

1 An extinct hummingbird species that never was: a cautionary tale about sampling issues 2 in molecular phylogenetics

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18 ABSTRACT

19
20 The Bogota Sunangel (*Helianzelus zusii*) was described based on a historical specimen lacking locality data as a striking
21 –and potentially extinct– new species of hummingbird more than two decades ago. However, it was considered a dubious
22 taxon by some researchers until a molecular study with strong species-level taxon sampling revealed its phylogenetic
23 affinities and validated its status as a distinct species. We reanalysed existing mitochondrial DNA data together with
24 a new data set sampling multiple populations of the Long-tailed Sylph (*Aglaiaocercus kingii*), a species broadly
25 distributed in the Andes of South America. In contrast to previous work, we found that *H. zusii* shares a haplotype
26 with specimens of *A. kingii* from the Eastern Cordillera of Colombia, which is phylogenetically nested within a clade
27 formed by populations of *A. kingii* from the Colombian Andes. These results suggest that *H. zusii* is not a distinct
28 species, but is most likely the result of hybridization between a female *A. kingii* and a male of another hummingbird
29 species. These findings highlight the importance of thorough taxonomic and geographic sampling when assessing the
30 likelihood of hybrid origin of an organism, particularly in cases potentially involving wide-ranging species in areas where
31 deep phylogeographic structure is likely.

32
33 Keywords: *Aglaiaocercus*, geographic sampling, *Helianzelus zusii*, hybridization, phylogeography.

35 INTRODUCTION

36
37 The selection of species and individuals for inclusion in molecular analyses critically affects inferences in various fields
38 of systematic biology including phylogenetics [1], phylogeography [2], and species delimitation [3]. Especially in areas
39 such as the Neotropical region where molecular analyses have recovered substantial within-species divergence and
40 unexpected affinities of populations [4], biases resulting from incomplete taxonomic or geographic sampling may
41 importantly compromise the results of analyses aimed at understanding phylogenetic relationships [5]. Here we document
42 surprising results revealing a case in which inferences regarding the validity of a potentially extinct and iconic species
43 of Neotropical bird were likely compromised because within-species variation was not accounted for in phylogenetic
44 analyses evaluating the alternative hypothesis that the only known specimen may represent a hybrid as opposed to a
45 distinct species.

46
47 Hummingbirds (Trochilidae) are well known for their propensity to hybridize, with numerous documented records of
48 interspecific hybridization, often involving species in different genera [6, 7]. Hybrid hummingbird specimens are
49 particularly common in natural history collections and have caused substantial taxonomic confusion because they were
50 often described as distinct species by museum-based ornithologists. Therefore, stringent protocols have been established

51 to diagnose hybrid hummingbird specimens and thus avoid treating hybrids as taxa [8]. The application of such protocols
52 has resulted in the diagnosis of numerous hybrids and, consequently, in the consideration of a large number of named
53 species as invalid. For example, G. R. Graves has authored no less than 17 papers on hybrid hummingbird diagnoses
54 since he first described his approach [8] until the present [9].

55
56 A notable exception to situations in which historical hummingbird specimens were described as distinct species but
57 were later determined to be hybrids is that of an atypical skin purchased in 1909 by Brother Nicéforo María in Bogotá,
58 Colombia, which was later sent to the Philadelphia Academy of Natural Sciences. Upon examining this specimen, which
59 had puzzled ornithologists for decades and lacked precise locality data, Graves [10] concluded that it was not an aberrant
60 individual of a known taxon and ruled out the possibility that it may represent a hybrid. Therefore, he designated the
61 specimen as the holotype of a new species, the Bogota Sunangel (*Helianzelus zusii*), which he described while noting it
62 may well have gone extinct due to habitat destruction, representing a “relic of a lost world” [10]. Despite the careful
63 consideration and rejection of alternative hypotheses for what this specimen might represent [10], its description as a
64 new species was received with skepticism by some researchers [11].

65
66 A study analyzing mitochondrial DNA (mtDNA) sequence data for the only known specimen of *H. zusii* largely settled
67 disagreements about its validity as a species [12]. Phylogenetic analyses remarkably indicated that the specimen is not
68 closely related to species of *Helianzelus*; rather, it was found to be included in a clade with two species in the genus
69 *Aglaiaocercus* from northern South America (Long-tailed Sylph *A. kingii* and Violet-tailed Sylph *A. coelestis*) and the
70 Gray-bellied Comet (*Taphrolesbia griseiventris*), a species in a monotypic genus endemic to semiarid scrub habitats in
71 north-central Peru [12]. In addition, sequence divergence between the *H. zusii* holotype and specimens of *Aglaiaocercus*
72 and *Taphrolesbia* was considered substantial (>5% and 3% p-distance, respectively), validating its status as a distinct
73 species [12]. Accordingly, the South American Classification Committee of the American Ornithological Society
74 presently treats *H. zusii* as valid species [13].

75
76 In 2011, news about observations of a striking hummingbird in montane forests of the Reserva Rogitama, located in
77 the Eastern Cordillera of the Andes in departamento Boyacá, Colombia, produced great excitement among ornithologists
78 and birding enthusiasts, who suspected it might correspond to *H. zusii*. However, after careful examination of a single
79 individual that was captured and released, it was concluded that the Rogitama hummingbird was not *H. zusii*; rather,
80 various phenotypic characters suggested that it was a hybrid, with *A. kingii* and Tyrian Metaltail (*Metallura*
81 *tyrianthina*) hypothesized to be its most likely parents [14]. Given clarity about the identity of the Rogitama bird, *H.*
82 *zusii* remains a lost taxon with no records other than the type specimen and is considered critically endangered if not
83 already extinct [15, 16].

84
85 Intrigued by the finding of the Rogitama bird, we obtained mtDNA sequence data from a feather sample of it to
86 compare it with data from other hummingbird taxa. Upon initial analyses, we were struck to find that the sequence we
87 obtained was remarkably similar to the published sequence of *H. zusii* available in GenBank [12] in the relatively few
88 nucleotide positions in which they overlapped. Considering that phenotypic traits clearly indicate that the Rogitama
89 bird is not *H. zusii*, we began to entertain a new hypothesis, namely that both of these birds are indeed hybrids, with
90 their similar mtDNA indicating a shared maternal species. Our ongoing work on the phylogeography of *A. kingii*, one
91 of the hypothesized parental species of the Rogitama hummingbird [14], allowed us to compare sequences of this species
92 from various regions with those of the Rogitama hummingbird, the holotype of *H. zusii*, and other closely related
93 hummingbird taxa to evaluate this hypothesis.

94
95 MATERIAL AND METHODS
96
97 Detailed analyses of the phylogeny and phylogeography of *Aglaiaocercus* and near relatives will be published elsewhere.
98 For the purpose of this study, we sequenced the ND2 mitochondrial gene (ND2) for 32 individuals of *A. kingii* and two
99 of *A. coelestis*. Sampling was designed to cover the distribution range of *A. kingii*, but was especially thorough in
100 montane regions of Colombia, considering the geographic origin of the Rogitama hummingbird and, hypothetically, of
101 *H. zusii* (Figure 1a, Appendix A). Details about the laboratory methods are described in the electronic supplementary

102 material. We combined our new data with published sequences of *H. zusii* (GenBank Accession GU166851), *T.*
103 *griseiventris* (GU166856), *Adelomyia melanogenys* (JF894047), and *Chalcostigma herrani* (EU042536), with the latter
104 two species designated as outgroups [12, 17]. New sequences were deposited in GenBank (accession numbers XXXX-
105 XXXX). Because we were unable to obtain complete ND2 sequences for the Rogitama hummingbird (we obtained 543
106 base pairs) and the available ND2 sequence of *H. zusii* is also incomplete, overlap in sequence data between these two
107 specimens was restricted only to 71 base pairs. Overlap with sequences of other specimens, however, was much more
108 extensive.

109

110 We estimated ND2 gene trees using maximum likelihood and Bayesian inference methods. Maximum-likelihood
111 reconstructions were conducted in RAxML-HPC version 8 [18] on XSEDE; we calculated nodal support using 1000
112 pseudoreplicates using the CIPRES Science Gateway [19]. The Bayesian analysis was carried out using MrBayes v.3.2.6
113 [20]. We ran four independent runs consisting of four MCMC chains for 10 million generations sampling every 1000
114 generations, discarding the first 25% of the sampled trees as burn in. We implemented a general-time reversible model
115 of nucleotide substitution with gamma-distributed rate heterogeneity among sites (GTR+G) in both analyses as selected
116 using the Akaike Information Criterion in jModelTest [21].

117 RESULTS AND DISCUSSION

118 Mitochondrial DNA data indicate that *A. kingii* exhibits considerable population structure, with distinct lineages
119 occupying different regions in its distribution range (Figure 1b). We found that sequences of the holotype of *H. zusii*
120 and the Rogitama hummingbird are nested within *Aglaiaocercus*, where they clearly belong in a clade formed by
121 individuals of *A. kingii* from the three Cordilleras of the Colombian Andes. Sequences of both “problem” birds are most
122 similar to sequences of individuals from the Eastern Cordillera. In fact, the ND2 sequence of the holotype of *H. zusii*
123 (181 bp) is identical to a sequence of *A. kingii* from Huila (IAvH BT1210; Appendix A) and differs in only one nucleotide
124 from sequences of *A. kingii* from Santander and Norte de Santander (Appendix A). Likewise, the ND2 sequence of the
125 Rogitama hummingbird is identical to sequences observed in individuals from Huila, Santander and Norte de Santander
126 (Colombia), and in individuals from eastern Ecuador and Peru. Indeed, the sequences of *H. zusii* and of the Rogitama
127 hummingbird are identical along the 71 nucleotide sites in which they overlap. Because of the short available sequences
128 of both *H. zusii* and the Rogitama hummingbird, we also analyzed the data considering only the 181 bp fragment we
129 had from *H. zusii* ND2, and results were the same. Phylogenetic analyses of the mtDNA data, however, were unable
130 to recover relationships among clades of *A. kingii* with strong support: major clades formed a polytomy with sequences
131 of *A. coelestis* and *T. griseiventris*, revealing a more complex pattern than suggested by previous phylogenetic studies
132 [12, 17].

133 We interpret the above the results to imply that, contrary to current views, the holotype of *H. zusii* is probably not a
134 representative of a valid species. Rather, it is most likely a hybrid; because mtDNA is maternally inherited, the data
135 suggest it resulted from a cross between a female *A. kingii* and another species of hummingbird. Given the
136 phylogeographic pattern observed, this hybridization event most likely took place in the Eastern Cordillera of the
137 Colombian Andes. Thus, in addition to resolving the status of an enigmatic specimen, our analysis helps to partly clarify
138 its geographic provenance, considering that “Bogota” trade skins may originate from multiple geographic areas in
139 northern South America [22]. Our results also confirm the hypothesis that the hybridization event producing the
140 Rogitama hummingbird involved a female *A. kingii* [14]. The phenotypic differences between the *H. zusii* and the
141 Rogitama hummingbird suggest that although they share *A. kingii* as female parent they were likely sired by males of
142 different species. However, the observation of phenotypic characters not present in either parental species as shown by
143 recent studies of vocalizations [14] and plumage [23] makes hybrid diagnosis in hummingbirds especially complicated.
144 In fact, our findings underscore the difficulty of diagnosing hummingbird hybrids using phenotypic characters even
145 when rigorous protocols are employed [8, 10]. We thus suggest that analyses of sequences of nuclear genes from the
146 holotype of *H. zusii* would be necessary to establish its other parental species with certainty.

147

148 To conclude, we note that our inferred phylogenetic relationships are entirely consistent with those inferred in the study
149 concluding that *H. zusii* is a valid taxon [12], with the radically different conclusion we reached becoming evident only
150 because of our increased taxonomic and geographic sampling. Specifically, because the authors of the earlier study

151 sampled a single individual per species of *Aglaiaocercus* (see also [17]), they were unable to detect that *H. zusii* has
152 *Aglaiaocercus* mtDNA and to uncover the complex pattern of genealogical relationships among populations of *A. kingii*
153 and with respect to other *Aglaiaocercus* and *Taphrolesbia*. Given the unexpected findings of this study and results of
154 other analyses [e.g. 5, 24, 25], we stress that addressing questions about the phylogeny and phylogeography of
155 Neotropical birds –even in cases involving questions about the affinities of a single specimen– requires comprehensive
156 sampling across taxonomy and geography, including multiple individuals per taxon and region.

157

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159

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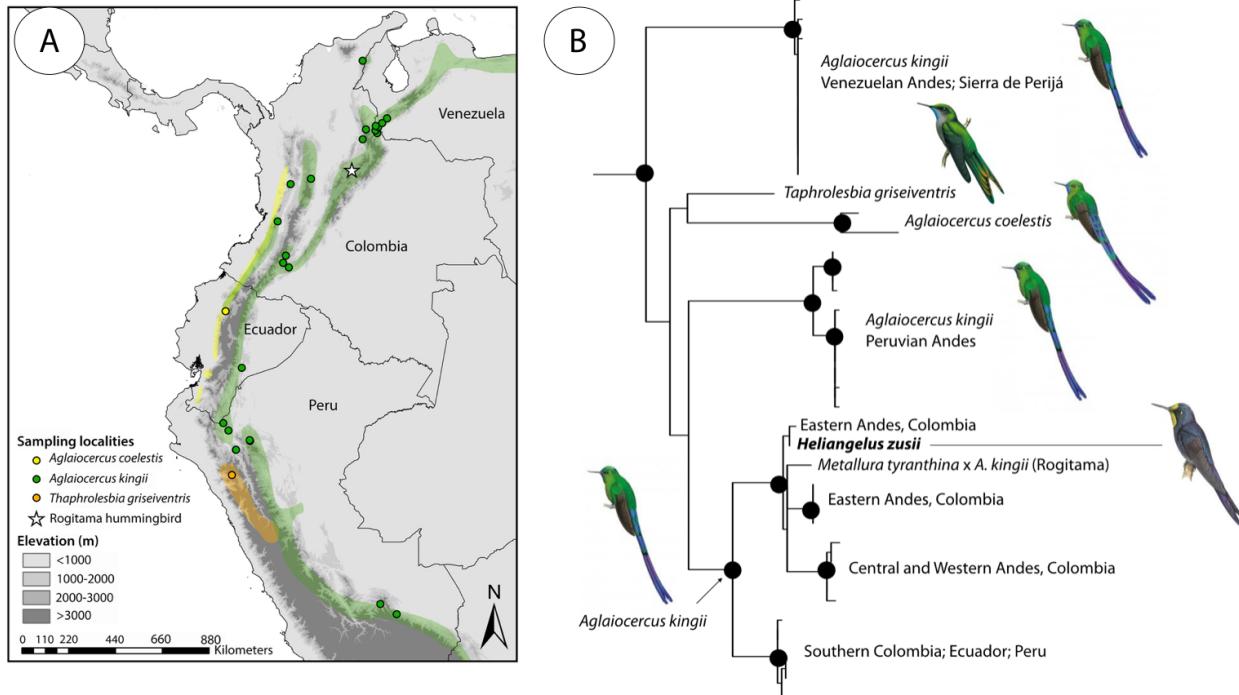
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228 302.

229



230
231

232 Figure 1. (A) Geographic ranges of *Aglaioecrus kingii*, *A. coelestis*, and *Taphrolesbia griseiventris* in northern South
233 America (polygons), and geographic provenance of specimens of these species and of the Rogitama hybrid hummingbird
234 included in molecular phylogenetic analyses (dots and star). (B) Phylogenetic relationships among species and
235 populations of *Aglaioecrus*, *Taphrolesbia*, the Rogitama hybrid hummingbird, and *Heliangelus zusii* based on sequences
236 of the ND2 mitochondrial gene. Strongly supported nodes (0.95 Bayesian posterior probability, 80% maximum-likelihood
237 bootstrap) are indicated with black dots. Although nodal support for deep branches is low, note that both the Rogitama
238 bird and *H. zusii* have haplotypes closely allied to those of *A. kingii* from the Eastern Andes of Colombia, indicating
239 they are both hybrids sharing *A. kingii* as female parent. Illustrations courtesy of Lynx Edicions; Handbook of the Birds
240 of the World, Vol. 15, 1999.

241 **Supplementary Material**

242

243 **Appendix A**

244

245 Tissue samples sequenced in this study for the mitochondrial ND2 gene. Collection acronyms: Colección Ornitológica
246 Phelps (COP), Instituto Alexander von Humboldt (IAvH), Instituto de Ciencias Naturales Colombia (ICN), Museo de
247 Zoología of the Universidad Católica del Ecuador (QCAZ), Louisiana State University Museum of Natural Science
248 (LSUMNH), Museum of Southwestern Biology University of New Mexico (MSB).

249

Taxon	Catalog number	Locality
<i>Aglaioecrus kingii</i>	COP (JM1113)	Venezuela, Táchira, P. N. El Tamá
<i>Aglaioecrus kingii</i>	COP (YPL205)	Venezuela, Táchira, P. N. El Tamá
<i>Aglaioecrus kingii</i>	COP (YPL226)	Venezuela, Táchira, P. N. El Tamá
<i>Aglaioecrus kingii</i>	COP (YPL233)	Venezuela, Táchira, P. N. El Tamá
<i>Aglaioecrus kingii</i>	COP (JM1053)	Venezuela, Táchira, P. N. Chorro El Indio
<i>Aglaioecrus kingii</i>	COP (YPL121)	Venezuela, Táchira, P. N. Páramos El Batallón y La Negra
<i>Aglaioecrus kingii</i>	ICN 37200 (JPL255)	Colombia, Cesar, Manaure
<i>Aglaioecrus kingii</i>	IAvH (SS1172)	Colombia, Casanare, Orocué
<i>Aglaioecrus kingii</i>	IAvH (SS1252)	Colombia, Casanare, Orocué
<i>Aglaioecrus kingii</i>	IAvH (AMC1018)	Colombia, Norte de Santander, Asiria
<i>Aglaioecrus kingii</i>	IAvH BT1780	Colombia, Norte de Santander, Cucutilla
<i>Aglaioecrus kingii</i>	IAvH BT1831	Colombia, Norte de Santander, Cucutilla
<i>Aglaioecrus kingii</i>	IAvH BT5331	Colombia, Santander, Piedecuesta
<i>Aglaioecrus kingii</i>	IAvH BT1210	Colombia, Huila, Acevedo, camino al pesebre
<i>Aglaioecrus kingii</i>	IAvH BT2330	Colombia, Huila, P. N. Puracé
<i>Aglaioecrus kingii</i>	IAvH BT7358	Colombia, Huila, San Agustín, Reserva Natural Los Yalcones
<i>Aglaioecrus kingii</i>	IAvH BT2435	Colombia, Valle, La Cumbre, cuenca alta del río Bitaco
<i>Aglaioecrus kingii</i>	IAvH BT2450	Colombia, Valle, La Cumbre, cuenca alta del río Bitaco
<i>Aglaioecrus kingii</i>	IAvH BT4070	Colombia, Risaralda, Pueblo Rico, P. N. Tatáma
<i>Aglaioecrus kingii</i>	IAvH BT4673	Colombia, Caldas, La Miel
<i>Aglaioecrus kingii</i>	LSUMNH B6216	Ecuador, Morona Santiago, W slope cordillera del Cutucú
<i>Aglaioecrus kingii</i>	LSUMNH B34830	Perú, Cajamarca, Cordillera del Condor, Picorana
<i>Aglaioecrus kingii</i>	LSUMNH 33579	Perú, Cajamarca, Nuevo Perú
<i>Aglaioecrus kingii</i>	MSB:Bird:32143	Perú, Amazonas, 4.5 km N Tullanya
<i>Aglaioecrus kingii</i>	MSB:Bird:32885	Perú, Amazonas, 4.5 km N Tullanya
<i>Aglaioecrus kingii</i>	MSB:Bird:32887	Perú, Amazonas, 4.5 km N Tullanya
<i>Aglaioecrus kingii</i>	LSUMNH 44328	Perú, San Martín, ca 22 km ENE Florida
<i>Aglaioecrus kingii</i>	LSUMNH B44400	Perú, San Martín, ca 22 km ENE Florida
<i>Aglaioecrus kingii</i>	LSUMNH B44447	Perú, San Martín, ca 22 km ENE Florida
<i>Aglaioecrus kingii</i>	MSB:Bird:27271	Perú, Cusco, San Pedro
<i>Aglaioecrus kingii</i>	MSB:Bird:33213	Perú, Cusco, Abra Bella Vista
<i>Aglaioecrus kingii</i>	MSB:Bird:33250	Perú, Cusco, Abra Bella Vista
<i>Aglaioecrus coelestis</i>	QCAZ (CARS159)	Ecuador, Pichincha, Estación Científica Río Guajalito (1900 m)
<i>Aglaioecrus coelestis</i>	QCAZ (CARS168)	Ecuador, Pichincha, Estación Científica Río Guajalito (1900 m)

250

251 **Laboratory Methods**

252

253 We isolated whole genomic DNA using the Dneasy Tissue Kit (Qiagen, Valencia, California) following manufacturer's
254 instructions or a phenol-chloroform method. Amplification of the subunit 2 of the protein-coding gene NADH (ND2,
255 1041 bp) was done using the polymerase chain reaction (PCR) in a 2720 termocycler (Applied Biosystems) and primers

256 L5219 and H6313 (Sorenson et al 1999). PCR conditions included an initial denaturation at 95°C for 8 min, followed
257 by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and an extension phase of 72°C for 60 s. These
258 cycles were ended with a final extension phase of 72°C for 10 m. The PCR reactions contained 1-2 µL of DNA template,
259 0.125 U of *Taq* polymerase (PROMEGA), 14.375 µL H₂O, 1.5 µL MgCl₂, 5 µL buffer solution, 1.25 µL of each primer,
260 and 0.5 µL dNTPs, in a total volume of 25 µL. These PCR products were purified in a 1% low-melting point agarose
261 gels and cycle-sequenced using 1 µL DNA template. Molecular work with the Rogitama hummingbird was conducted
262 in a separate lab at Universidad de los Andes and the data for *H. zusii* were obtained from the literature, implying that
263 contamination of samples is not a plausible explanation for our results.