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Museum genomics reveals the speciation history of *Dendrortyx* wood-partridges in the Mesoamerican highlands

Whitney L.E. Tsai[a], Claudio Mota-Vargas[b], Octavio Rojas-Soto[b], Ravi Bhowmik[a,c], Emily Y. Liang[a,c], James M. Maley[a], Eugenia Zarza[a,1], John E. McCormack[a,c,*]

[a] Moore Laboratory of Zoology, Occidental College, Los Angeles, CA 90041, United States
[b] Red de Biología Evolutiva, Instituto de Ecología A. C., Xalapa, Veracruz, Mexico
[c] Biology Department, Occidental College, Los Angeles, CA 90041, United States

A B S T R A C T

Natural history collections are increasingly valued as genomic resources. Their specimens reflect the combined efforts of collectors and curators over hundreds of years. For many rare or endangered species, specimens are the only readily available source of DNA. We leveraged specimens from a historical collection to study the evolutionary history of wood-partridges in the genus *Dendrortyx*. The three *Dendrortyx* species are found in the highlands of central Mexico and Central America south to Costa Rica. One of these species is endangered, and in general, *Dendrortyx* are secretive and poorly represented in tissue collections. We extracted DNA from historical museum specimens and sequenced ultraconserved elements (UCEs) and mitochondrial DNA (mtDNA) to assess their phylogeny and divergence times. Phylogenies built from hundreds to thousands of nuclear markers were well resolved and largely congruent with an mtDNA phylogeny. The divergence times revealed an unusually old avian divergence across the Isthmus of Tehuantepec in the Pliocene around 3.6 million years ago. Combined with other recent studies, our results challenge the general pattern that highland bird divergences in Mesoamerica are relatively young and influenced by the Pleistocene glacial cycles compared to the older divergences of reptiles and plants, which are thought to overlap more with periods of mountain formation. We also found evidence for monophyletic genetic lineages in mountain ranges within the widespread *D. macroura*, which should be investigated further with integrative taxonomic methods. Our study demonstrates the power of museum genomics to provide insight into the evolutionary histories of groups where modern samples are lacking.

1. Introduction

Natural history collections are experiencing a renaissance, as new methods and technologies extend the data that can be collected from museum specimens in modern directions (Soltis and Soltis, 2016; Webster, 2017). Advances are happening especially rapidly in the field of DNA research (McCormack et al., 2013; Burrell et al., 2015), where new sequencing platforms work especially well with the degraded DNA fragments extractable from historical museum specimens (Rowe et al., 2011; Nachman, 2013; McCormack et al., 2017). At the same time, there is an increasing need to describe the basic units of biodiversity quickly in the face of conservation threats (Rojas-Soto et al., 2010; Costello et al., 2013). Museum genomics provides one means to overcome a bottleneck in the process of biodiversity discovery by leveraging the efforts of centuries of collectors who travelled to far-flung places, collecting specimens that would be difficult, or even unethical, to collect today.

The Mesoamerican highlands represent one such ecoregion where next-generation sequencing of existing museum specimens could help speed up biodiversity research. The evergreen and cloud forests of this region host globally high levels of biodiversity (Myers et al., 2000), which are under multiple threats from habitat destruction and climate change (DeClerck et al., 2010; Ponce-Reyes et al., 2012; Rojas-Soto et al., 2012). New species are still being discovered in this region, and new investigations of already-described species are revealing that populations in isolated mountain ranges are often highly differentiated (Bryson Jr et al., 2011b; Jadin et al., 2011; Bryson Jr et al., 2012a, 2012b; Bryson Jr and Riddle, 2012; Navarro-Sigüenza et al., 2013; Rovito et al., 2013; Mastretta-Yanes et al., 2015; Bryson Jr et al., 2017; Caviedes-Solis and de Oca, 2018; Zarza et al., 2018; Venkatraman et al., 2019).

Wood-partridges in the genus *Dendrortyx* can potentially inform the biogeography of the Mesoamerican highlands because they have low dispersal and are distributed across the region (Fig. 1). Although the three species in the genus are distinct phenotypically, their level of DNA differentiation has never been investigated, likely because there are few modern specimens with associated frozen tissue samples. Older museum specimens, most of them collected more than 50 years ago, are likely the best source of DNA for *Dendrortyx* species which, realistically, will never be collected in high numbers again.

* Corresponding author at: Moore Laboratory of Zoology, Occidental College, Los Angeles, CA 90041, United States.
E-mail address: mccormack@oxy.edu (J.E. McCormack).
[1] Current affiliation: Grupo Académico de Biotecnología Ambiental, El Colegio de la Frontera Sur, Tapachula, Chiapas, Mexico 30700.

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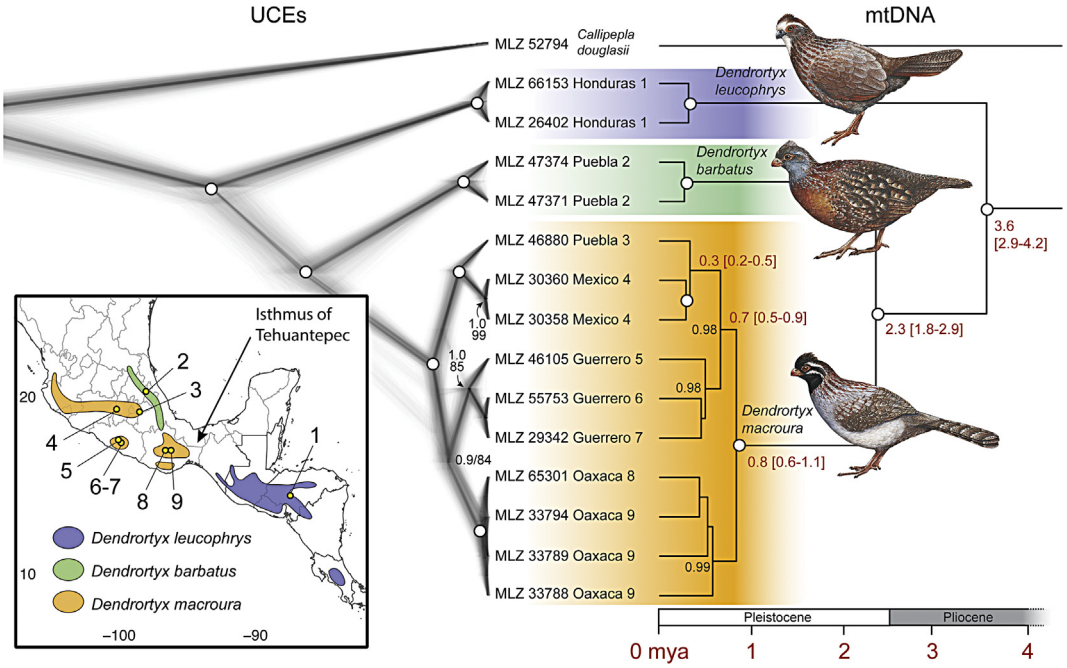

Fig. 1. Evolutionary history and geographic ranges (inset) of *Dendrortyx* wood-partridges. Yellow dots on the range map indicate sampling localities with numbers matching the labeling on the terminal tips of the phylogeny and the specimens in Table 1. At left, the nuclear DNA phylogeny is represented by the SNAPP tree, built from SNPs extracted from UCEs. At right, the mtDNA tree was inferred from the cyt b gene and calibrated to time using the molecular substitution rate from Weir and Schluter (2008). White dots on the nodes of the phylogenies indicate 1.0 posterior probability from Bayesian analysis. Nodes with black text on the UCE tree show posterior probability from the SNAPP analysis above and bootstrap value from the concatenated maximum-likelihood analysis below. Nodes without labeling have less than 0.80 posterior probability. Divergence times (with 95% highest probability densities in brackets) are shown in red for select nodes on the mtDNA tree. The roots of both trees have been truncated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Our goal was to assemble genomic DNA and mitochondrial DNA (mtDNA) from museum specimens of *Dendrortyx* species to assess (1) the evolutionary relationships among the three species, (2) genetic differentiation among the disjunct mountain populations of *D. macroura* within Mexico, and (3) divergence times in the genus, with the goal to place diversification in the context of the biogeography of the region. Prior studies have found wide variation among species in their divergence times across the Mesoamerican highlands (Ornelas et al., 2013). Generally, birds are thought to show younger divergence times compared to plants or reptiles, with their diversification linked mostly to habitat fragmentation during the Pleistocene glacial cycles (Barber and Klicka, 2010). However, some recent studies have challenged this trend, showing that some birds have older divergences that pre-date the glacial period (Maldonado-Sánchez et al., 2016; Venkatraman et al., 2019).

2. Materials and methods

2.1. Study species and sampling

Dendrortyx contains three species: the Long-tailed Wood-Partridge (*D. macroura*), which occurs across several disjunct mountain ranges in Mexico; the Bearded Wood-Partridge (*D. barbatus*), which is an endangered endemic to Mexico's Sierra Madre Oriental (Mota-Vargas and Rojas-Soto, 2012); and the Buffy-crowned Wood-Partridge (*D. leucophrys*), which occurs east of the Isthmus of Tehuantepec, from Chiapas, Mexico to Costa Rica. Using specimens in the Moore Laboratory of Zoology at Occidental College, we sampled as much of the range of *Dendrortyx* as possible (Fig. 1; Table 1): 10 individuals of *D. macroura* including subspecies *macroura* in Puebla (site 3), *griseipectus* in the state of México (site 4), *striatus* in Guerrero (sites 5–7), and *oaxacae* in Oaxaca (sites 8–9); two individuals of *D. barbatus*; and two individuals of *D. leucophrys*. We used an Elegant Quail (*Callipepla douglasii*) as the outgroup.

2.2. DNA data collection

We sequenced thousands of nuclear genomic loci anchored by ultraconserved elements (UCEs). UCEs are genomic regions that have high sequence identity among distantly related taxonomic groups, which allow them to act as genomic anchors in non-model species (McCormack et al., 2012). Phylogenetic information is then collected from DNA regions flanking the core UCE. We targeted 5060 genomic regions originally located and designed from an alignment of the chicken and *Anolis* lizard genomes (Faircloth et al., 2012).

To sequence UCEs, we first extracted genomic DNA from toe pads of historical museum skins using the standard protocol for a Qiagen DNeasy Blood and Tissue extraction kit with modifications, which included a longer incubation time and two final elution steps into a total of 100 µL. We took reasonable measures to avoid contamination, including using filter tips and sterilized work areas. We created libraries for each sample with a KAPA library preparation kit, attaching custom index tags to DNA from each sample (Glenn et al., 2016). We enriched each library for UCEs using RNA probes (MYbaits_Tetrapods-UCE-5 K kit, Mycroarray). We then quantified DNA concentrations for each sample using a Qubit and pooled the 15 libraries together in equimolar ratios prior to sequencing on one run of an Illumina MiSeq using 250 base-pair, paired-end sequencing.

2.3. Bioinformatic processing

We followed the PHYLUCE (Faircloth, 2015) pipeline to (1) perform quality control on the Illumina reads, (2) assemble UCE loci from the reads, and (3) align UCE loci for phylogenetic analysis. Briefly, we first binned reads by sample using their index tags. We then trimmed reads of index and adapter sequence and low-quality bases with Illumiprocessor (Faircloth, 2013) and Trimmomatic (Bolger et al., 2014). We assembled reads into contigs using the PHYLUCE script assemblo_abyss, in concert with the ABySS alignment tool (Simpson et al., 2009), using

Table 1
Dendrortyx museum specimens and summary statistics.

Catalog	Species subspecies	Fig. 1 Map Site	Country	State/Departmento	Locality	Latitude	Longitude	Reads	UCEs
MLZ:Bird:66153	*Dendrortyx leucophrys leucophrys*	1	Honduras	Francisco Morazan	Alto Cantoral	14.33	−87.4	8,98,635	2503
MLZ:Bird:26402	*Dendrortyx leucophrys leucophrys*	1	Honduras	Francisco Morazan	Alto Cantoral	14.33	−87.4	5,64,846	3025
MLZ:Bird:47374	*Dendrortyx barbatus*	2	Mexico	Puebla	Scapa, 3 mi NE Huauchinango	20.2111	−98.0341	5,26,771	3058
MLZ:Bird:47371	*Dendrortyx barbatus*	2	Mexico	Puebla	Scapa, 3 mi NE Huauchinango	20.2111	−98.0341	4,72,188	3295
MLZ:Bird:46880	*Dendrortyx macroura macroura*	3	Mexico	Puebla	El Venerable, 4 mi E Río Frío	19.3523	−98.6089	7,79,752	2692
MLZ:Bird:30360	*Dendrortyx macroura griseipectus*	4	Mexico	Mexico	Puerto Lengua de Vaca, 15 mi E Zitacuaro	19.4351	−100.1867	5,50,586	3039
MLZ:Bird:30358	*Dendrortyx macroura griseipectus*	4	Mexico	Mexico	Puerto Lengua de Vaca, 15 mi E Zitacuaro	19.4351	−100.1867	4,26,879	3145
MLZ:Bird:65301	*Dendrortyx macroura oaxacae*	8	Mexico	Oaxaca	5 km NW Cerro San Felipe	17.1765	−96.6724	4,80,803	3258
MLZ:Bird:33794	*Dendrortyx macroura oaxacae*	9	Mexico	Oaxaca	Totontepec	17.2570	−96.0287	5,37,810	3159
MLZ:Bird:33788	*Dendrortyx macroura oaxacae*	9	Mexico	Oaxaca	Totontepec	17.2570	−96.0287	3,39,742	3372
MLZ:Bird:33789	*Dendrortyx macroura oaxacae*	9	Mexico	Oaxaca	Totontepec	17.2570	−96.0287	4,59,592	3206
MLZ:Bird:46105	*Dendrortyx macroura striatus*	5	Mexico	Guerrero	Cerro Teotepec	17.4769	−100.1710	5,07,572	3081
MLZ:Bird:55753	*Dendrortyx macroura striatus*	6	Mexico	Guerrero	Omiltemi	17.5000	−99.6667	6,40,600	2782
MLZ:Bird:29342	*Dendrortyx macroura striatus*	7	Mexico	Guerrero	Coapango	17.5062	−99.6364	5,45,713	2907
MLZ:Bird:52794	*Callipepla douglasi teres*	–	Mexico	Jalisco	Puerto Vallarta	20.6189	−105.2301	4,60,421	3261

kmer = 40. Prior studies suggested ABySS as equal or superior to other alignment programs for UCE data (Zarza et al., 2018). We identified which contigs matched UCE loci using LASTZ (Harris, 2007) and the PHYLUCE script match_contigs_to_probes. Finally, we aligned UCE loci using MAFFT v7.13 (Katoh and Standley, 2013) by running the PHYLUCE script seqcap_align_2, using the default trimming algorithm.

2.4. Phylogeny using concatenated data & a species tree approach

We created a concatenated data matrix of UCE loci including the outgroup. We allowed some missing data, dropping a UCE locus if more than four individuals had missing data (i.e., a 70% missing data threshold). We analyzed this unpartitioned, concatenated data matrix with RAxML v8.0.19 (Stamatakis, 2014) using the GTRGAMMA model of evolution by searching for the best-scoring maximum likelihood (ML) tree, performing 1000 bootstrap searches, and reconciling the best ML tree with the bootstrap replicates. For species-tree estimation, we generated a gene tree for each alignment using RAxML and then used ASTRAL v5.6.3 (Mirarab and Warnow, 2015) to estimate a species tree, with support values generated through quartet frequencies.

2.5. Divergence dating

We used mtDNA data drawn from off-target sequencing reads to calibrate divergence times because there are no fossils to calibrate *Dendrortyx*, but the molecular clock rates with error have been well calibrated for avian bird *cytochrome oxidase b* (*cyt b*) gene (Weir and Schluter, 2008). We imported Illumina reads from each individual into Geneious v8.1.9 and aligned them to reference *cyt b* genes from close relatives (Genbank EU372675 and FR694138). For each individual, we created *cyt b* contigs from these matching reads, which we then aligned among individuals. We ran the aligned matrix in BEAST v2.2.1 (Bouckaert et al., 2014), using a strict molecular clock and divergence rate of 1.105% per lineage per million years (range: 0.765–01.445%), which is based on biogeographic calibrations for this gene in birds (Weir and Schluter, 2008). To assess the topology of the mtDNA tree with more data, we also ran an analysis including the ND2 gene. The topology was the same (Fig. S1), so we present only the *cyt b* phylogeny in the main text due to the robust molecular evolution rate estimate for this gene.

3. Calling and analyzing SNP data

We called SNPs within UCE loci using the assembled UCE contigs of the outgroup individual as the reference. We indexed and mapped the trimmed Illumina reads to the reference assembly with the BWA-mem algorithm, which is suitable for reads ranging from 70 bp to 1 Mbp (Li, 2013). After sorting reads with SAMtools (Li et al., 2009), we removed PCR duplicates with Picard (http://broadinstitute.github.io/picard/) and used GATK v3.2 (McKenna et al., 2010) to re-align the mapped reads around indels, call variants, quality filter, remove indels, and select only biallelic variants. We eliminated all positions with missing data, selected one SNP per contig, and converted variants to number format.

We generated a species tree from this SNP matrix in SNAPP 1.1.10 (Bryant et al., 2012), implemented in BEAST v2.2.1. We ran two instances of SNAPP for two million generations using default priors, saving the output every 1000 steps. We combined tree and parameter files from both runs with LogCombiner v2.1.3. We displayed the full set of likely species trees with Densitree v2.2.1 (Bouckaert, 2010), which is expected to show fuzziness in parts of the tree that conflict due to gene flow or other causes.

4. Results

We obtained 8.2 million reads total and between 339,742 to

898,635 reads per sample after initial quality control and adapter trimming. We assembled an average of 211,903 contigs per sample with an N50 between 557 and 685 bp. We recovered an average of 3052 UCE contigs per sample, forming a 70% complete aligned matrix of 10,788,562 base pairs. After SNP calling and variant filtration, we obtained a total of 1516 biallelic SNPs.

The SNAPP species tree and the maximum-likelihood phylogeny from concatenated UCE data agreed on the same topology where most nodes were strongly supported and the different geographic populations of *D. macroura* formed monophyletic lineages (Fig. 1). The ASTRAL species tree (Fig. S2), based on 352 loci, showed less resolution than the SNAPP tree, but did not conflict with it in any strongly supported nodes. The *cyt b* mtDNA tree had the same topology as the SNAPP tree and concatenated UCE tree, except that within *D. macroura*, the lineages in the Transvolcanic Belt and Guerrero were sister in the mtDNA tree, whereas the Guerrero and Oaxaca lineages were sister in the nuclear DNA trees. Divergence dating suggested the speciation events between the three species began in the Pliocene, with divergence within *D. macroura* continuing into the Pleistocene (Fig. 1).

5. Discussion

DNA studies using museum specimens are becoming more common as natural history collections are recognized as genomic resources (Bi et al., 2013; Nachman, 2013; Burrell et al., 2015; McCormack et al., 2017). Along with other recent studies, our results demonstrate that researchers can forsake the freezer entirely, completing whole projects using only historical museum specimens (Staats et al., 2013; Besnard et al., 2015). This does not mean that we should stop collecting specimens. On the contrary, these studies serve to highlight the value of specimens as scientific treasure troves that continue to yield new information and data sources the more they are studied and with technological advances. Plus, there are many questions beyond phylogeography and phylogenetics where modern specimens are needed, for example deep sampling of specific locations and time-series collections for documenting shifting baselines in our current biodiversity crisis (Johnson et al., 2011; Habel et al., 2014).

5.1. An especially old avian divergence across the Isthmus of Tehuantepec

One of the main conclusions of this study is that *Dendrortyx* represents a particularly old avian divergence across the Isthmus of Tehuantepec, an important barrier in southern Mexico that has shaped highland biodiversity in the region (Sullivan et al., 1997; Peterson et al., 1999). Prior work suggested that birds tend to have fairly recent Pleistocene histories of divergence across the Isthmus (Barber and Klicka, 2010; Rodríguez-Gómez et al., 2013), compared to what has been seen in other groups like plants (Ornelas et al., 2013) or reptiles (Castoe et al., 2009; Daza et al., 2010; but see Bryson Jr et al., 2011b for a younger reptile divergence). A multispecies comparative study found that birds diversified across the Isthmus in two pulses, with the older pulse dating to well within the Pleistocene around 1.6 million years ago (Barber and Klicka, 2010), which also roughly coincides with some single-species studies (González et al., 2011; Barrera-Guzmán et al., 2012). These divergence times in turn imply that habitat fragmentation during the Pleistocene glacial cycles was the primary diversifying agent for modern bird lineages because Mesoamerican mountain uplift was completed prior to this period in the Miocene and Pliocene (Barrier et al., 1998).

The oldest split in *Dendrortyx* (3.6 ± 0.7 million years ago) predates the Pleistocene and is older than all 10 of the single-species mean divergence estimates that comprised the Barber and Klicka (2010) comparative study. This result adds to an emerging counter-narrative of pre-Pleistocene avian divergences across the Isthmus, including *Aphelocoma unicolor* (Venkatraman et al., 2019), *Chlorospingus flavopectus* (Maldonado-Sánchez et al., 2016), and *Henicorhina leucophrys* (Cadena et al., 2019). These Pliocene bird divergences might have been influenced by tectonic activity in the late Miocene and early Pliocene, like the subduction and down-dropping of mountains that once spanned the Isthmus (Barrier et al., 1998), but it is difficult to rule out climatic drivers like global cooling during the Pliocene that would have restructured cloud forests and their communities.

5.2. Biogeography of Mesoamerican forests

Apart from the Isthmus, other divergences within *Dendrortyx* can inform the historical biogeography of Mesoamerican forests. For example, *D. barbatus* in the Sierra Madre Oriental and *D. macroura* in the Oaxacan Highlands, Tranvolcanic Belt, and Sierra Madre del Sur split around 2.3 (± 0.5) million years ago, around the Pliocene-Pleistocene transition and the initial onset of the glacial cycles. These two species have been described as sympatric in the eastern part of the Transvolcanic Belt, but their co-occurrence there is actually unlikely (Mota-Vargas et al., 2017). Something important appears to have happened between these mountain areas at this time, as some co-distributed species have similar divergence times, like *A. unicolor* (Venkatraman et al., 2019), *C. flavopectus* (Maldonado-Sánchez et al., 2016), and *A. wollweberi* (McCormack et al., 2011). Not all splits from this region are congruent, however, as the divergence within Mexican Pine Snakes (*Pituophis deppei*) is much older at 4.6 (± 1.5) million years ago (Bryson Jr et al., 2011a).

There is also a remarkable congruence in the emergence of a distinct Oaxacan Highlands lineage for both *Dendrortyx* and *A. unicolor* around 800,000 (± 200,000) years ago. The Oaxacan lineage of *C. flavopectus* is somewhat older (1.3 ± 0.5 million years ago), although the confidence intervals overlap (Maldonado-Sánchez et al., 2016). In general, the topology and timing of the splits between *Dendrortyx* and *A. unicolor* reflect simple vicariance between nearby mountain ranges, whereas other co-distributed species like *C. flavopectus* and the Northern Emerald-Toucanet (*Aulacorhynchus prasinus*) show more complex patterns involving multiple dispersal events across geographic barriers like the Isthmus of Tehuantepec (Bonaccorso et al., 2011; Maldonado-Sánchez et al., 2016). The co-divergence of *Dendrortyx* and *A. unicolor* might relate to their suspected low dispersal behavior and their more close association with high-elevation temperate forests.

5.3. Differentiation across the mountains of Mexico

One *Dendrortyx* species, the Long-tailed Wood-Partridge (*D. macroura*), is distributed across several disjunct mountain ranges in Mexico, offering an opportunity to investigate more recent differentiation across this region. Although we did not sample from all parts of its distribution, we did include four of the six subspecies, each of which was phylogenetically distinct in most trees. The ASTRAL species tree was an outlier and had low support, likely because of low locus counts due to missing data and low-information loci creating more noise compared to SNP data (Hosner et al., 2015; Manthey et al., 2016).

Better sampling and integrative taxonomy, including data from the phenotype and vocalizations would help further elucidate the divergence history within *D. macroura* and determine how distinctive these monophyletic lineages might be. Based on an ecological similarity test, Mota-Vargas and Rojas-Soto (2016) found that the disjunct subspecies of *D. macroura* had similar niches. In terms of their age, the oldest split within *D. macroura,* separating the Oaxaca lineage from the rest, dates to the onset of intense glacial cycling 800,000 years ago (± 0.2 million years). The formation of these Mexican highland lineages, therefore, is almost certainly linked to ice age habitat fragmentation.

5.4. Phylogenetic conflict and future studies

While the mtDNA and UCE trees agreed across nearly all nodes, there was one well-supported conflict within *D. macroura*. Nuclear trees

supported Guerrero + Oaxaca, whereas the mtDNA tree supported Guerrero + Transvolcanic Belt (Fig. 1). Although a minority of posterior trees in the SNAPP cloudogram supported the mtDNA topology, overall the SNAPP tree showed little visual evidence for the kind of mixed signal often observed when gene flow is influencing the divergence history (Zarza et al., 2016). The source of the discord must therefore await future study, but the evidence leans toward a biological cause rather than an analytical artifact or lack of data.

Finally, we included only two samples of the southern-most species *D. leucophrys*, both from the same location in Honduras. This species is distributed over a broad and complex mountain area in Central America, including a disjunct population in Costa Rica currently described as a subspecies, *D. l. hypospodius*. Denser sampling, including the Costa Rica lineage, is a priority for future research. One curiosity about the distribution of *D. leucophrys* is the lack of records just east of the Isthmus of Tehuantepec in western and central Chiapas, Mexico. There are no eBird observations and no museum specimens from this area where suitable habitat apparently exists and where the ecologically similar *A. unicolor* is found. *D. leucophrys* may occur sparsely in this region (Johnsgard, 1972), but its current population status there remains an open question.

6. Conclusions

Since the original sequencing of DNA from an extinct relative of the modern zebra (Higuchi et al., 1987), it has become apparent that the millions of natural history specimens housed in museums are genomic resources. This realization has opened new research avenues, with some studies focusing on extinct species (Hung et al., 2014), and other studies, like this one, focusing on species that are rare, endangered, or otherwise difficult to collect. In these particular cases, DNA from museum specimens offers the only way to assess biodiversity by leveraging the efforts of collectors over the last several hundred years. In this study, the legacy of these collectors reveals previously undescribed phylogenetic diversity in the Mesoamerican Highlands and shows that both the Pleistocene ice ages and events in the Pliocene were important to diversification of cloud forest birds.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.03.017.

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