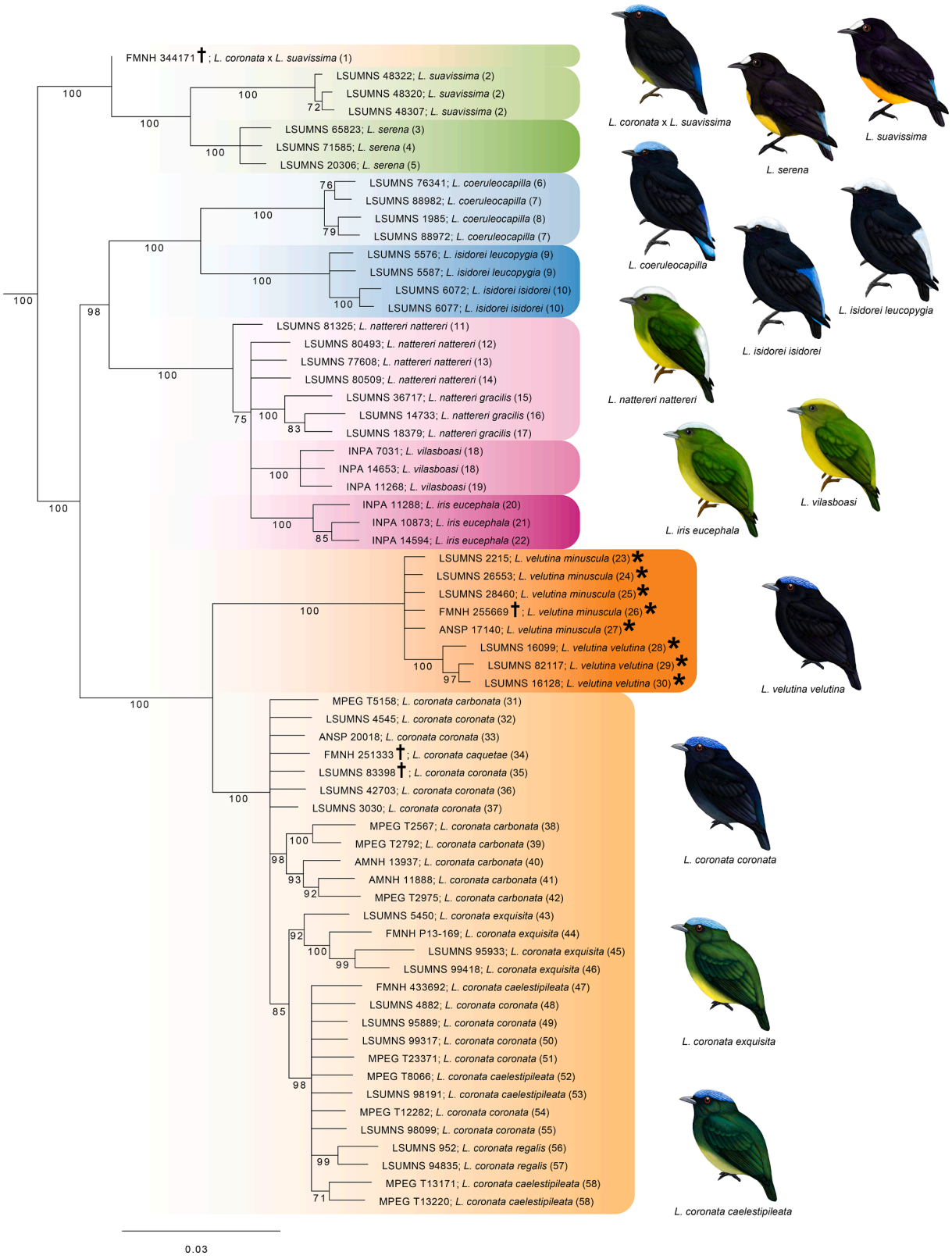

Fig. 2. Phylogeny of the genus *Lepidothrix* estimated using IQ-TREE based on the "linked", 75% complete dataset that included 5025 parsimony-informative SNPs. The *Cryptopipo holochlora* outgroup is not shown. Branch support reflects standard nonparametric bootstrap values, and all branches with a value < 70 are collapsed to a polytomy. Tip labels denote the sample source (ANSP: Academy of Natural Sciences of Drexel University; FMNH: Field Museum of Natural History; INPA: Instituto Nacional de Pesquisas da Amazônia; LSUMNS: Louisiana State University Museum of Natural Science; MPEG: Museu Paraense Emílio Goeldi), tissue number (or specimen catalog number in the case of four toepads), taxon name, and sampling locality, where numbers in parentheses correspond to localities in Fig. 1. Specimen catalog numbers for toepad samples are indicated with a cross, and asterisks at the end of tip labels highlight our use of the new names we recommend for *Lepidothrix coronata velutina* and *L. c. minuscula* (see Section 4.5).

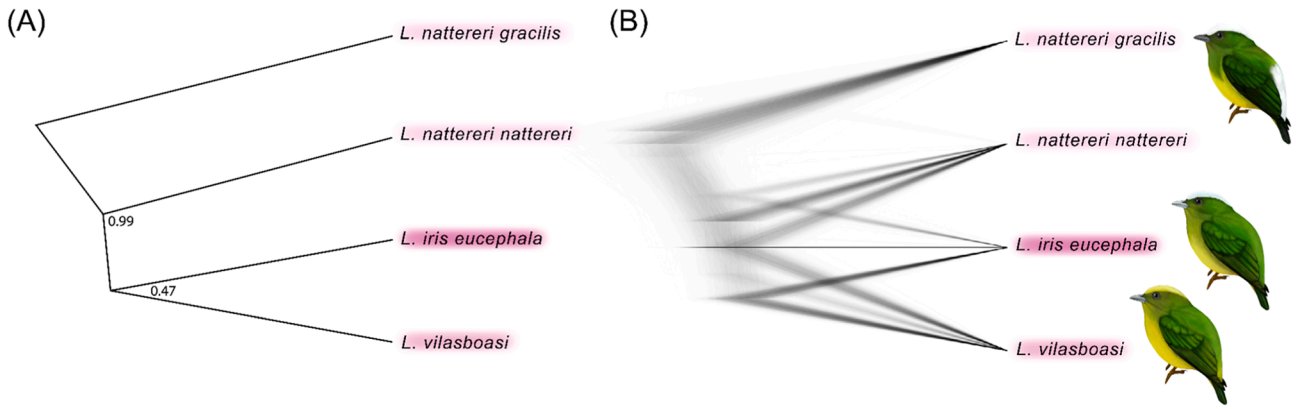

Fig. 3. Phylogenetic relationships of taxa in the *L. nattereri* + *L. vilasboasi* + *L. iris* clade estimated with SNAPP based on 990 SNPs. Males of *L. nattereri* show similar plumage in both subspecies. Label colors reflect those used in Figs. 1 and 2. A) Maximum clade credibility tree with posterior probabilities at nodes. B) Posterior distribution of trees showing alternative topologies.

TREE and SVDquartets trees (Fig. S4 and S6), which were based on the fewest SNPs of our genus-wide phylogenetic analyses (Tables S2–S3). Notably, trees with interspecific relationships that were poorly resolved (e.g., 75% complete "unlinked" IQ-TREE and SVDquartets trees: Fig. S3 and S5), retained strong support for this cross-Andes divergence.

Within Amazonian *L. coronata* we observed some support for a south-of-Amazon clade containing populations west (*L. c. exquisita*; Fig. 2, localities 43–46) and east of the Ucayali (*L. c. coronata* and *L. c. caelestipileata*; localities 47–58) as well as a north-of-Japurá clade (localities 38–42) although sample FMNH 251333 (locality 34) from the Japurá headwaters was not resolved within this clade (Fig. 2, Fig. S1–S2).

3.3. Ancestry of a rare hybrid

The SNMF analysis based on 1786 SNPs estimated an intermediate admixture coefficient for the *Lepidothrix coronata* × *L. suavissima* hybrid (Fig. 4A), while other individuals representing each population of parental species showed little evidence of hybridization. We identified 228 SNPs that were fixed between *L. coronata carbonata* and *L. suavissima*, and we found that 222 of these SNPs were heterozygous in the hybrid individual, resulting in a value of interspecific heterozygosity (0.97) only expected for F1 hybrids (Fig. 4B; Fitzpatrick, 2012). The six SNPs that were not heterozygous in the hybrid may result from loci that are not fully fixed between parental populations (despite being fixed in our sample of each population).

4. Discussion

4.1. Phylogenetics

Sequence capture of SNPs using RADcap (Hoffberg et al., 2016) collected sufficient phylogenetic information from thousands of loci to clarify historically difficult relationships within the genus *Lepidothrix*. Our phylogenetic results were generally well supported among SNP-filtering schemes and analytical paradigms at the species level, with a prominent exception being the relationships we inferred within the *L. nattereri* + *L. vilasboasi* + *L. iris* clade. At the population level, we identified several groups of taxa that reflected geography, including a consistent split between west-of-Andes and Amazonian populations of *L. coronata*, a pattern previously reported for this species group

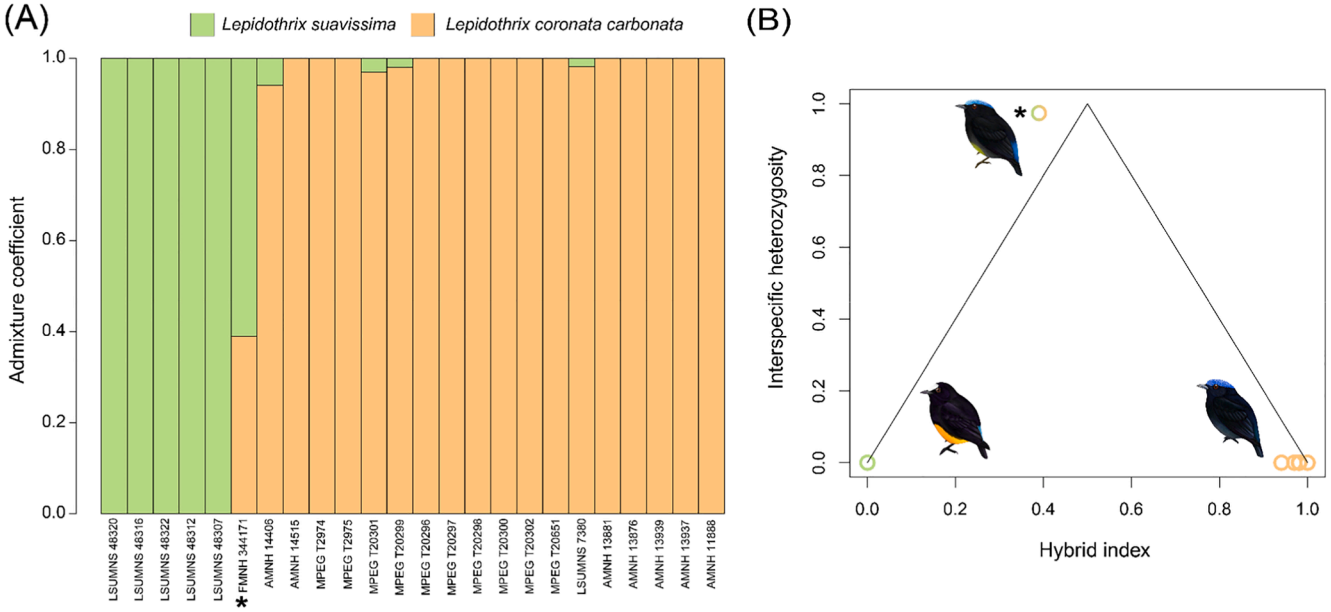

Fig. 4. Population genetic results for the ancestry of a previously documented *L. coronata* × *L. suavissima* hybrid, which is indicated by an asterisk in both panels. A) SNMF admixture coefficients showing an intermediate value for the *L. coronata* × *L. suavissima* hybrid (FMNH 344171). B) Triangle plot of interspecific heterozygosity and hybrid index showing the hybrid individual located near the apex of the triangle, the predicted location for F1 individuals.

(Cheviron et al., 2005; Reis et al., 2020) and for other widespread species groups found in lowlands on both sides of the Andes (d'Horta et al. 2013; Harvey and Brumfield, 2015; Milá et al., 2009). However, fully resolving geographic patterns among all the populations we sampled remained difficult. In most cases, further exploration of intraspecific relationships will depend on additional sampling and the use of population genetic approaches (e.g., Frichot et al., 2014; Pritchard et al., 2000) in place of phylogenetic approaches.

4.2. *Lepidothrix coronata* clade

We sampled across the genus *Lepidothrix* and particularly within the phenotypically variable and widespread *L. coronata* to assess the monophyly of *L. coronata* and its relationship to other taxa in the genus. We found that the 8 currently recognized subspecies of *L. coronata* (Dickinson and Christidis, 2014) formed a well-supported clade (Fig. 2; Fig. S1–S6). The placement of the *L. coronata* clade relative to other species in the genus was also consistent across data sets and methods, in contrast to conflicting or poorly supported results in recent analyses (Harvey et al., 2020; Leite et al., 2021; Ohlson et al., 2013). Unlike any result in our study, an ExaML tree estimated by Harvey et al. (2020) based on ultraconserved elements (UCEs) recovered *L. coronata* as sister to the *L. nattereri* + *L. vilasboasi* + *L. iris* clade but with low support. Leite et al. (2021) recovered this same relationship with UCEs in an ASTRAL species tree; however, using UCEs for concatenated trees (RAxML) and a different species tree (SVDquartets), they recovered a topology in agreement with that identified in our study. A tree based on mitochondrial ND2 and three nuclear introns estimated by Ohlson et al. (2013) was also consistent with our results but had low support. We consider our result of *L. coronata* as sister to the 5-species clade of *L. coeruleocapilla*, *L. isidorei*, *L. nattereri*, *L. vilasboasi*, and *L. iris*, to be robust given its consistency across analyses and agreement with the RAxML and SVDquartets trees estimated by Leite et al. (2021).

Researchers have repeatedly emphasized the need for further work to clarify systematics of *L. coronata* (Cheviron et al., 2005; Kirwan and Green, 2011; Snow, 2004) and have suggested that as many as 3 species-level clades, largely based on variation in male plumage coloration, may exist (Kirwan and Green, 2011; Ridgway, 1907): one clade of black-plumaged populations west of the Andes (*L. c. velutina* and *L. c. minuscula*), another of blackish-plumaged Amazonian populations (*L. c. coronata*, *L. c. caquetae*, *L. c. carbonata*), and a third of green-plumaged Amazonian populations (*L. exquisita*, *L. caelestipileata*, *L. regalis*). We found strong support for two major clades within *L. coronata*: one west of the Andes and another in Amazonia. This finding is consistent with most previous molecular phylogenies (Cheviron et al., 2005; Reis et al., 2020), but contrasts with one based on the mitochondrial cytochrome *b* gene that suggests west-of-Andes populations are sister to populations in Venezuela and northern Brazil (Smith et al. 2014). Within the Amazonian clade, the blackish-plumaged and green-plumaged populations were not reciprocally monophyletic and, consistent with previous work (Cheviron et al., 2005; Reis et al., 2020), were more closely related to nearby populations and those within the same interfluves, irrespective of male plumage color.

Although not resolved in every analysis, we observed some intra-Amazonian genetic structure in the concatenated phylogenies inferred from "linked" datasets (Fig. 2; Fig. S1–S2). We recovered a clade involving samples from north of the Japurá River (localities 38–42) that corresponded to clades identified in previous phylogeographic studies ("Venezuela clade" in Cheviron et al. 2005; clade "A" in Reis et al. 2020), but we were unable to resolve the relationship of this clade to other Amazonian clades. We also recovered a south-of-Amazon clade composed of two subclades separated by the Ucayali River (Fig. 2). Previous studies (Cheviron et al. 2005; Reis et al. 2020), in contrast, did not identify the Ucayali River as an important biogeographic barrier but rather suggested the presence of two south-of-Amazon clades that meet somewhere east of the Ucayali River. The clade we identified east of the Ucayali River involves black-plumaged (*L. c. coronata*) and green-plumaged populations (*L. c. caelestipileata* and *L. c. regalis*) that form a contact zone extending from central Peru northeast to the Madeira River (Haffer, 1970). Intergradation between these plumage forms has been documented east of the Ucayali River in Peru along a 130-km north/south transect in the southern Ucayali and northern Cuzco regions (Fig. S13; Moncrieff et al., 2020). Intergradation between *L. c. coronata* and *L. c. caelestipileata* has also been documented along the BR-319 highway in Brazil west of the lower Madeira River (de Abreu et al., 2018). Interestingly, these blackish and green plumage forms are found on opposite banks of the middle Juruá River with no sign of plumage intergradation (Del-Rio et al., 2021). Thus, the middle Juruá River seems to be an effective barrier for loci associated with plumage differences, even though we did not observe an association between this geographic boundary and our reduced-representation genetic data.

The clade we resolved west of the Ucayali River in Peru, corresponding to *L. c. exquisita*, has populations which extend south from the Marañón River to at least the left bank of the Tambo River in the Junín Region (AEM pers. observ.). Individuals in this clade are typically characterized by their bright green body plumage, bright yellow belly, and sky-blue crown (Fig. S14; Hellmayr, 1905). However, plumage is variable west of the Ucayali River, and the "true" *exquisita*-like plumage is largely restricted to central Peruvian foothills of the Junín, Ucayali, Pasco, and Huánuco regions in the vicinity of the type locality (Chuchurras, Pasco Region; AEM pers. observ.; Hellmayr, 1905; Stephens and Traylor 1983). North of these localities, individuals have a darker green body plumage, more muted yellow belly, and darker blue crown as seen in the Cordillera Escalera of San Martín, Peru, becoming very dark green in adult male birds along both banks of the Marañón River (Fig. S14). Despite the distinctive plumage around the type locality and monophyly of *L. c. exquisita*, we consider the current subspecies designation appropriate for this group given its vocal similarity to other Amazonian populations, fairly continuous variation towards darker phenotypes away from the type locality (phenotypes that can look very similar to *L. c. caelestipileata* and *L. c. regalis*), and relatively shallow genetic divergence from other Amazonian forms.

4.3. *Lepidothrix nattereri* + *L. vilasboasi* + *L. Iris clade*

The poor resolution of species-level relationships within the *L. nattereri* + *L. vilasboasi* + *L. iris* clade was consistent across different datasets and analytical paradigms. Recent studies of this clade have proposed a complex history of introgression among its members, with data suggesting that *L. vilasboasi* is a hybrid species and that there is ongoing gene flow between *L. nattereri* and *L. iris* (Barrera-Guzmán et al., 2018; Dias et al., 2018; Weir et al., 2015). Although our sampling was limited to regions outside the *L. nattereri-L. iris* hybrid zone (see Barrera-Guzmán et al., 2018; Weir et al., 2015), the abundance of conflicting topologies in the posterior distribution of SNAPP trees (Fig. 3B) suggests extensive allele sharing among *L. nattereri nattereri*, *L. iris eucephala*, and *L. vilasboasi* that is likely due to a combination of hybridization and incomplete lineage sorting. In IQ-TREE and SVDquartets trees, we found that *L. nattereri gracilis* samples (localities 15–17) consistently formed a clade (i.e., Fig. 2, S1–S2, S7–12), whereas *L. nattereri nattereri* samples (localities 11–14) did not form a clade in any trees, suggesting that our sampled populations of *L. nattereri nattereri*, which are geographically closer to *L. iris* and *L. vilasboasi*, may have experienced more hybridization and/or incomplete lineage sorting with other taxa than did our sampled populations of *L. nattereri gracilis*. We were unable to include samples of *L. iris iris* in this study, which is unfortunate because this taxon appears to have a history of introgression with *L. nattereri* (Dias et al., 2018). Additional geographic sampling and the collection of more complete genetic data using whole-genome resequencing will be important next steps for clarifying the evolutionary history of this clade.

4.4. Ancestry of a rare hybrid

The genetic evidence that the *L. coronata* × *L. suavissima* hybrid documented by Stotz (1993) was an F1 individual is consistent with expectations for a rare hybrid event. Where the two parental species come into contact, in southeastern Venezuela and extreme northern Brazil, they are separated by elevation (Stotz, 1993), with *L. suavissima* found on slopes and crests of tepuis (Kirwan and Green, 2011) and *L. coronata* found in the surrounding lowlands. Stotz (1993) hypothesized that a *L. suavissima* wandered downslope, eventually leading to the hybridization event with *L. c. carbonata* due, in part, to lack of available conspecific mates at the lowland site. Our findings are consistent with this hypothesis. Given the lack of evidence for introgression between the parental species, we suspect postmating reproductive barriers prevent successful backcrossing. However, more genetic sampling from the region of contact is needed to adequately assess the extent of hybridization between these two species.

4.5. Taxonomic implications for the Lepidothrix coronata clade

Many authors have suggested that the subspecies *L. coronata velutina* and *L. coronata minuscula*, together commonly referred to as the Velvety Manakin, may deserve species-level recognition (Hilty, 2021; Kirwan and Green, 2011; Ridgely and Tudor, 1994; Snow, 2004). However, factors including complex plumage variation among *L. coronata* populations (particularly in Amazonia), deep mitochondrial divergences between Amazonian populations, a lack of sufficient genetic data from the entire geographic range of *L. coronata*, and inconsistent phylogenetic placement of *L. coronata* with respect to other *Lepidothrix* species have delayed efforts to clarify the classification of the species group (Cheviron et al., 2005; Harvey et al., 2020; Kirwan and Green, 2011; Leite et al., 2021; Reis et al., 2020; Smith et al. 2014). Based on recent advances, including the molecular phylogenetic work in this study and the increased availability of audio recordings from across the geographic distribution of the group, we recommend the recognition of the Velvety Manakin (*L. velutina*) at the species level. We also suggest, following American Ornithological Society nomenclature guidelines, that the common name for Amazonian populations of the "Blue-crowned Manakin" be replaced with the name Blue-capped Manakin (Hellmayr, 1929) to avoid ambiguity in the usage of "Blue-crowned Manakin". Below we summarize our taxonomic recommendation:

- Species: *Lepidothrix velutina* (Berlepsch, 1883)
- Common name: Velvety Manakin
- Included taxa: *Lepidothrix velutina minuscula* (Todd, 1919)

4.5.1. Geographic range

From lowlands of central Costa Rica through Panama to northwestern Colombia and then south, west of the Andes, to northwestern Ecuador (Los Ríos); also, lower and middle Magdalena River valley of northern Colombia (Hilty, 2021; Kirwan and Green, 2011; Ridgely and Greenfield, 2001; Stiles and Skutch, 1989).

4.5.2. Molecular evidence

The mean sequence divergence between *L. velutina* and *L. coronata* at mitochondrial gene NADH dehydrogenase subunit 2 (ND2) is 4.25% (Table S4–S5; Supplemental Methods) with an estimated time of divergence 1.3–2.3 Ma (Cheviron et al., 2005; Reis et al., 2020). This sequence divergence is greater than that observed at ND2 between species of the *L. nattereri* + *L. vilasboasi* + *L. iris* clade (range 1.4–3.1%) and between *L. suavissima* and *L. serena* (3.7%), but it is smaller than that between *L. coronata* and *L. suavissima* (5.9%; Table S5), which appear to have postmating reproductive barriers between them. Based on the phylogenetic analyses in this study, support is consistent and high for the monophyly of *L. velutina* and *L. coronata* and for their evolutionary relationships with respect to each other and to other members of the genus.

4.5.3. Plumage

The body plumage of *L. velutina* is distinctly blacker than that of blackish-plumaged populations of *L. coronata* (*L. c. coronata*, *L. c. caquetae*, and *L. c. carbonata*), and *L. velutina* also has more extensive black on the forecrown. McCoy and Prum (2019) compared body feather barbule microstructure of *L. coronata* and *L. velutina* and suggest that the "super black" plumage of *L. velutina* is related to its sparsely distributed and strap-shaped barbules, which may promote multiple scattering of light among feathers and enhance melanin-based light absorption.

4.5.4. Voice

A review of audio recordings on xeno-canto (https://www.xeno-canto.org) and the Macaulay Library (https://www.macaulaylibrary.org) of the *L. coronata* species group revealed that both male advertisement songs and the primary call used by both sexes differ strikingly across the Andes but are otherwise similar over the geographic range of the group. Situating these observations within the literature on *L. coronata* vocalizations is complicated due to differing phonetic descriptions for the same vocalization types, general lack of referenced recordings or sonograms, and lack of locality information. Thus, we begin a review of *L. coronata* vocalizations building on work by Durães (2009), who wrote detailed vocal descriptions and provided associated sonograms for this species in eastern Ecuador.

Durães (2009) first described a "swee" call given by males and females of all ages. This upslurred whistle is commonly heard throughout Amazonian populations of *L. coronata* (e.g., Colombia: ML107942891; Ecuador: ML220839241; Venezuela: ML65742; Peru: ML190129, ML83075211; Brazil: ML117006, ML296626; and Bolivia: ML93867651; Fig. S15), but it is conspicuously absent from populations west of the Andes. The "swee" call is used in a variety of contexts including by males between advertisement songs and acrobatic displays and by both sexes while interacting or foraging (Durães, 2009).

Next, Durães (2009) described the advertisement song of males as consisting of a shorter, lower-pitched version of the "swee" call followed by two lower notes ("chí-wrr"). These latter two notes have been aptly described as a "froglike croak" (Schulenberg et al., 2010). As with the "swee" call, the "swee chí-wrr" song is commonly heard throughout Amazonian populations (e.g., Colombia: ML108320491; Ecuador: ML242117; Venezuela: ML65741; Peru: ML34194; Brazil: ML203897061, ML127687; and Bolivia: ML84796591; Fig. S16).

Comparing these vocalizations of *L. coronata* in Amazonia with those of *L. velutina* west of the Andes reveals several clear differences. First, the primary call and "most common utterance" (Skutch, 1969) given by both sexes west of the Andes is a "soft trilled "ti'ti't't't't't't't't", first note or two lower" (Hilty, 2021), and this call remains consistent throughout the range of *L. velutina* (e.g., Costa Rica: ML249370; Panama: ML202732201; Colombia: ML85450331; Ecuador: ML247953, ML139064; Fig. S17). Ridgley and Tudor (1994) noted that this trilled call "has not been heard in South America" (the Chocó region, a clear exception), and our review of audio recordings and voice descriptions reveal no such vocalization in Amazonian populations. Rather, the trilled call given west of the Andes appears to be fully replaced by the "swee" call in Amazonia. Further evidence of the analogous nature of these calls is that, in populations west of the Andes, a shortened version of the trill call generally begins the advertisement song of males "ti't't't't't, chu'WAK" (Hilty, 2021; Costa Rica: ML55245; Panama: XC437089; Colombia: XC400881; Ecuador: XC262304; Fig. S18) just as a "swee" call initiates the advertisement song in Amazonia ("swee chí-wrr"). The "chu'WAK" portion of the advertisement song west of the Andes has a similar frog-like quality and appearance on sonograms to the "chí-wrr" in Amazonia (Fig. S16, S18). In summary, primary calls and advertisement songs readily distinguish *L. velutina* from *L. coronata.*

Whether other less frequent vocalization types differ between these

species is not apparent from our review of audio recordings. Durães (2009) described a short "preew" call given by males during interactions with other males or females that appears to match vocalizations elsewhere in Amazonia (e.g., ML29564) and in Central America (e.g., ML302516). Among other vocalization types are also a "pee" call given during male display and copulation (Durães, 2009), an "emphatic rising "tr'r'riik" [given] repeatedly" (Hilty, 2021), and "a sharp *k'wek k'wek*, unaccompanied by other notes" (Stiles and Skutch, 1989).

We suggest that the vocal differences between *L. velutina* and *L. coronata* provide the best information available for assessment of reproductive barriers between these two species. The advertisement songs and primary calls of both *L. velutina* and *L. coronata* are given by males at their lekking territories (Durães, 2009; Skutch, 1969), and the "swee" call of *L. coronata* is often given during acrobatic display behaviors (Durães, 2009). Thus, differences in song and call in this species group are likely to function as prezygotic barriers. Furthermore, vocal variation tends to be limited within suboscine species, including manakins, and generally reflects underlying genetic variation as opposed to learned dialects (Kroodsma, 1984; Kroodsma and Konishi, 1991; Touchton et al., 2014; but see Kroodsma et al., 2013, Saranathan et al., 2007, and Trainer et al. 2002). Due to the allopatry of *L. velutina* and *L. coronata*, we consider whether the differences between vocalizations of these species are of similar degree as those between co-occurring species of *Lepidothrix* in the Andean foothills of Peru (Stotz, 1993): *L. coronata* and *L. coeruleocapilla*. The calls of these latter two species are similar upslurred, high-pitched whistles, and their songs also share a similar structure and "frog-like" quality, although *L. coeruleocapilla* does not have an introductory "swee" note before its two-noted song "*djew-HAI*" (Schulenberg et al. 2012). Given the trilled introduction to *L. velutina* advertisement songs, we consider their songs at least as different from those of *L. coronata* and *L. coeruleocapilla* as the songs of these latter two species are from each other. Primary calls are fundamentally different in *L. velutina* when compared to those of *L. coronata* and *L. coeruleocapilla* (sputtered trill vs. upslurred whistle). This comparative framework suggests that the distinctiveness of *L. velutina* vocalizations is greater than that necessary for maintenance of species boundaries between syntopic species in the same genus.

5. Conclusions

The frequency of recent species discoveries and "splitting" of polytypic bird taxa throughout the Neotropics (e.g., Cadena et al., 2020; Chesser et al., 2020; Lane et al., 2021; Moncrieff et al., 2018; Seeholzer et al., 2012; Whitney and Cohn-Haft, 2013) is a testament to the large gap between currently recognized species-level diversity and the diversity that actually exists (Lees et al., 2020). Even now, many putative species-level bird taxa are known to the scientific community, but limited resources often prevent the sampling and molecular phylogenetic work necessary to estimate evolutionary relationships and formally recommend species-level status. The increased sampling and phylogenetic methods we applied to study *Lepidothrix* manakins clarified relationships among taxa, highlighted the distinctiveness of the Velvety Manakin (*L. velutina*), and provided an improved framework for studying trait evolution across the genus.

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CRediT authorship contribution statement

Andre E. Moncrieff: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Brant C. Faircloth:** Conceptualization, Data curation, Methodology, Resources, Writing – review & editing. **Robb T. Brumfield:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

We deposited raw reads to the NCBI Sequence Read Archive under BioProjects PRJNA782327 and PRJNA787238. Sequence alignments and tree files from this project are archived on Dryad (https://doi.org/10.5061/dryad.j9kd51cds).

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Appendix A. Supplementary material

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