

## **Shallow evolutionary divergence between two Andean hummingbirds: Speciation with gene flow?**

**Running title:** Divergence with Gene Flow in Hummingbirds

Catalina Palacios<sup>1</sup>, Silvana García-R<sup>1</sup>, Juan Luis Parra<sup>2</sup>, Andrés M. Cuervo<sup>3</sup>, F. Gary Stiles<sup>4</sup>, John E. McCormack<sup>5</sup>, Carlos Daniel Cadena<sup>1</sup>

<sup>1</sup>Laboratorio de Biología Evolutiva de Vertebrados, Departamento de Ciencias Biológicas, Universidad de Los Andes, Carrera 1 No. 18 A 10, Bogotá, Colombia

<sup>2</sup>Grupo de Ecología y Evolución de Vertebrados, Instituto de Biología, Universidad de Antioquia, Calle 67 No. 53-108, Medellín, Colombia

<sup>3</sup>Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Villa de Leyva, Boyacá, Colombia

<sup>4</sup>Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia

<sup>5</sup>Moore Laboratory of Zoology, Occidental College, Los Angeles, California, USA

**Corresponding author:** Catalina Palacios

Laboratorio de Biología Evolutiva de Vertebrados, Departamento de Ciencias Biológicas, Universidad de Los Andes, Carrera 1 No. 18 A 10, J303, Bogotá, Colombia

dc.palacios10@uniandes.edu.co

### **Author contributions:**

All authors contributed with data, discussion and reviewing the manuscript. CP performed the analysis.

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1 **Shallow evolutionary divergence between two Andean hummingbirds: Speciation with gene**  
2 **flow?**

3 **Abstract**

4 Ecological speciation can proceed despite genetic interchange when selection counteracts  
5 homogenizing effects of migration. We tested predictions of this divergence-with-gene-flow  
6 model in *Coeligena helianthea* and *C. bonapartei*, two parapatric Andean hummingbirds with  
7 marked plumage divergence. We sequenced neutral markers (mtDNA and nuclear ultra  
8 conserved elements) to examine genetic structure and gene flow, and a candidate gene (*MC1R*) to  
9 assess its role underlying divergence in coloration. We also tested the prediction of Gloger's rule  
10 that darker forms occur in more humid environments, and compared ecomorphological variables  
11 to assess adaptive mechanisms potentially promoting divergence. Genetic differentiation between  
12 species was very low and coalescent estimates of migration were consistent with divergence with  
13 gene flow. *MC1R* variation was unrelated to phenotypic differences. Species did not differ in  
14 macroclimatic niches but were distinct in ecomorphology. Although we reject adaptation to  
15 variation in humidity as the cause of divergence, we hypothesize that speciation likely occurred  
16 in the face of gene flow, driven by other ecological pressures or by sexual selection. Marked  
17 phenotypic divergence with no neutral genetic differentiation is remarkable for Neotropical birds,  
18 and makes *C. helianthea* and *C. bonapartei* an appropriate system in which to search for the  
19 genetic basis of species differences employing genomics.

20

21 **Keywords:** Andes, ecological speciation, Gloger's rule, niche

22

## 23 **Introduction**

24 New species often arise when geographic isolation of populations allows for divergence via  
25 genetic drift or selection (Mayr 1963; Coyne and Orr 2004). Central to this speciation model are  
26 the ideas that geographic isolation restricts gene flow, thus allowing for differentiation, and that  
27 speciation without geographic isolation is unlikely because gene flow homogenizes populations  
28 (Coyne and Orr 2004). Alternatively, the divergence-with-gene-flow model proposes that  
29 speciation is possible without geographic isolation if selection is sufficiently strong to counteract  
30 the homogenizing effect of gene flow (Gavrilets 1999; Nosil 2008; Pinho and Hey 2010; Martin  
31 et al. 2013; Morales et al. 2017). Under this model, phenotypic differentiation may develop in the  
32 face of gene flow owing to divergent selection acting on traits directly associated with  
33 reproduction or on traits associated with those involved in reproduction through pleiotropic  
34 effects (Schluter 2001; Servedio 2016). Assortative mating or selection against hybrids may  
35 further facilitate the completion of reproductive isolation (Coyne and Orr 2004; Fitzpatrick et al.  
36 2009; Schluter 2009).

37 Several studies provide evidence that natural selection can generate phenotypic divergence  
38 among populations despite gene flow (e.g. Smith 1997; Morgans et al. 2014; Fitzpatrick et al.  
39 2015) and this could lead to speciation (Hey 2006; Nosil 2008). However, documenting  
40 speciation with gene flow is complicated because of the difficulty of determining whether shared  
41 genetic variation between species is a consequence of divergence in the presence of migration or  
42 rather an indication of post-speciation hybridization or incomplete lineage sorting of gene lineages  
43 due to recent divergence (Hey 2006; Pinho and Hey 2010). This difficulty has been partly  
44 overcome thanks to the development of coalescent-based tools to estimate migration since  
45 divergence between pairs of populations (Hey and Nielsen 2004, 2007; Beerli 2006; Kuhner  
46 2006; Durand et al. 2011). Some studies using such tools have found incomplete lineage sorting  
47 as the cause for lack of genetic differentiation (Nosil et al. 2009; Wall et al. 2013; Suh et al.  
48 2015), whereas others support population divergence despite gene flow (Green et al. 2010;  
49 Rheindt et al. 2014; Supple et al. 2015; Kumar et al. 2017). However, compelling evidence that  
50 population divergence has scaled up to the formation of different species in the face of gene flow  
51 remains limited. Nonetheless, the finding that the evolutionary histories of various organisms are

52 characterized by substantial cross-species genetic exchange (e.g. Novikova et al. 2016; Zhang et  
53 al. 2016; Kumar et al. 2017) implies that attention should be devoted to understanding the  
54 selective mechanisms maintaining species as distinct entities in the face of gene flow.

55 In birds, plumage traits are often targets of natural selection. This results in adaptations for  
56 foraging and flight efficiency (Zink and Remsen 1986), camouflage (Zink and Remsen 1986) or  
57 conspicuousness (Endler 1993), thermoregulation (Walsberg 1983), and protection against  
58 pathogens (Burt and Ichida 2004; Goldstein et al. 2004; Shawkey et al. 2007), among others.  
59 Because plumage traits are also critical in mate selection and species recognition, plumage  
60 divergence may drive lineage diversification (Price 2008; Servedio et al. 2011; Hugall and Stuart-  
61 Fox 2012; Maia et al. 2013). A frequently observed pattern in presumably adaptive plumage  
62 variation is Gloger's rule, which states that birds with darker plumage coloration occur in more  
63 humid environments than lighter-colored conspecifics (Burt and Ichida 2004). This pattern is  
64 often attributed to adaptation to reduce bacterial degradation of plumage in humid conditions  
65 where bacteria are most abundant, because melanin (the pigment responsible for black plumage  
66 color) confers resistance against these microbes (Goldstein et al. 2004; Peele et al. 2009; Amar  
67 et al. 2014). Because differences in melanic pigmentation can serve as cues for mate choice and  
68 species recognition (Uy et al. 2009), adaptive differentiation in plumage coloration might thus  
69 drive the origin of reproductive isolation. However, we are unaware of studies explicitly relating  
70 the evolution of melanic plumage coloration by natural selection to population divergence or  
71 speciation in the presence of gene flow (but see Rosenblum et al. 2017; Pfeifer et al. 2018 for  
72 examples of involving skin pigmentation in other animals).

73 Here, we test the divergence-with-gene-flow model of speciation as an explanation for the  
74 evolution of two Andean hummingbird species, *Coeligena helianthea* (Blue-throated Starfrontlet)  
75 and *Coeligena bonapartei* (Golden-bellied Starfrontlet). We studied these species because: (1)  
76 they have largely parapatric ranges in a topographically complex area of the Andes over which  
77 environmental conditions (hence selective pressures) may differ (Fig. 1). (2) They lack genetic  
78 differentiation in neutral markers (Parra et al. 2009; McGuire et al. 2014) as expected under  
79 divergence with gene flow. (3) They exhibit distinct phenotypic differences (plumage in *C.*  
80 *helianthea* is considerably darker than in *C. bonapartei*) and no hybrids have been reported even

81 where they coexist locally (except perhaps for a few old specimens; Fjeldså & Krabbe, 1990).  
82 And (4), because variation in melanic pigmentation may reflect adaptation to different  
83 environments, differentiation in plumage traits between these hummingbird species might have  
84 been driven by natural selection.

85 The apparent lack of genetic differentiation between *C. helianthea* and *C. bonapartei* (Parra et al.  
86 2009; McGuire et al. 2014) despite their distinct differences in potentially adaptive traits may  
87 reflect divergence with gene flow, contemporary hybridization, or incomplete lineage sorting  
88 (Hey 2006; Suh et al. 2015; Sonsthagen et al. 2016). We here evaluate predictions of the  
89 divergence-with-gene-flow model of speciation and consider the evolutionary mechanisms  
90 driving divergence between these species by first addressing the following questions: (1) does the  
91 lack of genetic differentiation between *C. helianthea* and *C. bonapartei* persist with a much  
92 larger and geographically extensive sampling and additional molecular markers relative to earlier  
93 work (Parra et al. 2009)?, and (2) are patterns of genetic variation consistent with a model of  
94 divergence in the face of gene flow? We next asked (3) is color divergence associated with  
95 genetic variation in the *MC1R* gene, a candidate underlying melanic coloration in various bird  
96 species and other vertebrates? To examine possible mechanisms through which natural selection  
97 might have driven population differentiation we examined whether phenotypic divergence may  
98 be attributable to adaptation to contrasting macro-environmental conditions by asking (4) is *C.*  
99 *helianthea* with darker plumage distributed in more humid environments as predicted by Gloger's  
100 rule? and (5) is there morphometric variation between species that may suggest adaptations to  
101 alternative microhabitats or resources?

102

## 103 **Materials and Methods**

### 104 **Study system**

105 *Coeligena helianthea* inhabits mostly the eastern slope of the Cordillera Oriental of the Northern  
106 Andes from western Meta in Colombia to the Táchira Depression in Venezuela, and comprises  
107 two subspecies: *C. h. helianthea* occupies most of the range, whereas *C. h. tamai* occurs in the  
108 Tamá Massif in the border between Colombia and Venezuela (Fig. 1). The distribution of

109 *Coeligena bonapartei* is not continuous and three subspecies are recognized: 1) *C. b. bonapartei*  
110 ranges along the western slope of the Cordillera Oriental in Cundinamarca, Boyacá, and western  
111 Santander in Colombia, 2) *C. b. consita* is restricted to the Serranía del Perijá, and 3) *C. b. eos* is  
112 endemic to the Cordillera de Mérida in the Venezuelan Andes (Hilty and Brown 1986; Hilty  
113 2003. Fig. 1). Some authors consider the Venezuelan taxon *C. b. eos* a distinct species (Del-  
114 Hoyo et al. 1999; Donegan et al. 2015), but it is currently treated as a subspecies of *C. bonapartei*  
115 (Remsen et al. 2017). Although the distributions of *C. bonapartei* and *C. helianthea* are not  
116 sympatric for the most part, the nominate subspecies co-occur regionally in Cundinamarca and  
117 Boyacá (Gutiérrez-Zamora 2008).

118 *C. helianthea* and *C. bonapartei* differ strikingly in plumage coloration. Although both species  
119 have bright green crowns and violet gorgets, males of *C. helianthea* are considerably darker, with  
120 a largely greenish black with a rose belly and aquamarine rump; males of *C. bonapartei* are  
121 largely golden green with fiery gold underparts and rump. Females are paler than males, but also  
122 differ distinctly in plumage, especially in their lower underparts (Hilty and Brown 1986; Parra  
123 2010). The differences in coloration between species may reflect variation in the melanin content  
124 of feathers (D’Alba et al. 2014), but may also be due to differences in the nanostructure of feather  
125 barbules (i.e. width of the air spaces or keratin layer), which interferes with light to generate the  
126 reflected colors (Greenewalt et al. 1960).

## 127 **Tissue samples and DNA sequencing protocols**

128 We collected specimens in Colombia and Venezuela, and obtained tissue samples from the  
129 collections of the Instituto Alexander von Humboldt (IAvH), the Museo de Historia Natural de la  
130 Universidad de los Andes (ANDES), and the Colección Ornitológica Phelps (Table S1). Our  
131 sampling included a total of 62 individuals: 38 specimens of *C. bonapartei* (12 *C. b. bonapartei*,  
132 5 *C. b. consita*, and 21 *C. b. eos*) and 24 specimens of *C. helianthea* (7 *C. h. helianthea*, 17 *C. h.*  
133 *tamai*). Subspecies were assigned based on taxonomic determination of museum specimens or by  
134 geography. We extracted DNA from tissue samples using either a QIAGEN DNeasy Tissue Kit  
135 (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions or a standard  
136 phenol/chloroform extraction protocol. For 60 specimens we amplified by PCR (Methods S1)  
137 and sequenced 1041 bp of the mitochondrial *ND2* gene, and used the data for range-wide

138 phylogeographic and population genetic analyses. We used published sequences of *C. lutetiae*  
139 (McGuire et al. 2007; Parra et al. 2009) and *C. orina* (McGuire et al. 2014) as outgroups in  
140 phylogenetic analyses.

141 We used a subset of 36 individuals to assess whether color differentiation between species is  
142 associated with nucleotide substitutions in the coding region of the *melanocortin-1 receptor* gene  
143 (*MC1R*), a locus responsible for melanic pigmentation in several birds and other vertebrates  
144 (Mundy 2005; Roulin and Ducrest 2013). We amplified by PCR (Methods S1) and sequenced  
145 788 bp of the 945 bp of the *MC1R* locus for 6 individuals of *C. h. helianthea*, 10 *C. h. tamai*, 8 *C.*  
146 *b. bonapartei*, 1 *C. b. consita* and 11 *C. b. eos*. All PCR products were cleaned and sequenced in  
147 both directions by MacroGen Inc. or at the sequencing facilities of the Universidad de los Andes.  
148 We assembled, edited, and aligned sequences of the *ND2* and *MC1R* genes using BioEdit 7.2.5  
149 (Hall 1999) and Geneious 9.1.5 (<http://www.geneious.com/>; Kearse et al., 2012), employing the  
150 MUSCLE algorithm and manual editing.

151 We also employed a sequence capture approach to acquire data from regions flanking  
152 ultraconserved elements (UCEs; Faircloth et al. 2012) for 1 individual of *C. h. helianthea*, 4 *C. h.*  
153 *tamai*, 1 *C. b. bonapartei*, and 1 *C. b. consita* to obtain a preliminary overview of genetic  
154 divergence between these taxa at a genomic level. We used a standard library preparation  
155 protocol (<http://ultraconserved.org/>; Faircloth & Glenn, 2012) and enriched the pool of samples  
156 for 5,060 UCE loci using the MYbaits\_Tetrapods-UCE-5K probes. We sequenced the pool after  
157 quantification using Illumina MiSeq. Following the PHYLUCE pipelines (Faircloth 2015), we  
158 used Illuminoprocessor (Faircloth 2013) and Trimmomatic (Bolger et al. 2014) to trim reads,  
159 discarded adapter contamination and low-quality bases, and assembled the reads into contigs  
160 using a kmer=50 and ABySS (Simpson et al. 2009). We aligned the contigs against the original  
161 UCE probes to identify contigs matching UCE loci using LASTZ (Harris 2007). Among the  
162 individuals, we aligned UCE loci using the default MAFFT v7.13 algorithm (Katoh and Standley  
163 2013). Finally, we pulled out UCE loci from the Anna's Hummingbird (*Calypte anna*) genome  
164 (Gilbert et al. 2014; Zhang et al. 2014) to use them as outgroup.

165 For phylogenetic analysis we used a concatenated alignment of 2,313 UCE loci shared at least  
166 among 3 individuals including the outgroup. Of these, 1,465 loci were present in all the



167 individuals (mean locus length = 615.1 bp, mean number of individuals per locus in the  
168 incomplete matrix = 7.3). We generated a second concatenated alignment of 1,604 loci shared  
169 among all *Coeligena* specimens (i.e. without the outgroup). Of these, 389 loci showed no  
170 variation, 75 had only indels (informative or not), 614 had singletons and indels, and 526 (32.8%)  
171 had informative sites (polymorphic sites with each variant represented in at least two  
172 individuals). We used the latter 526 loci for population genetic analyses aimed at assessing gene  
173 flow.

#### 174 **Phylogenetic and Population Genetic Analysis**

175 We used maximum-likelihood and Bayesian inference methods to reconstruct phylogenies from  
176 the CIPRES Portal (<http://www.phylo.org/>) or locally. We conducted maximum-likelihood  
177 analyses in RAxML (Stamatakis 2014) using the GTR+GAMMA model and non-parametric  
178 bootstrapping under the autoMRE stopping criterion for *ND2* and UCE data. We conducted  
179 Bayesian analyses in Mr.Bayes v3.2 (Ronquist et al. 2012) using a single partition and the HKY  
180 model for *ND2* data, which was the best fit according to JModelTest 2.1.7 (Posada 2008; Darriba  
181 et al. 2012). For UCE data we used 16 partitions and the models for each of these suggested by  
182 CloudForest analysis (Crawford and Faircloth 2011). The MCMC parameters consisted of two  
183 runs with four chains ran for 15 million generations sampling every 100 generations for the *ND2*  
184 data, and ran for 25 million generations sampling every 500 generations for the UCE data. We  
185 discarded the first 10% generations as burn-in before estimating the consensus tree and posterior  
186 probabilities. Convergence and effective sample sizes of parameter estimates were examined  
187 using Tracer 1.6 (Rambaut et al. 2016).

188 To further examine relationships among *ND2* haplotypes, we used an alignment of 885 bp for  
189 which complete data were available for all individuals to construct a haplotype network in  
190 Network 5.0.0.1 (<http://www.fluxus-engineering.com/>; Bandelt, Forster, & Röhl, 1999). To  
191 examine genetic structure between species, we calculated *Fst* with R package hierfstat (Goudet  
192 and Jombart 2015) and AMOVAs with R package ade4 (Dray and Dufour 2007) assessing  
193 significance using 10,000 permutations (Script S1). Also, we used the program Structure 2.3.4  
194 (Pritchard et al. 2000) to assess population structure using UCE data. We performed 20 runs for  
195 each value of *K* from *K*=1 to *K*=5, using a burnin period of 10,000 steps and 100,000 repetitions.

196 We followed Pritchard et al. (2000) to calculate the probability of different values of  $K$  using the  
197 mean ln likelihood value calculated over the 20 runs as  $\text{prob}(K=n) = (\text{eln}K=n) /$   
198  $(\text{eln}K=1 + \dots + \text{eln}K=n)$ .

### 199 **Testing for Divergence with Gene Flow**

200 We used Migrate 3.2.1 (Beerli 2009) to examine whether lack of genetic differentiation observed  
201 between *C. helianthea* and *C. bonapartei* is more likely a result of speciation in the face of gene  
202 flow or rather a consequence of hybridization following secondary contact. In addition to  
203 estimating parameters such as effective population size scaled by mutation rate ( $\theta$ ) and migration  
204 scaled by mutation rate ( $M$ ), Migrate can estimate parameters for different time bins, allowing  
205 one to estimate migration at different moments through time. If there has been gene flow between  
206 species after speciation, then posterior distributions of migration estimated as  $M=(m/\mu)$  should  
207 exclude values of zero. Given non-zero estimates of migration, divergence-with-gene-flow  
208 predicts higher values of  $M$  close to the time of divergence, whereas post-speciational gene flow  
209 (i.e. recent hybridization) predicts higher values of  $M$  near the present.

210 We used two data sets for Migrate analyses. First, we employed a *ND2* alignment of 885 bp  
211 (excluding all the positions with missing data) including 22 individuals of *C. helianthea* and 17  
212 individuals of *C. b. bonapartei/consita* (i.e. excluding *C. b. eos*, which we found to be genetically  
213 distinct; see below). Second, we used an alignment of 591 SNPs (368 informative sites) derived  
214 from 296 UCE loci; we used only those UCE loci having data for all seven individuals and  
215 considered only sites where SNPs showed variation between at least two individuals. Because  
216 inference of gene flow requires using markers that have evolved neutrally, we first confirmed that  
217 both data sets meet this assumption by calculating Tajima's  $D$  using DNAsp 5.1 (Librado and  
218 Rozas 2009).

219 We determined prior maximum values for the parameters  $\theta$  and  $M$  for each species and each data  
220 set based on several test runs. In final analyses aimed to estimate gene flow using both *ND2* and  
221 UCE data, we set prior values to 0.1 for  $\theta$  for both species, and to 1,000 for  $M$  from *C.*  
222 *bonapartei* to *C. helianthea* and to 600 for  $M$  from *C. helianthea* to *C. bonapartei*. We ran

223 Migrate in the CIPRES Portal (<http://www.phylo.org/>) using a long chain of 300 million steps  
224 (sampling 100,000 steps recorded every 3,000 steps) with a burn-in of 100,000 steps.

### 225 ***MCIR* gene analyses**

226 We compared variable sites in *MCIR* sequences between our study species and translated  
227 sequences to aminoacids to check for synonymous and non-synonymous substitutions. As  
228 references for comparisons we used sequences of *Calypte anna* and Chimney Swift (*Chaetura*  
229 *pelagica*) predicted from genome annotations (Zhang et al. 2014). Because these comparisons  
230 revealed no variation potentially implied in phenotypic variation (see results), we did not conduct  
231 any additional analysis.

### 232 **Examining the selective regime: niches and ecomorphological differentiation**

233 We tested the hypothesis that natural selection underlies the phenotypic divergence in color  
234 between *C. helianthea* and *C. bonapartei* through macroclimatic differences in the regions  
235 occupied by these species. Specifically, we tested the prediction of Gloger's rule that *C.*  
236 *helianthea* (with darker plumage) occurs in environments with more humid conditions than *C.*  
237 *bonapartei*, and examined whether other macroclimatic conditions that may promote adaptation  
238 differ between environments occupied by these hummingbirds. We examined ecological  
239 differentiation among *C. helianthea*, *C. b. bonapartei/consita* and *C. b. eos* (which we found to  
240 be genetically distinct; see below) using occurrence data, environmental variables, and  
241 measurements of niche overlap (Broennimann et al. 2012). In addition to the locality data  
242 associated with specimens included in molecular analyses, we obtained occurrence data from  
243 eBird (<http://ebird.org/content/ebird/>), Vertnet (<http://vertnet.org/>), GBIF (<http://www.gbif.org/>),  
244 Xeno-canto (<http://www.xeno-canto.org/>), and the ornithological collection of the Instituto de  
245 Ciencias Naturales of the Universidad Nacional de Colombia  
246 (<http://www.biovirtual.unal.edu.co/en/>), for a total of 242 records. After eliminating duplicates  
247 and excluding non-reliable locations we retained 196 records for analysis: 85 of *C. helianthea*, 75  
248 of *C. b. bonapartei/consita*, and 36 of *C. b. eos*.

249 To delimit the accessible areas for each species we used ecoregions as defined by Dinerstein et al.  
250 (Dinerstein et al. 2017). We used all the ecoregions with occurrence records as the environmental

251 background available for the analysis of niche overlap. We obtained climatic data from  
252 WorldClim (<http://www.worldclim.org/> Hijmans et al. 2005), CliMond  
253 (<https://www.climond.org/> Kriticos et al., 2012), and EarthEnv (<http://www.earthenv.org/cloud>  
254 Wilson and Jetz 2016). We selected and excluded variables highly correlated to others  
255 (Threshold: 0.7) using the package usdm (Naimi 2015) in R (R Core Team 2016). We conducted  
256 niche overlap analyses using 11 variables: three related to temperature, three related to  
257 precipitation, four related to cloudiness, and one related to air moisture (Table S2).

258 We extracted climatic data from 10,000 points from the background environment and from the  
259 196 occurrence records and performed a principal component analysis (PCA) to summarize  
260 climatic variation using the R package ade4 (Dray and Dufour 2007). With the two first PCA  
261 axes, we plotted the densities of each taxon in climatic space relative to the background using R  
262 package ecospat (Broennimann et al. 2016). We also used this package to estimate the D statistic  
263 (Warren et al. 2008) to quantify niche overlap ( $D = 0$  indicates different niches, and  $D = 1$   
264 indicates identical niches), and we performed similarity tests (1,000 iterations) to assess whether  
265 niches are less similar (niche divergence) than expected by chance given background climatic  
266 variation (Script S2). Significant niche divergence with the darker *C. helianthea* occupying more  
267 humid areas would be consistent with adaptive divergence following Gloger's rule, whereas no  
268 significant differences in niches would suggest that adaptation to distinct climatic conditions  
269 cannot account for phenotypic differentiation between species.

270 We also assessed whether there is morphometric differentiation between species which may  
271 reflect adaptation to different microhabitats or food resources (Stiles 2008) by measuring 17 traits  
272 related to beak, wing, tail and leg morphology (Table S3). We measured morphological variables  
273 from 35 live individuals (17 females and 18 males) of *C. h. helianthea* and 46 individuals (23  
274 females and 23 males) of *C. b. bonapartei*. Using these data we asked whether individuals of  
275 different species and sexes are distinguishable in multivariate space employing linear  
276 discriminant analysis (LDA) using R package MASS (Venables and Ripley 2002). We also built  
277 ANOVA models to test for mean differences in individual variables among species and sexes  
278 simultaneously (Script S3).

279

## 280 Results

### 281 Does lack of genetic differentiation between *C. helianthea* and *C. bonapartei* persist with 282 greater sampling and additional markers?

283 We found low genetic differentiation between *C. b. bonapartei* and *C. b. consita*, but both taxa  
284 were markedly differentiated from *C. b. eos*. Therefore, in the following we treat *C. b. bonapartei*  
285 and *C. b. consita* as a single group, which we refer to as *C. b. bonapartei/consita*. Divergence in  
286 *ND2* of *C. helianthea* and *C. b. bonapartei/consita* relative to *C. b. eos* was high, with significant  
287 *Fst* values of 0.56 and 0.52, respectively ( $p < 0.001$  in both cases), and relatively high fractions of  
288 genetic variance (59.4% and 52.0%, respectively) existing between groups in AMOVA. In  
289 contrast, *ND2* data showed little to no differentiation between *C. helianthea* and *C. b.*  
290 *bonapartei/consita*. Although differentiation as measured by *Fst* was significant ( $p = 0.03$ ), the  
291 *Fst* value was very low (0.07) and only 1.7% of the variance was partitioned between these two  
292 taxa in AMOVA, with 98.3% of the variance existing among individuals within taxa.

293 Phylogenetic analyses showed that *C. b. eos* forms a strongly supported clade (posterior  
294 probability  $PP = 1.0$ , maximum-likelihood bootstrap  $MLbs = 87\%$ ), which is sister to a  
295 moderately supported clade ( $PP = 0.77$ ,  $MLbs = 69\%$ ) formed by *C. helianthea* and *C. b.*  
296 *bonapartei/consita* (Fig. 2A). Within the latter clade, relationships among populations appeared  
297 to be determined more by geography than by current species-level taxonomy: most sequences of  
298 the northern subspecies *C. b. consita* and *C. h. tamai* formed a strongly supported clade ( $PP =$   
299  $1.0$ ,  $MLbs = 85\%$ ), whereas the majority of sequences of southern subspecies *C. b. bonapartei*  
300 and *C. h. helianthea* formed another moderately supported clade ( $PP = 0.94$ ,  $MLbs = 61\%$ ).

301 Haplotype networks confirmed the above findings (Fig. 2B): (1) *C. helianthea* and *C. b.*  
302 *bonapartei/consita* shared haplotypes, whereas *C. b. eos* did not share any haplotypes with the  
303 other taxa; and (2) haplotype groups were more consistent with geography than with taxonomy.  
304 However, networks showed that the latter pattern is not perfect because two individuals of *C. b.*  
305 *bonapartei* (from the south) had the haplotype most common in the north, one *C. h. tamai* (from  
306 the north) had the haplotype most common in the south, and one *C. b. bonapartei* had an  
307 intermediate haplotype.

308 UCE nuclear markers also did not reveal genetic differentiation between *C. helianthea* and *C.*  
309 *bonapartei*. The UCE phylogeny shows a well-supported clade including all sequences of *C.*  
310 *helianthea* nested within a clade in which the two earliest diverging branches were the two  
311 specimens of *C. bonapartei* (Fig. 2C). Additionally, population genetic structure between species  
312 in UCE markers was not significant ( $F_{st} = 0.2$ ,  $p = 0.5$ ), and the most likely number of genetic  
313 clusters in the data set according to Structure was  $K = 1$  ( $\text{prob}_{(K=1)} = 0.8$ ). Support for larger  
314 values of  $K$  was much lower and clusters defined assuming different values of  $K$  never  
315 corresponded to groups defined by species identity (Fig. S1).

### 316 **Are patterns of genetic variation consistent with divergence in the face of gene flow?**

317 Our data sets fit the assumption of neutrality, allowing one to use them for gene flow inference:  
318 Tajima's  $D$  values were not significant for either marker (*ND2*  $D = -0.68$   $p > 0.1$ , UCEs  $D = 0.22$   
319  $> 0.1$ ). The analysis of both *ND2* and UCE data suggested that there has been gene flow between  
320 *C. helianthea* and *C. bonapartei* after their divergence. Mean estimates of migration ( $M = m/\mu$ )  
321 were in all cases different from zero:  $M = 725.1$  from *C. helianthea* to *C. bonapartei* and 446.1  
322 from *C. bonapartei* to *C. helianthea* for *ND2*, and  $M = 869.9$  from *C. helianthea* to *C. bonapartei*  
323 and 555.4 from *C. bonapartei* to *C. helianthea* for UCE data. However, the posterior probability  
324 distributions of  $M$  estimated from the *ND2* data were wide: 95% credibility intervals ranged from  
325 284.7 to 1,000 from *C. helianthea* to *C. bonapartei*, and from 0.0 to 628 from *C. helianthea* to *C.*  
326 *bonapartei* (Fig. 3A). In contrast, posterior distributions of  $M$  estimated from UCE data were  
327 narrowly concentrated around the mean: 95% credibility intervals ranged from 782.0 to 958.7  
328 from *C. helianthea* to *C. bonapartei* and from 482.0 to 626.7 from *C. bonapartei* to *C. helianthea*  
329 (Fig. 3C), rejecting scenarios of no migration after divergence.

330 Estimates of migration through time further supported that divergence occurred and has been  
331 maintained in the face of gene flow as predicted by the divergence-with-gene-flow model of  
332 speciation. Our analyses indicated that migration between *C. helianthea* and *C. bonapartei*  
333 continued after their initial divergence (i.e. the estimated time of their most common recent  
334 ancestor, Fig. 3B,D). Whereas *ND2* data suggested that gene flow has continued until the present  
335 (Fig. 3B), the UCE data suggested that gene flow likely ceased at approximately half the time  
336 passed since these species last shared a common ancestor (Fig. 3D).



337 **Is color divergence associated with genetic variation in *MC1R*?**

338 Of the 36 *Coeligena* individuals sampled for *MC1R*, 32 shared a haplotype (excluding ambiguous  
339 positions). Genetic variation at *MC1R* was limited to three individuals of *C. helianthea* and one  
340 individual of *C. bonapartei*, and involved changes in four sites. Only one change was non-  
341 synonymous (Ser275 [AGC] → Arg275 [AGG] at nucleotide site 825), but it was present in a  
342 single *C. helianthea* (Andes-BT 1126) with typical plumage coloration. These results reveal no  
343 association between *MC1R* genotype and species-specific color phenotypes in *C. helianthea* and  
344 *C. bonapartei*.

345 **Is *C. helianthea* with darker plumage distributed in more humid environments as predicted  
346 by Gloger's rule?**

347 We found no support for the prediction that the more darkly colored *C. helianthea* occurs in more  
348 humid environments than *C. b. bonapartei/consita*: the climatic niches of these taxa overlap  
349 considerably ( $D = 0.65$ , Fig. 4A) and we found no evidence for significant niche divergence  
350 relative to background climate ( $p = 0.99$ ). Niche overlap between *C. b. eos* and *C. b.*  
351 *bonapartei/consita* and *C. helianthea* was considerably lower ( $D = 0.07$  and  $0.10$ , respectively,  
352 Fig. 4B), but relative to the background niche differences were not significant ( $p = 0.70$  and  $0.76$ ,  
353 respectively).

354 **Is there morphometric variation between species that may suggest adaptations to  
355 alternative microhabitats or resources?**

356 Morphometric data showed differences between *C. h. helianthea* and *C. b. bonapartei* and  
357 between females and males of each taxon: LDA analysis distinguished species/sex with a low  
358 classification error of 1.2%. The two most relevant variables in the LD function were wing  
359 loading (coefficients: LD1 = 240.2, LD2 = 287.8, and LD3 = 120.0), and wing taper  
360 (coefficients: LD1 = -39.7, LD2 = -42.6, and LD3 = -23.4). ANOVA models showed significant  
361 differences in 12 morphological variables between species, and in 15 variables between sexes  
362 (see Fig. S2). The three variables that differed the most between species and sexes were length of  
363 extended wing (ANOVA coefficients: -3.4 species and 5.9 sex), total culmen (ANOVA  
364 coefficients: 2.3 species and 2.2 sex), and length of tail (ANOVA coefficients: 1.0 species and

365 3.7 sex). *Coeligena b. bonapartei* has longer wings, shorter bills and shorter tails than *C. h.*  
366 *helianthea* ( $p = < 0.001$  in all cases), and females have shorter wings, longer bills and shorter tails  
367 than males in both species ( $p = < 0.001$  in all cases). Our analyses further revealed that the  
368 magnitude of morphometric differences between sexes varied by species. For example, females  
369 of *C. helianthea* are the smallest of the four groups (i.e. combinations of species and sexes), but  
370 males of *C. helianthea* are the largest.

371

## 372 **Discussion**

373 *C. helianthea* and *C. bonapartei* are sister species of hummingbirds from the Northern Andes that  
374 differ distinctly in plumage coloration, but we found a striking lack of genetic differentiation  
375 between them in a mitochondrial gene (*ND2*) and in 1,604 UCE markers broadly scattered across  
376 the genome. The strong phenotypic differences between *C. helianthea* and *C. bonapartei* in the  
377 absence of neutral genetic differentiation are remarkable for Neotropical birds, and make these  
378 species an appropriate system in which to search for the genetic basis and adaptive significance  
379 of phenotypic differences involved in speciation (see Campagna et al. 2017). However, we found  
380 no evidence that *MC1R* (a candidate gene associated with melanic pigmentation in a variety of  
381 vertebrates) underlies phenotypic variation, and found no support for the hypothesis that Gloger's  
382 rule (adaptation to geographic variation in humidity) or other macroclimatic niche differences are  
383 associated with phenotypic divergence between these species. Nonetheless, coalescent estimates  
384 of migration indicate that *C. helianthea* and *C. bonapartei* diverged in the presence of gene flow,  
385 suggesting that phenotypic differences likely originated under selective pressures strong enough  
386 to offset the homogenizing effects of migration. Our finding that *C. h. helianthea* and *C. b.*  
387 *bonapartei* differ in morphometric traits related to habitat and resource use is consistent with the  
388 hypothesis that natural selection may have played a role in their divergence. In addition, as we  
389 discuss below, phenotypic divergence may have been maintained in the face of gene flow  
390 because of sexual selection.

391 Although shallow genetic divergence between species may also result from processes including  
392 incomplete lineage sorting or contemporary hybridization after secondary contact, our coalescent



393 analyses are consistent with a scenario where *C. helianthea* and *C. b. bonapartei/consita* (i.e.  
394 excluding *C. b. eos*) have exchanged genes since their divergence from a common ancestor. Our  
395 inference of gene flow is likely robust because both *ND2* and UCE data fit neutrality (Hey and  
396 Nielsen 2004). Thus, our data are consistent with the hypothesis that speciation occurred in the  
397 face of gene flow, with divergence in loci underlying phenotypic differences between species  
398 likely maintained by some form of selection. Future work should conduct analyses examining  
399 other markers across the genome because this may allow reducing uncertainty in the estimation  
400 of population genetic parameters (Hey and Nielsen 2004), may help rule out other processes such  
401 as incomplete lineage sorting (Suh et al. 2015), and may allow one to identify the genetic basis of  
402 phenotypic differences (Bourgeois et al., 2016, Toews et al. 2016, Campagna et al. 2017).

403 We found no variation between species in the coding region of *MC1R*, a gene associated with  
404 variation in plumage coloration in several other birds (Theron et al. 2001; Doucet et al. 2004;  
405 Mundy 2004; Baião et al. 2007; Gangoso et al. 2011). Thus, as with other studies showing no  
406 association between plumage coloration and variation in *MC1R* (MacDougall-Shackleton et al.  
407 2003; Cheviron et al. 2006; Haas et al. 2009), our work suggests that differences in coloration  
408 between *C. helianthea* and *C. bonapartei* are controlled by other genes such as agonists or  
409 antagonists of *MC1R* in the melanin metabolic pathway, regions regulating the expression of  
410 *MC1R* or other genes (Theron et al. 2001), or genes controlling the shape of the keratin medullar  
411 matrix of the feather barb's spongy layer, which determines light scattering to produce structural  
412 colors (Shawkey et al. 2014).

413 We found no support for Gloger's rule because the darker *C. helianthea* does not occur in more  
414 humid environments than the more lightly colored *C. bonapartei*. Nevertheless, adaptation to  
415 different environmental conditions may occur at a finer scale, where habitat differences might  
416 select for plumage traits that, for instance, stand out from the background augmenting signal  
417 efficacy (Endler 1993; Brumfield and Braun 2001). Indeed, we found that the species differ in  
418 morphometric traits (e.g. *C. bonapartei* has longer wings and shorter tails than *C. helianthea*)  
419 typically associated with use of different microhabitats or foraging behaviors. Variation in such  
420 traits can affect flight speed or the relative ability to maneuver in open vs closed environments  
421 (Altshuler et al. 2010; Ortega-Jimenez et al. 2014). To the extent that morphological differences

422 may reflect adaptations to different resources between species (Altshuler and Dudley 2002) and  
423 between sexes within species of hummingbirds (Temeles and Kress 2010), our data are consistent  
424 with a role for selection driving ecomorphological divergence, but the adaptive value of  
425 phenotypic variation, if any, remains to be discovered. Considering that *C. bonapartei* often  
426 occurs along forest edges whereas *C. helianthea* is more frequently found in forest interior (Hilty  
427 and Brown 1986), studies of the functional consequences of phenotypic differences would be  
428 especially useful to assess any potential role of natural selection in driving and maintaining  
429 divergence.

430 Knowledge of the timing of speciation might allow one to make inferences about historical  
431 processes that could have promoted divergence between *C. helianthea* and *C. bonapartei*. We can  
432 place an upper boundary on the divergence time between these species based on their divergence  
433 from *C. lutetiae*, which is 1.6% divergent in *ND2* sequences (Parra et al., 2009). Assuming 2%  
434 divergence in mtDNA is approximately equivalent to one million years of isolation (Weir and  
435 Schluter 2008), *C. helianthea* and *C. bonapartei* likely split within the past ~ 800,000 years. This  
436 time period involves some of the last Pleistocene glaciations when high-altitude environments  
437 were uninhabitable and forests likely retreated, resulting in the isolation and divergence of  
438 populations (Vuilleumier 1969; Ramírez-Barahona and Eguiarte 2013). It thus remains possible  
439 that different selective regimes promoted speciation in these hummingbirds if their divergence  
440 occurred across environments with contrasting climatic conditions in the Pleistocene even if they  
441 occupy similar environments at present. Although such a hypothesis might be partly testable by  
442 modeling historical climates and potential distributions, however, one would still be faced with  
443 the question of what evolutionary forces might maintain *C. helianthea* and *C. bonapartei* as  
444 distinct given that they occur in regional sympatry in the same macroenvironments in the present.

445 An alternative explanation for the origin and maintenance of phenotypic distinctiveness in  
446 plumage, given the strong sexual dichromatism in *C. helianthea* and *C. bonapartei*, is that their  
447 differentiation may have proceeded in the face of gene flow due to sexual selection (Price 1998,  
448 2008). Sexual selection is thought to be a powerful force driving speciation in birds and other  
449 organisms (Campagna et al. 2012, 2017; Harrison et al. 2015), and some examples exist of  
450 speciation due to sexual selection with gene flow (Servedio 2016). Of direct relevance to our

451 system, a study comparing sexually selected (i.e. gorget and crown coloration) and non-sexually  
452 selected traits among *Coeligena* species found that sexual selection may be an important driver of  
453 phenotypic differentiation, but that it is probably insufficient for speciation to be completed  
454 unless it acts in concert with natural selection (Parra 2010; see also Servedio and Boughman  
455 2017). To assess the plausibility of the hypothesis that sexual selection is involved in the  
456 divergence and speciation of *C. helianthea* and *C. bonapartei*, one should test for associations  
457 among components of males' fitness, signaling traits (i.e. coloration, songs), and female  
458 preferences. Genomic analyses examining whether there are genetic and signatures of selection  
459 acting on regions associated with sexual traits (Charlesworth 2009; Huang and Rabosky 2015;  
460 Kirkpatrick 2017) would further help to test the hypothesis of divergence driven by sexual  
461 selection.

462 In conclusion, our study provides evidence that the formation of two species of Andean  
463 hummingbirds likely occurred in the face of gene flow, suggesting some form of selection played  
464 a role maintaining phenotypic differences and driving speciation. However, because the main  
465 selective mechanism we examined (i.e. adaptation to contrasting macroclimatic conditions)  
466 appears not to operate in *C. helianthea* and *C. bonapartei*, we conclude that ecological pressures  
467 that we did not consider directly or sexual selection were likely involved in their divergence.  
468 Future studies should thus aim to test predictions of hypotheses of natural and sexual selection  
469 acting on this system. Regardless of the selective processes involved, in line with previous  
470 research, our study suggests that selection has played an important role in maintaining phenotypic  
471 differences that could lead to speciation in tropical montane birds (Cadena et al. 2011; Winger  
472 and Bates 2015). Finally, the shallow genetic divergence that we observed between these species  
473 suggest that their genomes are unlikely to have been substantially affected by processes occurring  
474 after speciation (e.g. post-speciation divergence by drift), which makes this system especially  
475 promising for work on the genomics of speciation. Studies aiming to understand the genetic  
476 underpinnings of species differences employing genomic approaches (e.g. Campagna et al. 2017;  
477 Stryjewski and Sorenson 2017) will be an important complement to our increasing knowledge of  
478 the geographic and ecological context of speciation in tropical montane birds.

479

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489

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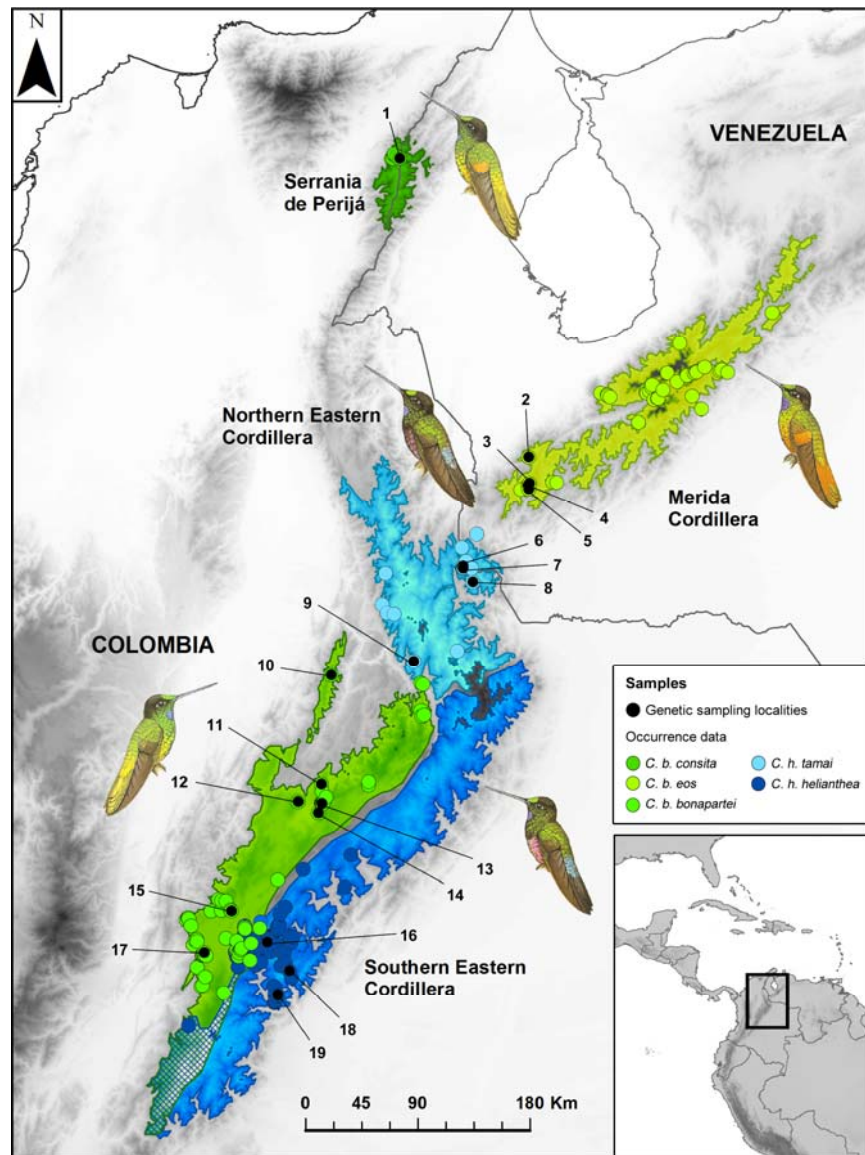
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816 **Figures**

817 **Figure 1.** Geographical distribution and sampled localities of *C. helianthea* and *C. bonapartei*.  
818 Black dots correspond to localities of specimens sampled for genetic markers. Colored dots  
819 correspond to occurrence data obtained from public data bases (see Material and Methods). Both  
820 were used for niche overlap analysis. Polygons correspond to the likely distributions of the  
821 subspecies according to elevational limits (Ayerbe-Quiñones 2015) and occurrence data.

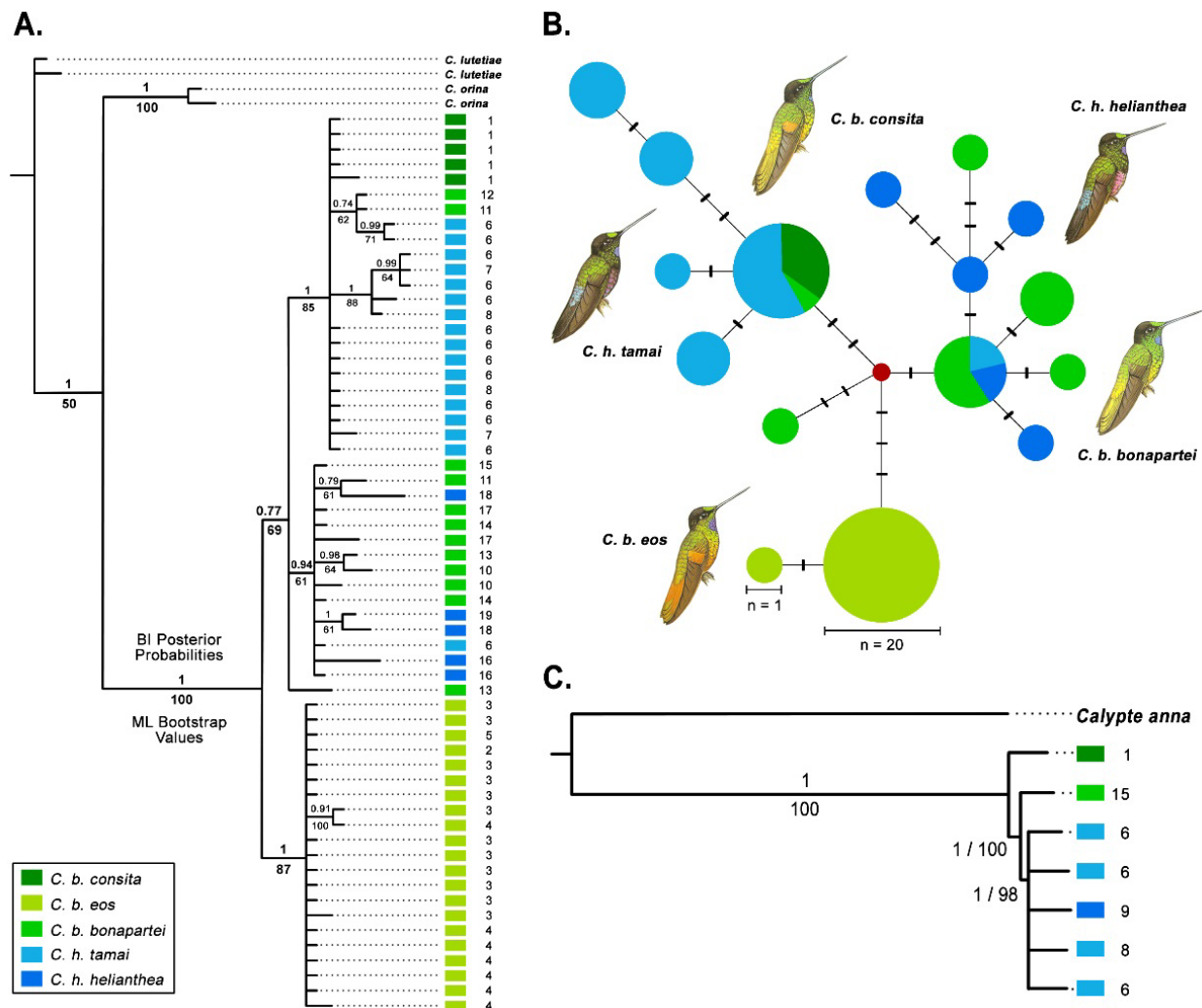


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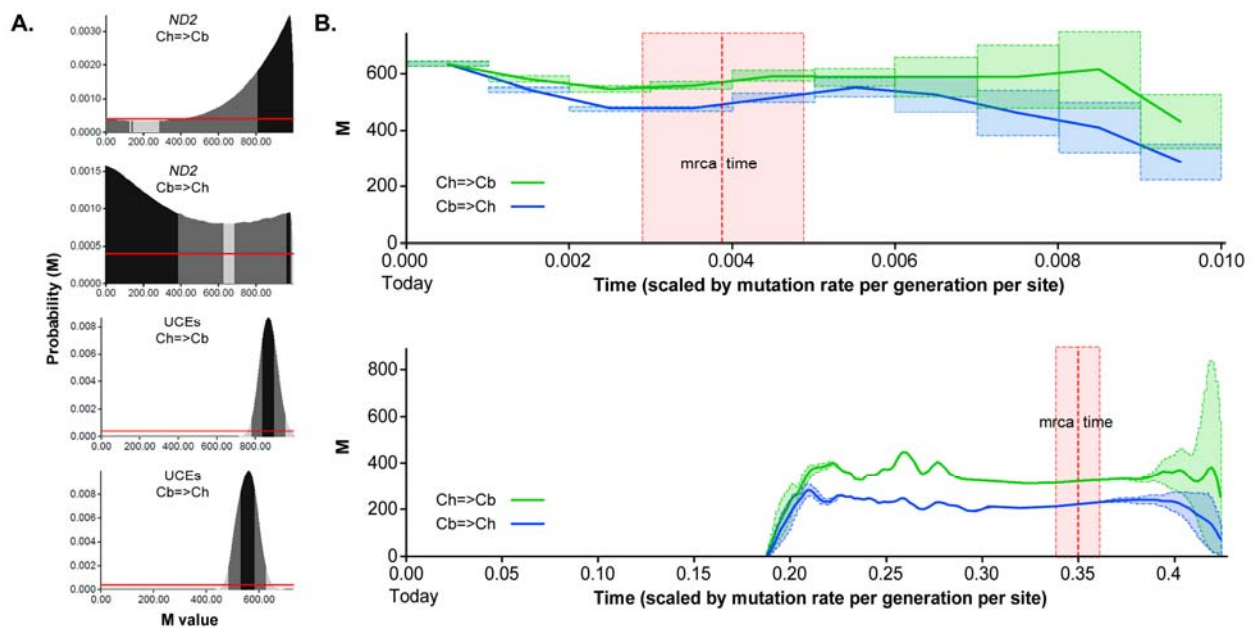
824 **Figure 2.** *ND2* phylogenetic reconstructions and haplotype network show lack of divergence  
 825 between *C. helianthea* and *C. b. bonapartei/consita*. The *ND2* gene trees (A) and haplotype  
 826 networks (B) show *C. helianthea* and *C. b. bonapartei/consita* in a single clade separate from a  
 827 *C. b. eos* clade. Most specimens of the northern subspecies *C. h. tamai* and *C. b. consita* cluster  
 828 together, whereas southern subspecies *C. h. helianthea* and *C. b. bonapartei* form another cluster,  
 829 suggesting that population structure more strongly reflects geography (i.e. north-south  
 830 differentiation) than taxonomy based on plumage phenotype. The phylogenetic reconstruction  
 831 based on UCE loci shows *C. helianthea* nested within *C. b. bonapartei/consita* (C). Numbers at  
 832 the right of the individuals in the tips of the trees correspond to the sampled localities (Table S1).



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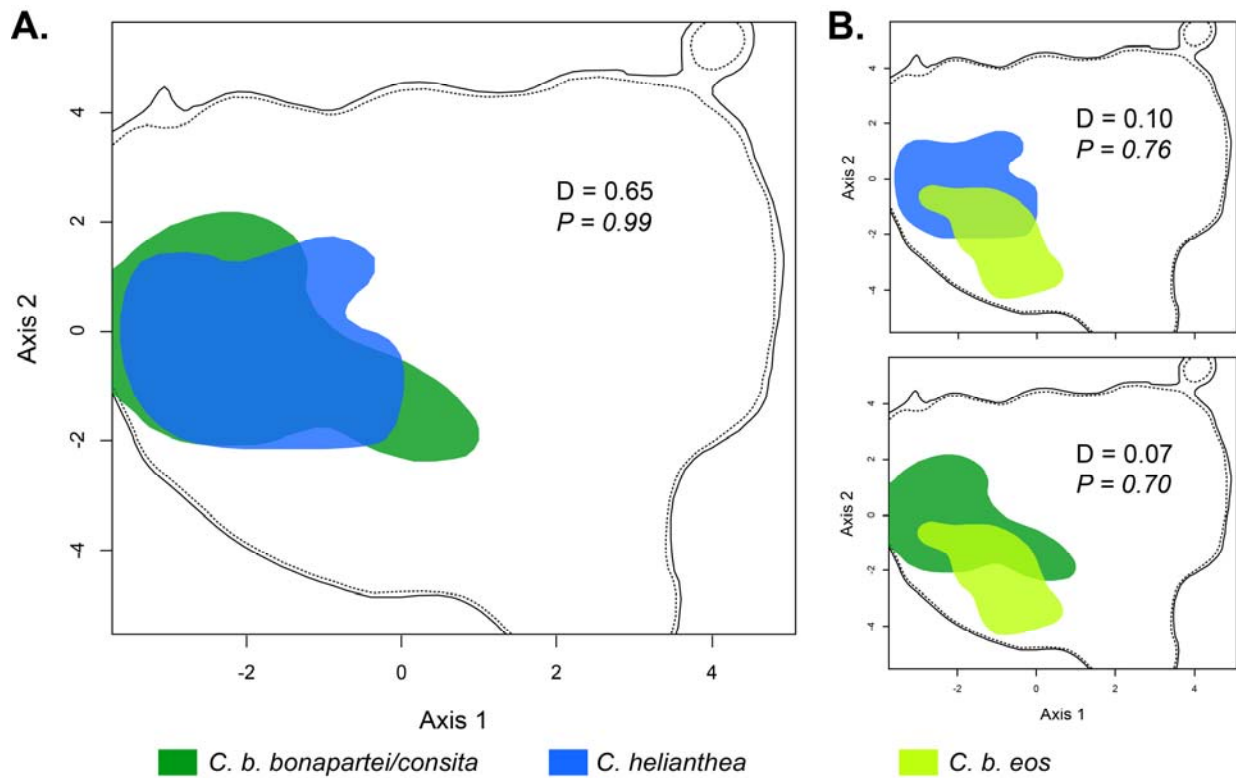
835 **Figure 3.** Migration parameter estimates suggest gene flow after the divergence between *C.*  
 836 *helianthea* to *C. bonapartei*. Posterior distributions of the migration parameter  $M=m/\mu$  from *C.*  
 837 *helianthea* to *C. bonapartei* and vice versa (A) estimated based on *ND2* (top) and *UCE* (bottom)  
 838 data; colors correspond to the limits of the intervals accumulating 50% (black), 75% (dark gray)  
 839 and 95% (light gray) of the probability density. The red horizontal line corresponds to the prior,  
 840 which is constant. Migration parameter  $M$  estimated value (y axis) from *C. helianthea* to *C.*  
 841 *bonapartei* (green) and vice versa (blue) changing through time (scaled by mutation rate per  
 842 generation per site, 0 = today) (B) for the *ND2* (upper panel) and *UCE* (bottom panel) data sets.  
 843 Dashed boxes in green and blue depict ca. 1.96 of standard error of the estimated value of  $M$ . The  
 844 red vertical dashed lines and boxes correspond to the mean value and one standard deviation,  
 845 respectively, of the estimated time of the most recent common ancestor of the species.



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848 **Figure 4.** *C. helianthea* and *C. b. bonapartei/consita* do not differ in climatic niches thus do not  
849 support Gloger's rule. The climatic niches of *C. helianthea* and *C. b. bonapartei/consita* overlap  
850 considerably ( $D = 0.65$ ) (A). The climatic niche of *C. b. eos* overlaps very little with *C.*  
851 *helianthea* ( $D = 0.10$ ) and *C. b. bonapartei/consita* ( $D = 0.07$ ) climatic niches (B). Nevertheless,  
852 relative to the background the differences between the niches are not significant in any case  
853 ( $p > 0.1$ ).



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