DNA from a 100-year-old holotype confirms the validity of a potentially extinct hummingbird species

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We used mtDNA sequence data to confirm that the controversial 100-year-old holotype of the Bogotá sunangel (*Heliangelus zusii*) represents a valid species. We demonstrate that *H. zusii* is genetically well differentiated from taxa previously hypothesized to have given rise to the specimen via hybridization. Phylogenetic analyses place *H. zusii* as sister to a clade of mid- to high-elevation Andean species currently placed in the genera *Taphrolesbia* and *Agelaiocercus*. *Heliangelus zusii*, presumed extinct, has never been observed in nature by biologists. We infer that the species occupied a restricted distribution between the upper tropical and temperate zones of the northern Andes and that it was most probably driven to extinction by deforestation that accompanied human population growth during the nineteenth and early twentieth centuries. We demonstrate the feasibility of obtaining DNA from nearly microscopic tissue samples from old hummingbird specimens and suggest that these methods could be used to resolve the taxonomy of dozens of avian taxa known only from type specimens.

Keywords: ancient DNA; Andes Mountains; hummingbird; hybrid; extinction

1. INTRODUCTION

Cataloguing the biota of biologically unexplored regions often follows closely on the heels of human population expansion and subsequent waves of anthropogenically caused extinctions (Fuller 2001). Many species that were rare when first encountered by biologists have not been observed since the type specimens were collected. Species known only from unique specimens are rightfully viewed with caution because they may represent hybrids or aberrant phenotypes. Living populations of these biological mysteries are occasionally ‘rediscovered’ in nature many decades or even centuries after their scientific descriptions, providing hope to conservationists and new data to

systematists (Cohn-Haft 1993; Bauer *et al.* 2000; Buzzetti & Carlos 2005; Mauro 2005; Lane *et al.* 2006; Round *et al.* 2007). More frequently, the mysteries remain unsolved, hinting at an unknown history of hybridization or of possible extinction. For these cases, ancient DNA (aDNA) methods provide the means for resolving disputes regarding the validity of species known only from unique holotypes. We have used this approach to investigate the contentious case of a spectacular 100-year-old hummingbird specimen, the holotype and the only known specimen of the Bogotá sunangel *Heliangelus zusii*.

In 1947, Brother Nicéforo María presented a specimen he purchased in 1909 in Bogotá, Colombia, to Rodolphe Meyer de Schauensee at the Academy of Natural Sciences of Philadelphia (ANSP). Meyer de Schauensee was perplexed by the specimen (ANSP 159261) and solicited the opinions of many leading ornithologists of his day, including John Zimmer, James Peters and Alexander Wetmore. There was no consensus among the experts, and Meyer de Schauensee referred the specimen to *Neolesbia nehrkorni*, a taxon based on two specimens now thought to be hybrids (Hinkelmann *et al.* 1991). Graves (1993) conducted an exhaustive analysis of plumage and morphology, comparing the specimen to all hummingbird taxa housed in several of the largest museums. Following a well-established method of hybrid diagnosis (Graves 1990), he concluded that the specimen represented the only known example of a valid species, which he named *H. zusii*. This conclusion was controversial, in part, because the exact provenance of the specimen is unknown and because hummingbirds are known to hybridize extensively (McCarthy 2006). The taxon has not been accepted by many authorities (Schuchmann 1999).

2. MATERIAL AND METHODS

(a) Laboratory procedures

Skin cells were scraped from the feet of ANSP 159261 using a sterile scalpel blade at the National Museum of Natural History, Smithsonian Institution. Extraction and PCR setup (with negative controls and replications) were performed in the dedicated aDNA laboratory at the New York State Museum, where no previous work on hummingbirds had been performed. We employed a modified version of the silica-based DNA extraction method of Höss & Pääbo (1993). We designed six PCR primer pairs to target short segments of the mitochondrial *ND2* and *ND4* genes and their flanking tRNAs. Extraction protocol, primer sequences and PCR conditions are described in the electronic supplemental material. In a separate laboratory at University of California, Berkeley, we obtained sequences from frozen tissues of 95 hummingbird species and from five swifts (Apodidae), a treeswift (Hemiprocridae) and an owl-nightjar (Aegothelidae), which were used to root phylogenetic trees. Taxon sampling within the hummingbird family Trochilidae included species from all nine major trochilid clades and all 18 genera within the ‘coquette’ clade (Lophornithini) (McGuire *et al.* 2008), including an additional 16 species beyond those included in McGuire *et al.* (2007) because we expected *H. zusii* to be nested within that group. Methods and primers for amplification and sequencing from frozen samples are available in McGuire *et al.* (2007). Specimen details and GenBank (NCBI) accession numbers are in the electronic supplementary material, table S1.

(b) Phylogenetic analyses

Four of the six primer pairs produced PCR products from the mitochondrial *ND4*, *ND2*, *tRNA-His* and *tRNA-Ser* genes, totalling 356 base pairs (bp) of concatenated sequence. Phylogenies were reconstructed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian approaches to analyse both a taxonomically broad sample of hummingbirds for only those characters for which we had data for *H. zusii* (95 hummingbird species plus outgroups, 356 bp), and a larger alignment of 4133 bp of the above

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Figure 1. Consensus Bayesian phylogeny of select hummingbird species showing the relationship of *H. zusii* to other high-elevation coquettes. Numbers at nodes are Bayesian posterior probabilities/ML bootstrap support (1000 replicates)/MP Bremer support. Trees were rooted with an outgroup comprising swifts, a treeswift and an owlet-nightjar.

mitochondrial genes plus the nuclear β -fibrinogen intron 7 and adenylate kinase 1 intron 5 genes for 33 species of montane coquettes, one lowland coquette, *H. zusii* and outgroups. This combination of analyses allowed us to initially place *H. zusii* within the broad phylogenetic context of all hummingbird lineages, and then to bring as much data as possible to bear on the relationship of *H. zusii* to its closest relatives. We used the program Nona (Goloboff 1995) for MP and to compute Bremer support values (Bremer 1994). ML tree searches and bootstrapping (1000 replicates) were run in PHYL (Guindon & Gascuel 2003). Bayesian analysis using MrBAYES (Huelsenbeck & Ronquist 2001) included two runs of 10 million generations, sampling every 10 000 generations and excluding the first one million generations as burn-in. Details of substitution model selection are given in the electronic supplementary material.

3. RESULTS AND DISCUSSION

Initial analysis of the complete taxon sample (356 bp) placed *H. zusii* (ANSP 159261) unambiguously

within the high-elevation group of coquettes (McGuire et al. 2008) in a clade consisting of species placed in *Taphrolesbia* and *Agelaiocercus*, but did a poor job recovering well-established, higher-level hummingbird clades. Our MP, ML and Bayesian analyses of the more character-rich (4133 bp) alignment of 35 coquette species plus outgroups provided strong support for the hypothesis that *H. zusii* represents a distinct lineage allied with *Taphrolesbia* and *Agelaiocercus* (figure 1), with high Bremer support (6), ML bootstrap (95%) and Bayesian posterior probabilities (1.00) for the critical node linking *H. zusii* to its sister taxa. ML branch lengths (not shown) as well as pairwise genetic distances indicate that *H. zusii* is not a hybrid, but is a distinct taxon exhibiting

Table 1. Genetic distances between *H. zusii* and closely related coquette genera. (Distances are uncorrected per cent sequence divergences, including mean (number of species sampled) and range. —, sequence not available.)

	<i>Helianthus</i>	<i>Agelaiocercus</i>	<i>Adelomyia</i>	<i>Taphrolessia</i>
ND2 + ND4 (245 bp)	13.86 (6) 12.25–17.42	5.51 (2) 5.31–5.71	9.80 (1)	3.28 (1)
tRNA-His + tRNA-Ser (112 bp)	4.24 (6) 2.81–4.74	3.84 (2) 3.84–3.84	1.92 (1)	—

species-level divergence from all sampled species (at least 3.3% at ND2 + ND4, 245 bp) and is 12.3–17.4% divergent from six species of *Helianthus* (table 1).

In rejecting the hybrid hypothesis for *H. zusii*, we feel certain that all potential parental species were sampled. Although we did not sample *Agelaiocercus berlepschi* (Venezuelan sylph), this taxon is morphologically similar to *Agelaiocercus kingi*, and the two are considered conspecific by many authorities. The three species of *Agelaiocercus* (sylphs) undoubtedly form a monophyletic group based on morphology. The 19 species of coquettes not sampled are in the genera *Lophornis*, *Discosura*, *Sephanoides*, *Oreotrochilus*, *Helianthus*, *Chalcostigma*, *Metallura* and *Phlogophilus*; all these genera form well-supported clades that are genetically divergent from the clade containing *H. zusii*, and only *Chalcostigma* is not monophyletic (figure 1; McGuire *et al.* 2007). Robust support for the affinity of *H. zusii* with the *Taphrolessia*–*Agelaiocercus* clade necessitates generic reassignment of *zusii*. We defer this action pending a general review of generic limits within the Trochilidae.

Our findings permit us to more accurately circumscribe the probable geographical range and habitat of *zusii*. Tens of thousands of hummingbird trade skins were exported from Bogotá for the millinery trade in the nineteenth century (Doughty 1975), and nearly all refer to species restricted to the northern Andean region of Colombia and adjacent Ecuador (Berlioz & Jouanin 1944). Graves (1993) speculated that *zusii* originated from the Eastern Cordillera of the Colombian Andes within a few hundred kilometres of Bogotá, or possibly in the Central Cordillera, in cloud forest between 1400 and 2200 m (altitude above sea level). The fact that only a single specimen is known despite the extensive collection of showy hummingbirds suggests that it had a relictual or restricted geographical distribution when collected. The three species of *Agelaiocercus* inhabit humid Andean forest (900–3000 m) from the Coastal Range of Venezuela south to northern Bolivia, whereas *Taphrolessia griseiventris* occurs in a semi-arid region (2750–3170 m) of northern Peru, characterized by cacti and other xerophytic plants (Collar *et al.* 1992). Assuming that the ecology of *zusii* falls between these extremes, the search for *zusii* should be expanded to include semi-arid habitat in the Andes as high as 3200 m from northwestern Venezuela south of northern Peru. We presume that *zusii* is extinct, but hummingbird species continue to be discovered (Fitzpatrick *et al.* 1979; Graves 1980; Cortes-Diogo *et al.* 2007), suggesting that *zusii* may

persist in an unexplored region of the Andes, such as an outlying cordillera (e.g. *Helianthus regalis*) or an isolated peak (e.g. *Ramphomicron dorsale*).

aDNA from unique types may help resolve long-standing taxonomic problems in ornithology and other disciplines. Our finding that mtDNA from ANSP 159261 is highly divergent from potential parental species rules out a hybrid origin and precludes sequencing nuclear DNA, which is much more difficult to obtain from century-old archival material (Kirchman 2009). The use of silica-based techniques, originally developed for extractions from organic remains of Pleistocene origin, should enable genetic characterization of other extremely small samples of archival material such as those we have obtained from the tiny feet of a hummingbird museum specimen.

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