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RESEARCH ARTICLE

Shallow genetic divergence and distinct phenotypic differences between two Andean hummingbirds: Speciation with gene flow?

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ABSTRACT

Ecological speciation can proceed despite genetic interchange when selection counteracts the homogenizing effects of migration. We tested predictions of this divergence-with-gene-flow model in *Coeligena helianthea* and *C. bonapartei*, 2 parapatric Andean hummingbirds with marked plumage divergence. We sequenced putatively neutral markers (mitochondrial DNA [mtDNA] and nuclear ultraconserved elements [UCEs]) to examine genetic structure and gene flow, and a candidate gene (MC1R) to assess its role underlying divergence in coloration. We also tested the prediction of Gloger's rule that darker forms occur in more humid environments, and examined morphological variation to assess adaptive mechanisms potentially promoting divergence. Genetic differentiation between species was low in both ND2 and UCEs. Coalescent estimates of migration were consistent with divergence with gene flow, but we cannot reject incomplete lineage sorting reflecting recent speciation as an explanation for patterns of genetic variation. MC1R variation was unrelated to phenotypic differences. Species did not differ in macroclimatic niches but were distinct in morphology. Although we reject adaptation to variation in macroclimatic conditions as a cause of divergence, speciation may have occurred in the face of gene flow driven by other ecological pressures or by sexual selection. Marked phenotypic divergence with no neutral genetic differentiation is remarkable for Neotropical birds, and makes *C. helianthea* and *C. bonapartei* an appropriate system in which to search for the genetic basis of species differences employing genomics.

Keywords: Andes, ecological speciation, gene flow, Gloger's rule, MC1R, niche overlap

Baja divergencia genética y diferencias fenotípicas marcadas entre dos colibríes altoandinos ¿Especiación con flujo génico?

RESUMEN

La especiación ecológica puede ocurrir en presencia de flujo génico si la selección contrarresta el efecto homogeneizador de la migración. Evaluamos predicciones del modelo de divergencia con flujo génico en *Coeligena helianthea* y *C. bonapartei*, dos especies parapátricas de colibríes altoandinos con diferencias marcadas en su coloración. Secuenciamos marcadores putativamente neutrales (ADN mitocondrial [mtADN] y elementos ultraconservados nucleares [UCEs]) para evaluar estructura genética y flujo génico, y secuenciamos un gen candidato (MC1R) para evaluar su papel en la divergencia en coloración. También evaluamos la regla de Gloger, que señala que organismos de coloraciones más oscuras habitan ambientes más húmedos, y examinamos variación morfológica para analizar posibles mecanismos adaptativos que podrían haber promovido la divergencia entre estas especies de colibríes. Encontramos baja diferenciación genética entre las especies tanto en ND2 como en UCEs. Los estimadores de migración fueron consistentes con el modelo de divergencia con flujo génico, pero no podemos rechazar la posibilidad de que los patrones de variación genética reflejen separación incompleta de linajes debida a especiación reciente. La variación en MC1R fue también muy baja y no estuvo asociada con las diferencias en coloración. Las dos especies no se diferencian en el nicho macroclimático que ocupan, aunque son distintas morfológicamente. Aunque rechazamos la variación en condiciones macroclimáticas como una causa de la divergencia en estos colibríes, estas especies podrían haberse diferenciado en presencia de flujo génico debido a otras presiones de selección ecológicas o por selección sexual. La marcada diferenciación fenotípica sin diferenciación genética en marcadores neutrales que documentamos es excepcional en aves neotropicales y hace de *C. helianthea* y *C. bonapartei* un sistema ideal para buscar las bases genéticas de la divergencia entre especies utilizando genómica.

Palabras clave: Andes, especiación ecológica, flujo génico, MC1R, regla de Gloger, superposición de nicho

INTRODUCTION

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

Several studies provide evidence that natural selection can promote phenotypic divergence among populations despite gene flow (e.g., Smith 1997, Morgans et al. 2014, Fitzpatrick et al. 2015) and this may lead to speciation (Hey 2006, Nosil 2008). However, documenting speciation with gene flow is complicated because of the difficulty in determining whether shared genetic variation among species is a consequence of divergence in the presence of migration, hybridization ocurring after speciation, or incomplete lineage sorting due to recent or rapid divergence (Hey 2006, Pinho and Hey 2010). This difficulty has been partly overcome thanks to the development of tools to estimate migration between pairs of populations under alternative demographic scenarios (Hey and Nielsen 2004, 2007, Beerli 2006, Kuhner 2006, Durand et al. 2011). Some studies using such tools have found incomplete lineage sorting as the cause for lack of genetic differentiation (Nosil et al. 2009, Wall et al. 2013, Suh et al. 2015), whereas others support population divergence despite gene flow (Green et al. 2010, Rheindt et al. 2014, Supple et al. 2015, Kumar et al. 2017). However, compelling evidence that population divergence has scaled up to the formation of different species in the face of gene flow remains limited. Nonetheless, the finding that the evolutionary histories of various organisms are characterized by substantial cross-species genetic exchange (e.g., Novikova et al. 2016, Zhang et al. 2016, Kumar et al. 2017) implies that attention should be devoted to understanding the selective mechanisms maintaining species as distinct entities in the face of gene flow.

In birds, plumage traits are often targets of natural selection. This results in adaptations for foraging and flight efficiency (Zink and Remsen 1986), camouflage (Zink and Remsen 1986) or conspicuousness (Endler 1993), thermoregulation (Walsberg 1983), and protection against pathogens (Burtt and Ichida 2004, Goldstein et al. 2004, Shawkey et al. 2007), among others. Because plumage traits are also critical in mate selection and species recognition, plumage divergence may drive lineage diversification (Price 2007, Servedio et al. 2011, Hugall and Stuart-Fox 2012, Maia et al. 2013). A frequently observed pattern in presumably adaptive plumage variation is Gloger's rule, which states that birds with darker plumage coloration occur in more humid environments than lighter-colored conspecifics (Delhey 2019). This pattern is often attributed to adaptation to reduce bacterial degradation of plumage in humid conditions where bacteria are most abundant because melanin (the pigment responsible for black plumage color) confers resistance against these microbes (Goldstein et al. 2004, Peele et al. 2009, Amar et al. 2014). Because differences in melanic pigmentation can serve as cues for mate choice and species recognition (Uy et al. 2009), adaptive differentiation in plumage coloration might thus drive the origin of reproductive isolation. However, we are unaware of studies explicitly relating the evolution of melanic plumage coloration by natural selection to population divergence or speciation in the presence of gene flow (but see Cooper and Uy 2017; see also Rosenblum et al. 2017, Pfeifer et al. 2018 for examples involving skin pigmentation in other animals).

Here, we test the divergence-with-gene-flow model of speciation as an explanation for the evolution of 2 Andean hummingbird species, Blue-throated Starfrontlet (*Coeligena helianthea*) and Golden-bellied Starfrontlet (*C. bonapartei*). We studied these species because (1) they have largely parapatric ranges in a topographically complex area of the Andes over which environmental conditions, hence selective pressures, may differ (Figure 1); (2) they seemingly lack genetic differentiation in neutral markers (Parra et al. 2009, McGuire et al. 2014) as expected under divergence with gene flow; (3) they exhibit distinct phenotypic differences (plumage in *C. helianthea* is considerably darker than in *C. bonapartei*) and no hybrids have been reported even where they coexist locally (except perhaps for a few old specimens; Fjeldså and Krabbe 1990); and (4) because variation in melanic pigmentation may reflect adaptation to different environments, divergence in plumage traits between these hummingbird species might have been driven by natural selection.

The apparent lack of genetic differentiation between *C. helianthea* and *C. bonapartei* (Parra et al. 2009, McGuire et al. 2014) despite their distinct differences in traits potentially under selection may reflect divergence with gene flow, contemporary hybridization, or incomplete lineage

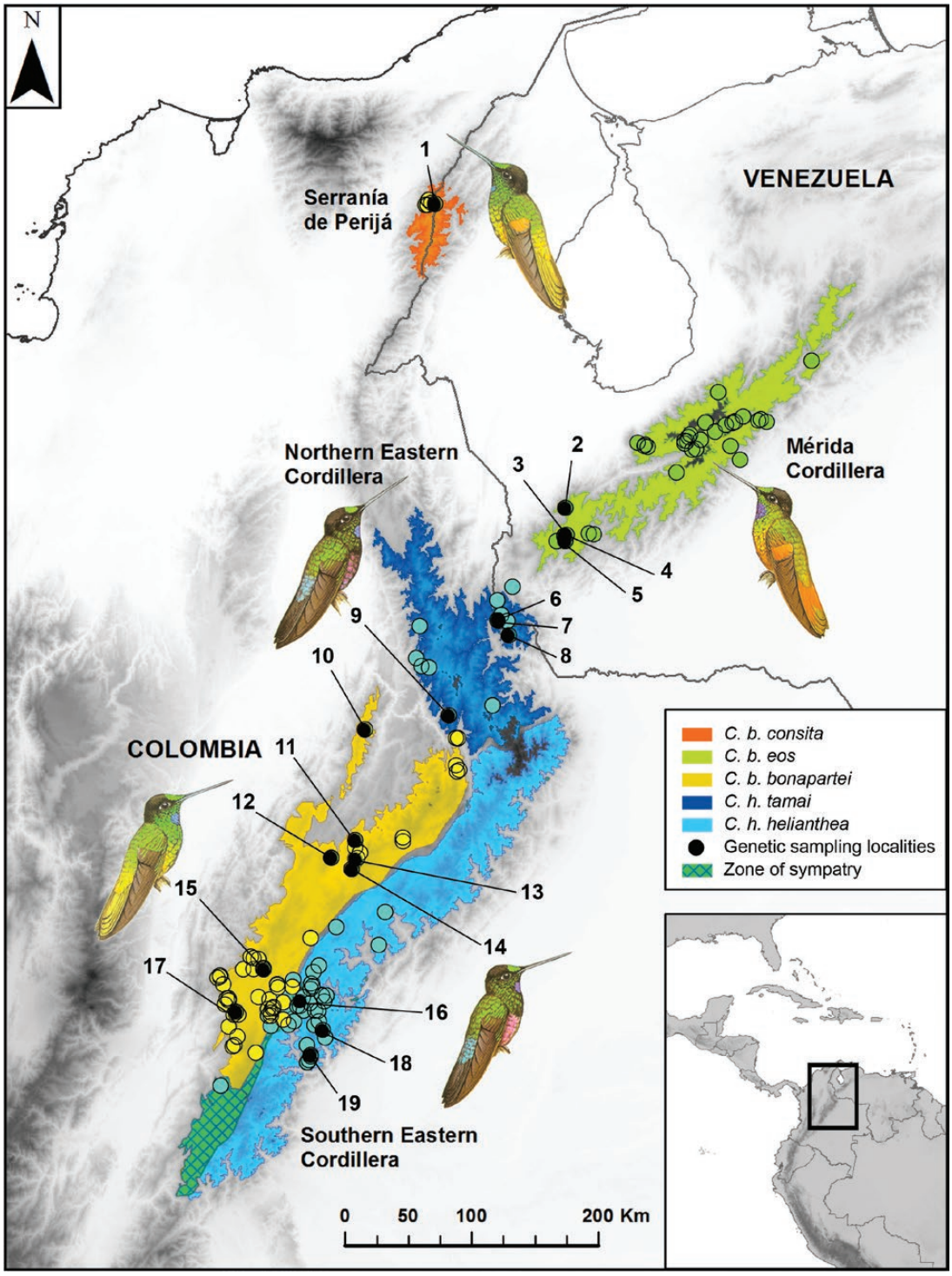

FIGURE 1. Geographical distribution and sampled localities of *C. helianthea* and *C. bonapartei*. Black dots correspond to localities of specimens sampled for genetic markers. Colored dots correspond to occurrence data obtained from public databases. All localities were used for niche overlap analyses. Polygons correspond to the likely distributions of the subspecies according to elevational limits (Ayerbe-Quiñones 2015) and occurrence data. The teal polygon in the south corresponds to the region where species are sympatric. Illustrations courtesy of Lynx Edicions (del Hoyo et al. 2018).

sorting (Hey 2006, Suh et al. 2015, Sonsthagen et al. 2016). Here, we evaluate predictions of the divergence-with-gene-flow model of speciation and consider evolutionary mechanisms driving divergence between these species by first addressing the following questions: (1) Does the lack of genetic differentiation between *C. helianthea* and

C. bonapartei persist with a much larger and geographically extensive sampling and additional molecular markers relative to earlier work (Parra et al. 2009)? and (2) Are patterns of genetic variation consistent with a model of divergence in the face of gene flow? Also, (3) Is color divergence associated with genetic variation in the melanocortin-1 receptor (MC1R) gene, a candidate underlying melanic coloration in various bird species and other vertebrates? To examine possible mechanisms through which natural selection might have driven population differentiation we examined whether phenotypic divergence may be attributable to adaptation to contrasting macroenvironmental conditions by asking (4) Is *C. helianthea* with darker plumage distributed in more humid environments as predicted by Gloger's rule? and (5) Is there morphometric variation between these species that may suggest adaptation to alternative microhabitats or resources?

MATERIALS AND METHODS

Study System

Coeligena helianthea inhabits mostly the eastern slope of the Cordillera Oriental of the Northern Andes from western Meta in Colombia to the Táchira Depression in Venezuela, and comprises 2 subspecies: *C. h. helianthea* occupies most of the range, whereas *C. h. tamai* occurs in the Tamá Massif in the border between Colombia and Venezuela (Figure 1). The distribution of *C. bonapartei* is not continuous and 3 subspecies are recognized: *C. b. bonapartei* ranges along the western slope of the Cordillera Oriental in Cundinamarca, Boyacá, and western Santander in Colombia; *C. b. consita* is restricted to the Serranía del Perijá; and *C. b. eos* is endemic to the Cordillera de Mérida in the Venezuelan Andes (Hilty and Brown 1986, Hilty 2003; Figure 1). Some authors consider *C. b. eos* a distinct species (Donegan et al. 2015, del Hoyo et al. 2018), but it is currently treated as a subspecies of *C. bonapartei* (Remsen et al. 2018). Although the distributions of *C. helianthea* and *C. bonapartei* are not sympatric for the most part, the nominate subspecies co-occur regionally in Cundinamarca and Boyacá (Gutiérrez-Zamora 2008).

Coeligena helianthea and *C. bonapartei* differ strikingly in plumage coloration. Although both species have bright green crowns and violet gorgets, males of *C. helianthea* are considerably darker, with a largely greenish back with a rose belly and aquamarine rump; males of *C. bonapartei* are largely golden green with fiery gold underparts and rump. Females are paler than males, but also differ distinctly in plumage, especially in their lower underparts (Hilty and Brown 1986, Parra 2010). The differences in coloration between species may reflect variation in the melanin content of feathers (D'Alba et al. 2014), differences in the nanostructure of feather barbules (Greenewalt et al. 1960), or both.

Tissue Samples and DNA Sequencing Protocols

We collected specimens in Colombia and Venezuela, and obtained tissue samples from the collections of the Instituto Alexander von Humboldt (IAvH), the Museo de Historia Natural de la Universidad de los Andes (ANDES), and the Colección Ornitológica Phelps (Supplementary Material Table S1). Our sampling included a total of 62 individuals: 38 specimens of *C. bonapartei* (12 *C. b. bonapartei*, 5 *C. b. consita*, and 21 *C. b. eos*) and 24 specimens of *C. helianthea* (7 *C. h. helianthea* and 17 *C. h. tamai*). Subspecies were assigned based on taxonomic determination of museum specimens or by geography. We extracted DNA from tissue samples using either a QIAGEN DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions or a standard phenol/chloroform extraction protocol. For 60 specimens we amplified by PCR (Appendix) and sequenced 1,041 base pairs (bp) of the mitochondrial ND2 gene, and used the data for range-wide phylogeographic and population genetic analyses (GenBank accession numbers: MG874354–874409). We used published sequences of *C. lutetiae* (McGuire et al. 2007, Parra et al. 2009) and *C. orina* (McGuire et al. 2014) as outgroups in phylogenetic analyses.

We used a subset of 36 individuals to assess whether color differentiation between species is associated with nucleotide substitutions in the coding region of the MC1R gene, a locus responsible for melanic pigmentation in several birds and other vertebrates (Mundy 2005, Roulin and Ducrest 2013). We amplified by PCR (Appendix) and sequenced 788 bp of the 945 bp of the MC1R locus for 6 individuals of *C. h. helianthea*, 10 *C. h. tamai*, 8 *C. b. bonapartei*, 1 *C. b. consita*, and 11 *C. b. eos* (GenBank accession numbers: MG880079–880114). All PCR products were cleaned and sequenced in both directions by Macrogen or at the sequencing facilities of the Universidad de los Andes. We assembled, edited, and aligned sequences of the ND2 and MC1R genes using BioEdit 7.2.5 (Hall 1999) and Geneious 9.1.5 (http://www.geneious.com/; Kearse et al. 2012), employing the MUSCLE algorithm (Edgar 2004) and manual editing.

We also employed a sequence capture approach to acquire data from regions flanking ultraconserved elements (UCEs; Faircloth et al. 2012) for a small sample of 1 individual of *C. h. helianthea*, 4 *C. h. tamai*, 1 *C. b. bonapartei*, and 1 *C. b. consita* to obtain a preliminary overview of genetic divergence between these taxa at a genomic level. We used a standard library preparation protocol (http://ultraconserved.org/; Faircloth and Glenn 2012) and enriched the pool of samples for 5,060 UCE loci using the MYbaits_Tetrapods-UCE-5K probes. We sequenced the pool after quantification using 250 bp paired-end

Illumina MiSeq. Following the PHYLUCE pipelines (Faircloth 2015), we used Illuminoprocessor (Faircloth 2013) and Trimmomatic (Bolger et al. 2014) to trim reads, discarded adapter contamination and low-quality bases, and assembled the reads into contigs using a kmer = 50 and ABySS (Simpson et al. 2009). We aligned the contigs against the original UCE probes to identify contigs matching UCE loci using LASTZ (Harris 2007). Among the individuals, we aligned unphased sequences of UCE loci using the default MAFFT 7.13 algorithm (Katoh and Standley 2013). Finally, we pulled out UCE loci from the Anna's Hummingbird (*Calypte anna*) genome (Zhang et al. 2014a, 2014b) to use them as an outgroup.

For phylogenetic analyses we used a concatenated alignment of 2,313 UCE loci shared at least among 3 individuals including the outgroup. Of these, 1,465 loci were present in all the individuals (mean locus length = 615.1 bp, mean number of individuals per locus in the incomplete matrix = 7.3). We generated a second concatenated alignment of 1,604 loci shared among all *Coeligena* specimens (i.e. without the outgroup). Of these, 389 loci showed no variation, 75 had only indels (informative or not), 615 had singletons and indels, and 525 (32.8%) had informative sites (polymorphic sites with each variant represented in at least 2 individuals). We used the latter 525 loci or a subset of these for population genetic analyses (Appendix), but because our sample size was low we treated the results from these data as preliminary and interpreted them with caution.

Phylogenetic and Population Genetic Analysis

We used maximum-likelihood (ML) and Bayesian inference methods to reconstruct phylogenies from the CIPRES Portal (http://www.phylo.org/) or locally. We selected a single partition and the TNR substitution model as the best-fit for our ND2 data according to the corrected Akaike Information Criterion (AIC_c) in PartitionFinder 2.1.1 (Guindon et al. 2010, Lanfear et al. 2017). To analyze UCE data we used a concatenated alignment of all 2,313 loci, for which we specified 16 partitions and nucleotide substitution models for each partition following CloudForest analysis (Crawford and Faircloth 2011). We conducted ML analyses in RAxML (Stamatakis 2014) using the GTR+GAMMA model and non-parametric bootstrapping under the autoMRE stopping criterion for ND2 and UCE data. We conducted Bayesian analyses in Mr.Bayes 3.2 (Ronquist et al. 2012). The MCMC parameters consisted of 2 runs with 4 chains run for 15 million generations sampling every 100 generations for the ND2 data and run for 25 million generations sampling every 500 generations for the UCE data. We discarded the first 10% generations as burn-in before estimating the consensus tree and posterior probabilities. To account for heterogeneity among gene trees, we also conducted a species-tree analysis of our dataset of 525 UCE loci using the program ASTRAL (Zhang et al. 2018; details in Appendix).

We also conducted Bayesian analyses in BEAST 2 (Bouckaert et al. 2014) using the ND2 data to estimate divergence times using a strict clock model of evolution, assuming a Yule model prior or a coalescent constant-size population prior. We used a substitution rate of 2.5% divergence per million year for ND2 (Smith and Klicka 2010). MCMC runs consisted of 50 million generations, sampling trees every 1,000 generations, and discarding the first 15,000 trees (30%) as burn-in. Convergence and effective sample sizes of parameter estimates for Mr.Bayes and Beast 2 results were examined using Tracer 1.7.1 (Rambaut et al. 2018).

To further examine relationships among ND2 haplotypes, we used an alignment of 885 bp, for which complete data were available for all individuals, to construct a median-joining haplotype network in Network 5.0.0.1 (http://www.fluxus-engineering.com/; Bandelt et al. 1999). To examine genetic structure between species, we calculated F_{st} with *R* package Hierfstat (Goudet and Jombart 2015, R Core Team 2017) and AMOVAs with *R* package Ade4 (Dray and Dufour 2007, R Core Team 2017) assessing significance using 10,000 permutations (Supplementary Material Script S1). Also, we used Structure 2.3.4 (Pritchard et al. 2000) to assess population structure using our 293 SNP dataset from UCE loci; because our sample size was limited, we consider population genetic analyses using UCEs as preliminary and thus do not report on them in detail in the main text (Appendix).

Testing for Divergence with Gene Flow

To examine whether there has been gene flow between *C. helianthea* and *C. bonapartei*, we used Migrate 3.2.1 (Beerli 2009) to estimate the following demographic parameters: effective population size scaled by mutation rate (θ), time scaled by the mutation rate (T), and migration scaled by mutation rate ($M = (m / \mu)$). If there has been gene flow between species after speciation, then the posterior distributions of M should exclude values of zero. We also tried to distinguish scenarios of divergence with gene flow and hybridization following secondary contact using the option of parameter estimation for different moments through time in Migrate, but because results were unreliable we chose not to report on these analyses (Appendix).

For Migrate analyses we employed a ND2 alignment including 23 individuals of *C. helianthea* and 17 individuals of *C. b. bonapartei/consita* (i.e. excluding *C. b. eos*, which we found to be genetically distinct; see Results). We also used 293 SNPs from our UCE data for Migrate analyses (Appendix). Because inference of gene flow requires neutrally evolving markers, we first confirmed that our datasets met this assumption by calculating Tajima's D using DNAsp 5.1 (Librado and Rozas 2009). We determined

prior maximum values for the parameters θ and *M* for each species based on several test runs. In final analyses aimed to estimate gene flow we set prior values to 0.15 for θ for both species, and to 1,000 for *M* in both directions. We ran Migrate in the CIPRES Portal (http://www.phylo.org/) using a long chain of 3,000 million steps (sampling 1,000,000 steps recorded every 3,000 steps) with a burn-in of 1,000,000 steps.

MC1R Gene Analyses

We compared variable sites in MC1R sequences between our study species and translated sequences to aminoacids to check for synonymous and non-synonymous substitutions. As reference for comparisons we used sequences of Anna's Hummingbird and Chimney Swift (*Chaetura pelagica*) predicted from genome annotations (Zhang et al. 2014b). Because these comparisons revealed no variation potentially implied in phenotypic variation (see Results), we did not conduct any additional analysis.

Examining the Selective Regime: Niches and Morphological Differentiation

We tested the hypothesis that natural selection underlies phenotypic divergence in color between *C. heliathea* and *C. bonapartei* through macroclimatic differences in the regions occupied by these species. Specifically, we tested the prediction of Gloger's rule that *C. helianthea* (with darker plumage) occurs in environments with more humid conditions than *C. bonapartei*, and examined whether other macroclimatic conditions that may promote adaptation differ between environments occupied by these hummingbirds. We examined ecological differentiation among *C. helianthea*, *C. b. bonapartei/consita*, and *C. b. eos* (which we found to be genetically distinct; see Results) using occurrence data, environmental variables, and measurements of niche overlap (Broennimann et al. 2012). In addition to the locality data associated with specimens included in molecular analyses, we obtained occurrence data from eBird (http://ebird.org/content/ebird/), Vertnet (http://vertnet.org/), the Global Biodiversity Information Facility (http://www.gbif.org/), Xeno-canto (http://www.xeno-canto.org/), and the ornithological collection of the Instituto de Ciencias Naturales of the Universidad Nacional de Colombia (http://www.biovirtual.unal.edu.co/en/), for a total of 242 records. After eliminating duplicates and excluding non-reliable locations we retained 196 records for analysis: 85 of *C. helianthea*, 75 of *C. b. bonapartei/consita*, and 36 of *C. b. eos* (Supplementary Material Table S2).

To delimit the accessible areas for each species we used ecoregions as defined by Dinerstein et al. (2017). We used all the ecoregions with occurrence records as the environmental background available for the analysis of niche overlap. We obtained climatic data from WorldClim (http://www.worldclim.org/; Hijmans et al. 2005), CliMond (https://www.climond.org/; Kriticos et al. 2012), and EarthEnv (http://www.earthenv.org/cloud; Wilson and Jetz 2016). We clipped layers for ecoregions and climatic variables to our study area (i.e. longitude: −76° to −70°, latitude: 3° to 12°) and excluded variables highly correlated to others ($r > 0.70$) within this area using the package usdm in *R* (Naimi 2015, R Core Team 2017). We conducted niche overlap analyses using 11 variables: 3 related to temperature, 3 related to precipitation, 4 related to cloudiness, and 1 related to air moisture (Supplementary Material Table S3).

We extracted climatic data from 10,000 points from the background environment and from the 196 occurrence records and performed a principal component analysis (PCA) to summarize climatic variation using the package ade4 in *R* (Dray and Dufour 2007, R Core Team 2017). With the first 2 PCA axes, we plotted the densities of each taxon in climatic space relative to the background using the package ecospat in *R* (Broennimann et al. 2016, R Core Team 2017). We also used this package to estimate the *D*-statistic (Warren et al. 2008) to quantify niche overlap ($D = 0$ indicates different niches, and $D = 1$ indicates identical niches), and we performed similarity tests (1,000 iterations) to assess whether niches are less similar (niche divergence) than expected by chance given background climatic variation (Supplementary Material Script S2). Significant niche divergence with the darker *C. helianthea* occupying more humid areas would be consistent with adaptive divergence following Gloger's rule, whereas no significant differences in niches would suggest that adaptation to distinct climatic conditions cannot account for phenotypic differentiation between species.

We also assessed whether there is morphometric differentiation between species, which may reflect adaptation to different microhabitats or food resources (Stiles 2008) by measuring 17 traits related to beak, wing, tail, and leg morphology (Supplementary Material Table S4). We measured morphological variables from 35 live individuals (17 females and 18 males) of *C. h. helianthea* and 46 individuals (23 females and 23 males) of *C. b. bonapartei*. Using these data we asked whether individuals of different species and sexes are distinguishable in multivariate space employing linear discriminant (LD) analysis using the package MASS in *R* (Venables and Ripley 2002, R Core Team 2017). We built ANOVA models to test for mean differences in all individual variables among species and sexes simultaneously. Because a few of the variables were not normally distributed according to Shapiro-Wilk tests, we used Kruskal-Wallis tests for comparisons involving such variables. Shapiro-Wilk tests, Kruskal-Wallis tests, and ANOVAs were performed using basic functions in *R* (R Core Team 2017).

RESULTS

Does Lack of Genetic Differentiation Between *C. helianthea* and *C. bonapartei* Persist With Greater Sampling and Additional Markers?

We found low genetic differentiation between *C. b. bonapartei* and *C. b. consita*, but both taxa were markedly differentiated from *C. b. eos*. Therefore, hereafter we treat *C. b. bonapartei* and *C. b. consita* as a single group, which we refer to as *C. b. bonapartei/consita*. Divergence in ND2 of both *C. helianthea* and *C. b. bonapartei/consita* relative to *C. b. eos* was high, with significant F_{st} values of 0.56 and 0.52, respectively ($P < 0.001$ in both cases), and relatively high fractions of genetic variance (59.4% and 52.0%, respectively) existing between groups in AMOVA. By contrast, ND2 data showed little to no differentiation between *C. helianthea* and *C. b. bonapartei/consita*. Although differentiation as measured by F_{st} was significant ($P = 0.03$), the F_{st} value was low (0.07) and only 1.7% of the variance was partitioned between these 2 taxa in AMOVA, with 98.3% of the variance existing among individuals within taxa.

Phylogenetic analyses using ND2 data showed that *C. b. eos* forms a strongly supported clade (posterior probability PP = 1.0, maximum-likelihood bootstrap [MLbs] = 87%), which is sister to a clade lacking strong support (PP = 0.84, MLbs = 69%) formed by *C. helianthea* and *C. b. bonapartei/consita* (Figure 2A). Within the latter clade, relationships among populations appeared to be determined more by geography than by current species-level taxonomy: most sequences of the northern subspecies *C. h. tamai* and *C. b. consita* formed a strongly supported clade (PP = 1.0, MLbs = 85%), whereas the majority of sequences of southern subspecies *C. h. helianthea* and *C. b. bonapartei* formed another moderately supported clade (PP = 0.95, MLbs = 61%).

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

Likewise, UCE nuclear markers for 7 individuals did not reveal genetic differentiation between *C. helianthea* and *C. bonapartei* (no data were available for *C. b. eos*). The phylogeny estimated using 2,313 concatenated UCE loci showed a well-supported clade including all sequences of *C. helianthea* nested within a clade in which the earliest diverging branches were the samples of (1) *C. b. consita* and (2) *C. b. bonapartei* (Figure 2C). The same topology was obtained with the species-tree analysis which considers gene-tree heterogeneity (Appendix). Because our dataset of 293 SNPs obtained from UCEs met the assumption of neutrality (Tajima's $D = 1.5$; $P > 0.1$), we were able to use them for analyses of population genetic structure. Differentiation between species in these markers was not significant ($F_{st} = 0.2$, $P = 0.5$), and the most likely number of genetic clusters estimated in Structure was 1 ($K = 1$, $\text{prob}_{(k=1)} = 0.99$), although we caution that our sample was small.

We estimated that divergence between the clade formed by *C. helianthea* and *C. bonapartei* and its sister group formed by *C. lutetiae* and *C. orina* occurred ~0.40 mya (95% CI: 0.27 to 0.58 mya) using a Yule model prior. Using a coalescent constant-size population prior, the time of divergence was 0.74 mya (95% CI: 0.48 to 1.01 mya; Appendix Figure 5). Because the latter divergence time is more consistent with published estimates based on broader phylogenetic frameworks (Parra et al. 2009, McGuire et al. 2014), we focus on estimates using the coalescent constant-size population prior. Given this model, the time of divergence between the *C. helianthea* + *C. b. bonapartei/consita* clade and *C. b. eos* was estimated at 0.31 mya (95% CI: 0.17 to 0.46 mya). Because *C. helianthea* and *C. b. bonapartei/consita* were not reciprocally monophyletic in the *ND2* gene tree, we were unable to date their divergence, but it must be more recent than the time of their divergence from *C. b. eos*.

Are Patterns of Genetic Variation Consistent With Divergence in the Face of Gene Flow?

Our ND2 dataset fit the assumption of neutrality (Tajima's $D = -0.68$, $P > 0.1$), which allowed us to use it for gene flow inference. The analyses suggested that there has been gene flow from *C. helianthea* to *C. b. bonapartei/consita*, whereas gene flow in the other direction could not be estimated reliably. Mean estimates of migration ($M = m / \mu$) were in all cases different from zero: $M = 725.1$ from *C. helianthea* to *C. b. bonapartei/consita* and 446.1 from *C. b. bonapartei/consita* to *C. helianthea*. However, the estimated posterior probability distributions of M were wide: 95% CI: 284.7 to 1000 from *C. helianthea* to *C. b. bonapartei/consita*, and 95% CI: 0.0 to 628 from *C. helianthea* to *C. b. bonapartei/consita* (Figure 3). However, analyses of UCE markers with our limited sample were inconclusive as to whether patterns of variation are best explained by gene flow between *C. helianthea* and *C. b. bonapartei/consita*, or by incomplete lineage sorting (Appendix).

Is Color Divergence Associated With Genetic Variation in MC1R?

Of the 36 *Coeligena* individuals sampled for MC1R, 32 shared a haplotype (excluding ambiguous positions). Genetic variation at MC1R was limited to 3 individuals of *C. helianthea* and 1 individual of *C. bonapartei*, and involved changes in 4 sites. Only 1 change was non-synonymous [Ser275

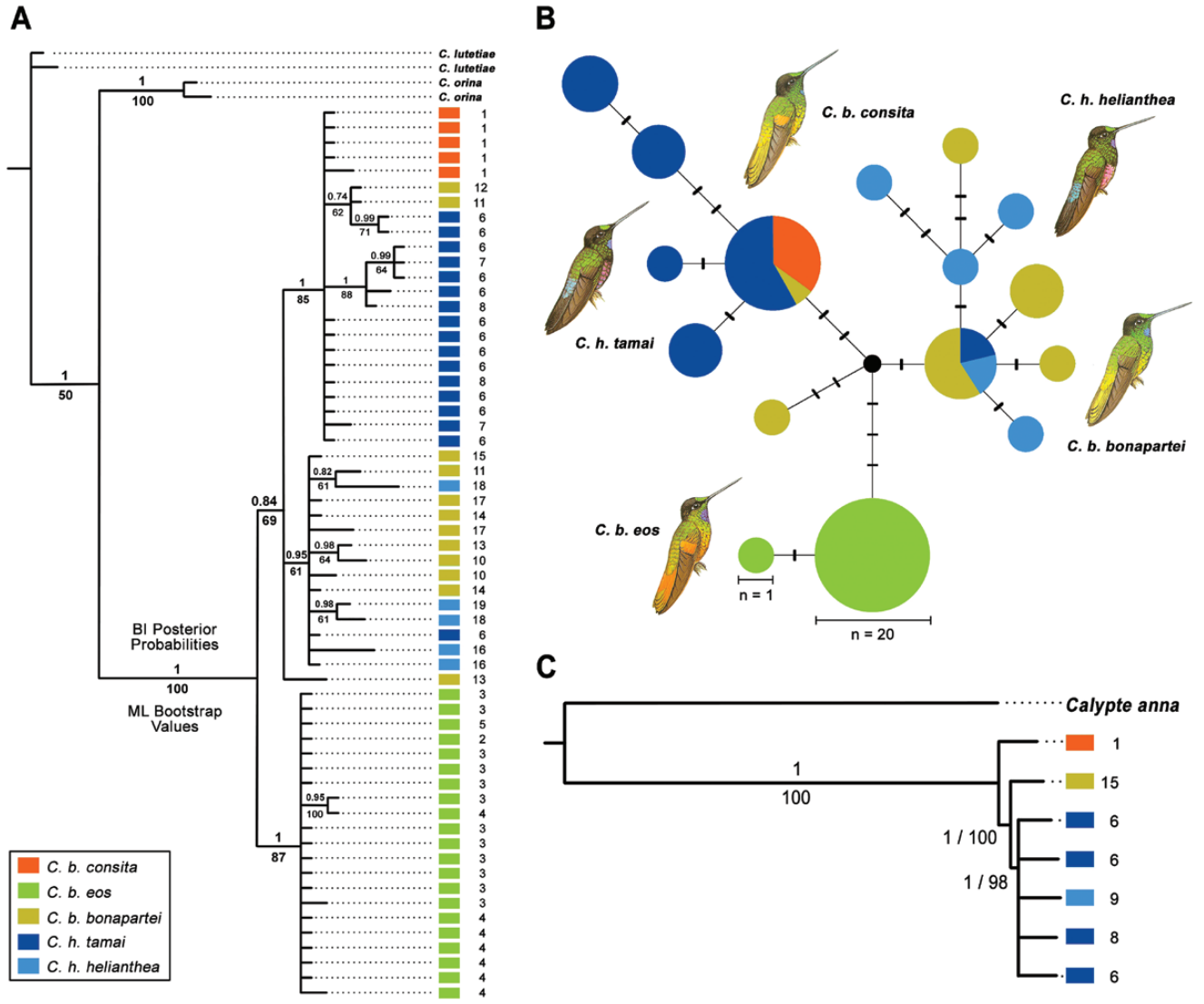

FIGURE 2. ND2 phylogenetic reconstructions and haplotype network show lack of divergence between *C. helianthea* and *C. b. bonapartei/consita*. The ND2 (1,041 bp) gene tree (**A**) and ND2 (885 bp) haplotype network (**B**) show *C. helianthea* and *C. b. bonapartei/consita* in a single group separate from *C. b. eos*. Most specimens of the northern subspecies *C. h. tamai* and *C. b. consita* cluster together, whereas southern subspecies *C. h. helianthea* and *C. b. bonapartei* form another group, suggesting that population structure more strongly reflects geography (i.e. north-south differentiation) than taxonomy based on plumage phenotype. The phylogenetic reconstruction based on UCEs (2,313 loci shared by at least 3 individuals including the outgroup, **C**) shows *C. helianthea* nested within *C. b. bonapartei/consita*. Numbers to the right of the individuals in the tips of the trees correspond to the sampling locality (Supplementary Material Table S1). Illustrations courtesy of Lynx Edicions (del Hoyo et al. 2018).

(AGC) → Arg275 (AGG) at nucleotide site 825], but it was present in a single *C. helianthea* (Andes-BT 1126) with typical plumage coloration. These results reveal no association between MC1R genotype and species-specific color phenotypes in *C. helianthea* and *C. bonapartei*.

Is *C. helianthea* With Darker Plumage Distributed in More Humid Environments as Predicted by Gloger's Rule?

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

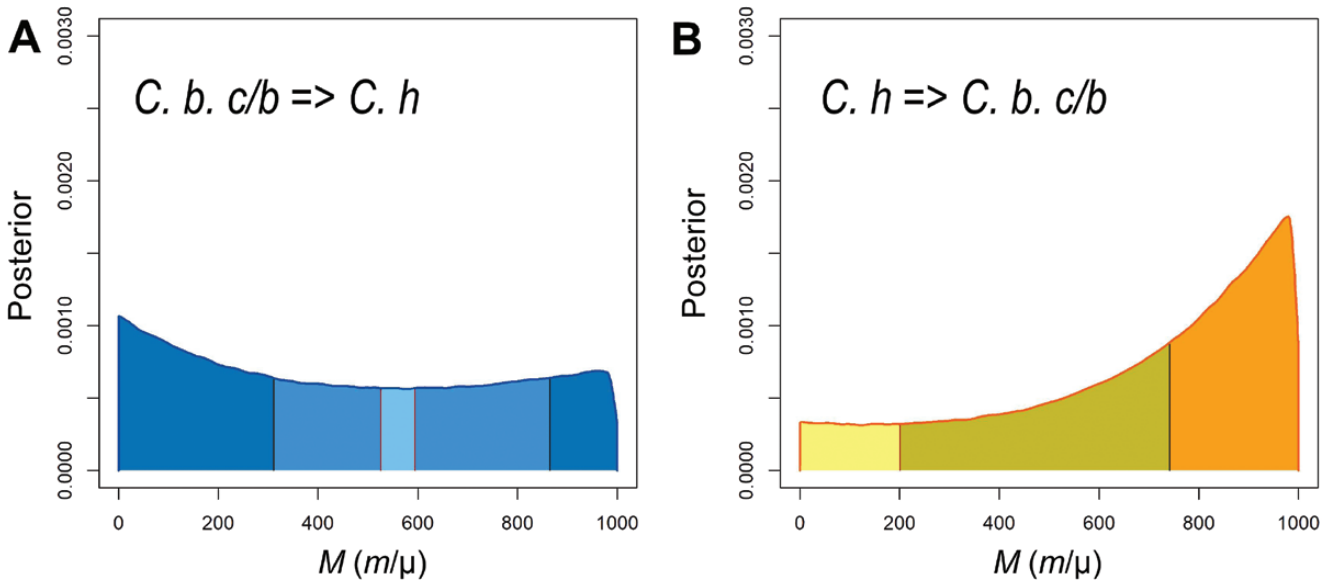

FIGURE 3. Posterior probability distributions of the migration parameter $M = m / \mu$ based on ND2 data (1,041 bp) suggest gene flow from *C. helianthea* to *C. b. bonapartei* (**B**), but gene flow could not be estimated reliably from *C. b. bonapartei* to *C. helianthea* (**A**). Colors and lines correspond to the limits of the intervals accumulating 50% (darker colors) and 95% (medium colors) of the probability density.

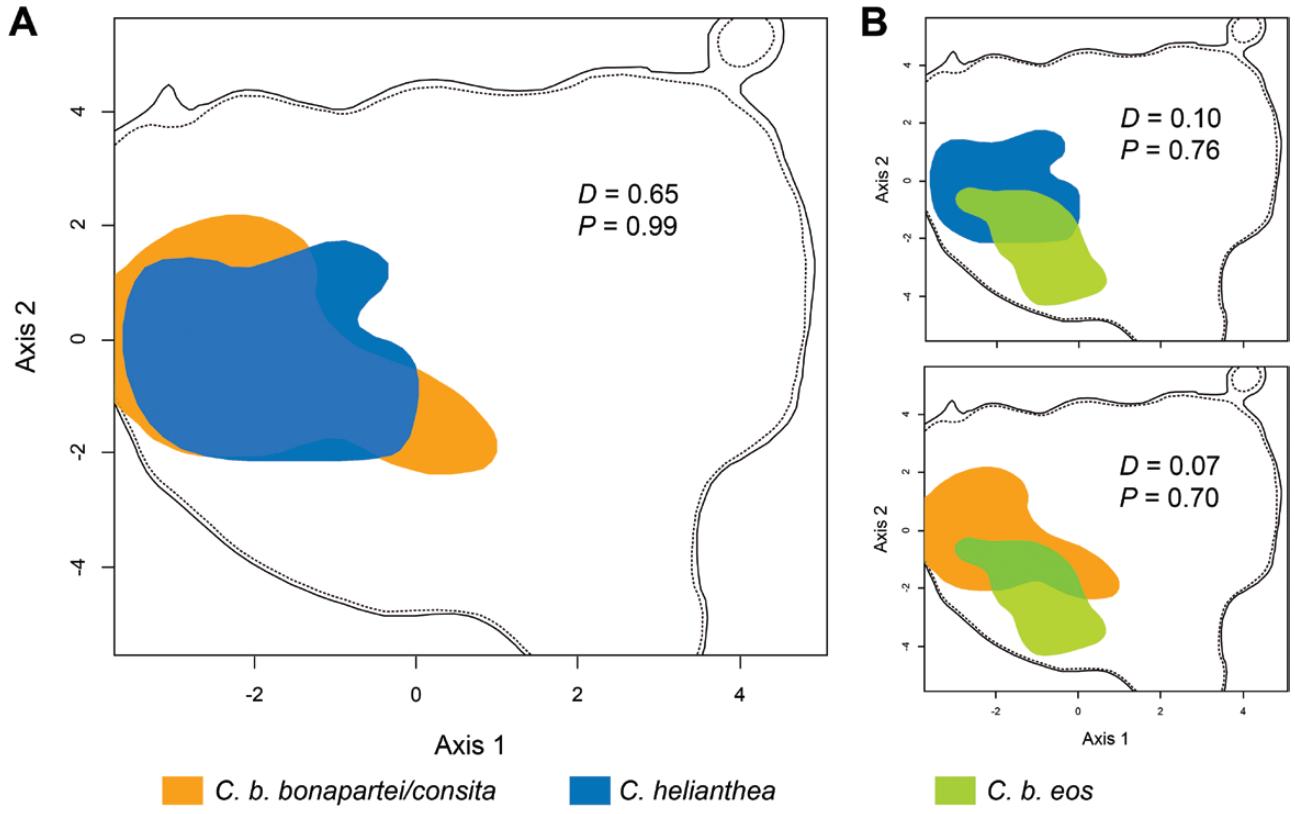

FIGURE 4. Because *C. helianthea* and *C. b. bonapartei/consita* do not differ in climatic niches, their phenotypic differences are not attributable to Gloger's rule. The climatic niches of *C. helianthea* and *C. b. bonapartei/consita* overlap considerably ($D = 0.65$) (**A**). The climatic niche of *C. b. eos* overlaps little with *C. helianthea* ($D = 0.10$) and *C. b. bonapartei/consita* ($D = 0.07$) climatic niches (**B**). Nevertheless, relative to the background (solid and dashed black lines represent 100% and 95% of the available background environment, respectively) the differences between the niches are not significant in any case ($P > 0.1$).

Is There Morphometric Variation Between Species That May Suggest Adaptations to Alternative Microhabitats or Resources?

Morphometric data showed differences between *C. h. helianthea* and *C. b. bonapartei* and between females and males of each taxon: LD analysis distinguished species/sex with a low classification error of 1.2%. The 2 most relevant variables in the LD function were wing loading (coefficients: LD1 = 240.2, LD2 = 287.8, and LD3 = 120.0), and wing taper (coefficients: LD1 = −39.7, LD2 = −42.6, and LD3 = −23.4). ANOVA or Kruskal-Wallis tests showed significant differences in 11 morphological variables between species, and in 14 variables between sexes (Appendix Figure 8). The 3 variables that differed the most between species and sexes were length of extended wing (ANOVA coefficients: −3.4 species and 5.9 sex), total culmen (ANOVA coefficients: 2.3 species and 2.2 sex), and length of tail (ANOVA coefficients: 1.0 species and 3.7 sex). *Coeligena b. bonapartei* has longer wings, shorter bills, and shorter tails than *C. h. helianthea* ($P \leq 0.001$ in all cases), and females have shorter wings, longer bills, and shorter tails than males in both species ($P \leq 0.001$ in all cases). Our analyses further revealed that the magnitude of morphometric differences between sexes varied by species. For example, females of *C. helianthea* are the smallest of the 4 groups (i.e. combinations of species and sexes), but males of *C. helianthea* are the largest.

DISCUSSION

Coeligena helianthea and *C. bonapartei* are closely related species of hummingbirds from the Northern Andes differing distinctly in plumage coloration, but we found a striking lack of genetic differentiation between them in a mitochondrial gene (ND2) and in 2,313 UCE markers broadly scattered across the genome (Figure 2). Considering that low genomic divergence is typically associated with low differentiation in coloration in other Andean birds (Winger 2017), the strong phenotypic differences between *C. helianthea* and *C. bonapartei* in the absence of neutral genetic differentiation are remarkable, and make these species an appropriate system in which to search for the genetic basis and adaptive significance of phenotypic differences involved in speciation (see Campagna et al. 2017). However, we found no evidence that MC1R (a candidate gene associated with melanic pigmentation in a variety of vertebrates) underlies phenotypic variation, and found no support for the hypothesis that Gloger's rule (adaptation to geographic variation in humidity) or other macroclimatic niche differences (Figure 4) are associated with phenotypic divergence between these species. Nonetheless, our finding that *C. h. helianthea* and *C. b. bonapartei* differ in morphometric traits (Appendix Figure 8) potentially related to habitat and resource use is consistent with the hypothesis that natural selection may have played a role in their divergence. In addition, as we discuss below, phenotypic divergence in coloration with little genetic differentiation may reflect sexual selection.

Because coalescent estimates of migration based on ND2 and UCE data suggested that *C. helianthea* and *C. b. bonapartei/consita* may have experienced migration (Figure 3 and Appendix Figure 7), their phenotypic divergence might have arisen or been maintained by selection in the face of gene flow. However, preliminary analyses with UCE data did not allow us to rule out incomplete lineage sorting as an explanation for the low genetic divergence between these species, although we note that divergence with gene flow and incomplete lineage sorting are not mutually exclusive explanations of shallow genetic differentiation (Kutschera et al. 2014), especially when speciation occurs rapidly due to selection (Suh et al. 2015, McLean et al. 2016). Because our sample size for UCE markers was admittedly small, more extensive data and analyses of genome-wide variation are required to reach more definitive conclusions. Complete genome analyses may help clarify the influence of introgression and incomplete lineage sorting on patterns of genetic variation (Suh et al. 2015), may allow reducing uncertainty in the estimation of population genetic parameters (Hey and Nielsen 2004), and may allow identifying the genetic basis of phenotypic differences (Bourgeois et al. 2016, Toews et al. 2016, Campagna et al. 2017).

We found no variation between species in the coding region of MC1R, a gene associated with variation in plumage coloration in several other birds (Theron et al. 2001, Mundy 2004, Doucet et al. 2004, Baião et al. 2007, Gangoso et al. 2011). Nevertheless, other studies have shown no association between plumage coloration differences and variation in the coding region of MC1R (MacDougall-Shackleton et al. 2003, Cheviron et al. 2006, Haas et al. 2009). As in these latter studies, our work suggests that differences in coloration between *C. helianthea* and *C. bonapartei* are controlled by other genetic mechanisms which may include genes agonists or antagonists of MC1R in the melanin metabolic pathway, regions regulating the expression of MC1R or other genes (Theron et al. 2001), and genes controlling traits of feather structure influencing the production of structural colors (Shawkey et al. 2003).

We found no support for Gloger's rule because the darker *C. helianthea* does not occur in more humid environments than the more lightly colored *C. bonapartei* (Figure 4). Nevertheless, adaptation to different environmental conditions may occur at a finer scale, where habitat differences might select for plumage traits that, for instance, stand out from the background augmenting signal efficacy (Endler 1993, Brumfield and Braun 2001). Indeed, we found that the species differ in morphometric traits (e.g., *C. bonapartei* has longer wings and shorter tails than *C. helianthea*; Appendix Figure 8) typically

associated with use of different microhabitats or foraging behaviors. Variation in such traits can affect flight speed or the relative ability to maneuver in open vs. closed environments (Altshuler et al. 2010, Ortega-Jimenez et al. 2014). To the extent that morphological differences may reflect adaptations to different resources between species (Altshuler and Dudley 2002) and between sexes within species of hummingbirds (Temeles and Kress 2010), our data are consistent with a role for selection driving morphological divergence, but the adaptive value of phenotypic variation, if any, remains to be discovered. Considering that *C. bonapartei* often occurs along forest edges whereas *C. helianthea* is more frequently found in forest interior (Hilty and Brown 1986), studies of the functional consequences of phenotypic differences would be especially useful to assess any potential role of natural selection in driving and maintaining divergence.

Knowledge of the timing of speciation also might allow one to make inferences about historical processes that could have promoted divergence between *C. helianthea* and *C. bonapartei*. We estimated that the most recent common ancestor of these species diverged from *C. b. eos* between 0.17 and 0.46 mya (Appendix Figure 5). Therefore, divergence between *C. helianthea* and *C. bonapartei* must be more recent, potentially coinciding with some of the last glaciations of the Pleistocene when high-altitude environments were uninhabitable and forests likely retreated, resulting in the isolation and divergence of populations (Vuilleumier 1969, Ramírez-Barahona and Eguiarte 2013). Under this scenario, *C. helianthea* and *C. bonapartei* may have diverged in allopatry and their lack of genetic differentiation could be a result of hybridization after secondary contact, with the existence of 2 groups of haplotypes reflecting geography (north and south) more than taxonomy (i.e. plumage phenotype; Figure 2) reflecting divergence in allopatry followed by range expansions by both species and subsequent hybridization in both areas. It thus remains possible that different selective regimes promoted speciation in these hummingbirds if their divergence occurred across environments with contrasting climatic conditions in the Pleistocene even if they occupy similar environments at present. Although such a hypothesis might be partly testable by modeling historical climates and potential distributions, one would still be faced with the question of what evolutionary forces might maintain *C. helianthea* and *C. bonapartei* as distinct given that they occur in regional sympatry in the same macroenvironments in the present.

Aside from natural selection, another plausible explanation for the origin and maintenance of phenotypic distinctiveness in plumage, given the strong sexual dichromatism in *C. helianthea* and *C. bonapartei*, is that their differentiation was promoted by sexual selection (Price 1998, 2007). Sexual selection is thought to be a powerful force driving speciation in birds and other organisms (Campagna et al. 2012, 2017; Harrison et al. 2015), and some examples exist of speciation due to sexual selection with gene flow (Servedio 2016). Of direct relevance to our system, a study comparing sexually selected (i.e. gorget and crown coloration) and non-sexually selected traits among *Coeligena* species found that sexual selection may be an important driver of phenotypic differentiation, but that it is probably insufficient for speciation to be completed unless it acts in concert with natural selection (Parra 2010; see also Servedio and Boughman 2017). To assess the plausibility of the hypothesis that sexual selection is involved in the divergence and speciation of *C. helianthea* and *C. bonapartei*, one should test for associations among components of males' fitness, signaling traits (i.e. coloration, songs), and female preferences. Genomic analyses examining whether there are genetic and signatures of selection acting on regions associated with sexual traits (Charlesworth 2009, Huang and Rabosky 2015, Kirkpatrick 2017) would further help to test the hypothesis of divergence driven by sexual selection.

Another explanation for our results showing no genetic differentiation despite marked phenotypic differences and patterns of population genetic structure better reflecting geography than plumage phenotype is that *C. helianthea* and *C. bonapartei* are not different species but rather morphs within a single species which have become differentially sorted in different areas. While this possibility is intriguing and parapatric populations lacking neutral genetic differences yet exhibiting distinct plumages are treated as conspecific in other birds (e.g., Joseph et al. 2006, Poelstra et al. 2014; but see Aguillon et al. 2018), we stress that with the exception of a handful of old specimens there is no current evidence of hybridization that would suggest that *C. helianthea* and *C. bonapartei* are conspecific. Furthermore, because plumage differences between them are quite striking in the context of differences among undisputed species in the genus and other hummingbirds (Remsen et al. 2018), we believe they are best treated as distinct species given existing evidence.

In conclusion, our study provides evidence that the formation of 2 species of Andean hummingbirds likely occurred recently, rapidly, and possibly in the face of gene flow, suggesting that some form of selection played a role maintaining phenotypic differences and driving speciation. However, because the main selective mechanism we examined (i.e. adaptation to contrasting macroclimatic conditions) appears not to operate in *C. helianthea* and *C. bonapartei*, we conclude that ecological pressures that we did not consider directly or sexual selection may have been involved in their divergence. Future studies should thus aim to test predictions of hypotheses of natural and sexual selection acting on this system. Regardless of the selective processes involved, in line with previous research,

our study suggests that selection may play an important role in maintaining phenotypic differences that could lead to speciation in tropical montane birds (Cadena et al. 2011, Winger and Bates 2015). Finally, the shallow genetic divergence that we observed between these species suggests that their genomes are unlikely to have been substantially affected by processes occurring after speciation (e.g., post-speciation divergence by drift), which makes this system especially promising for work on the genomics of speciation. Studies aiming to understand the genetic underpinnings of species differences employing genomic approaches (e.g., Campagna et al. 2017, Stryjewski and Sorenson 2017) will be an important complement to increasing knowledge of the geographic and ecological context of speciation in tropical montane birds.

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Ethics statement: We followed the ethics protocols of our institutions and countries. We did not cite any particular protocol since we used sample tissues from collections.

Author contributions: S.G.R., C.D.C., and J.L.P. conceived the idea. C.P. and S.G.R. performed the experiments and analyzed the data. A.M.C., F.G.S., J.E.M., and C.D.C. contributed substantial materials, resources, and funding. All authors contributed to the writing of the paper led by C.P. and C.D.C.

Data deposits: Genetic data are available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

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APPENDIX

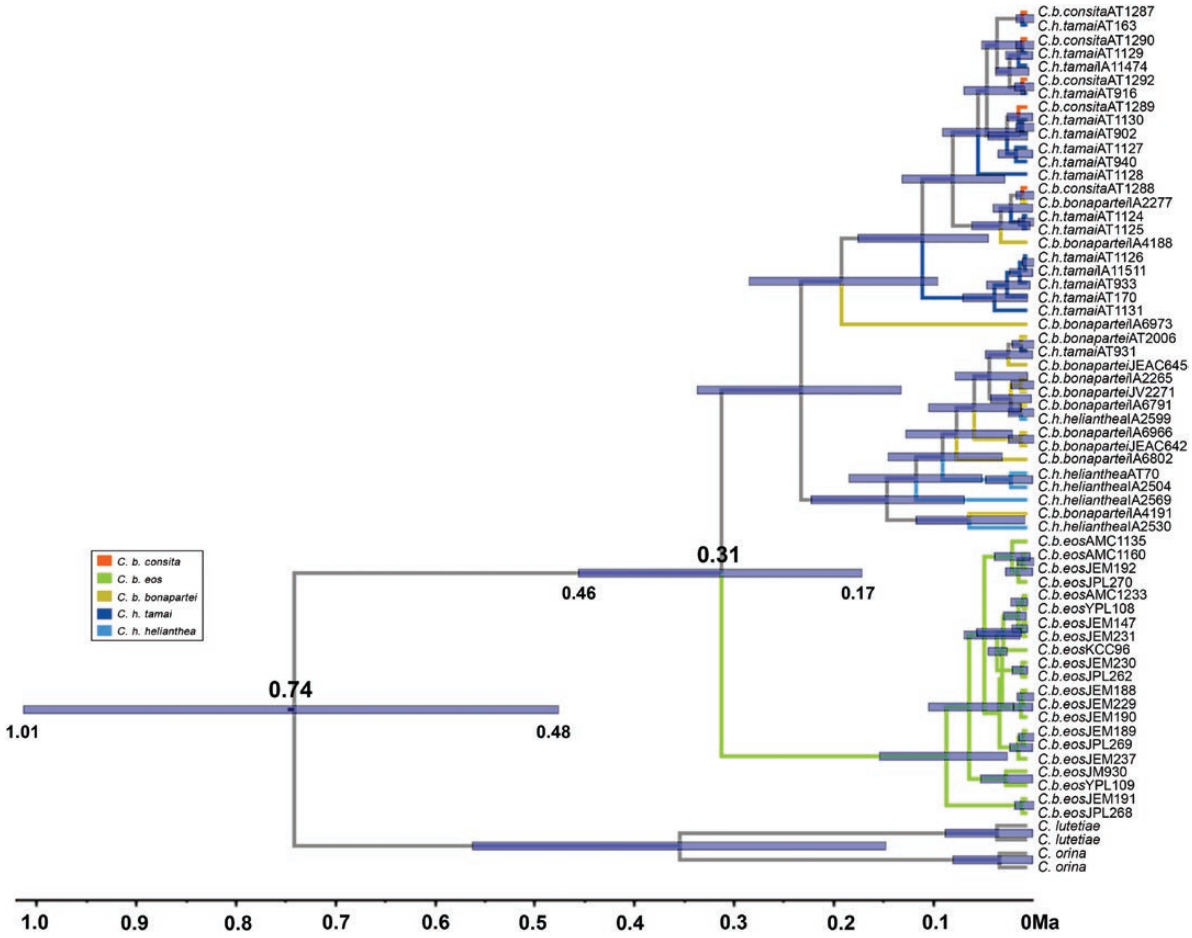
Methods and Results

DNA samples and protocols. PCR mixes to amplify the ND2 gene were prepared to a final volume of 25 μL and contained 2 μL of DNA, 1.0X of Taq buffer, 3.0 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.48 μM of each primer L5215 and H6313 (Sorenson et al., 1999), and 1 U of Taq DNA polymerase recombinant (Invitrogen, São Paulo, Brazil). Amplification conditions were as follows: 94°C for 5 min for initial denaturation, 35 cycles of 94°C for 45 s for denaturation, 20 cycles of touch down –0.5°C fr=om 62°C to 52°C plus 15 cycles of 52°C for 45 s for annealing, and 72°C for 1 min for extension, and a final extension of 72°C for 7 min.

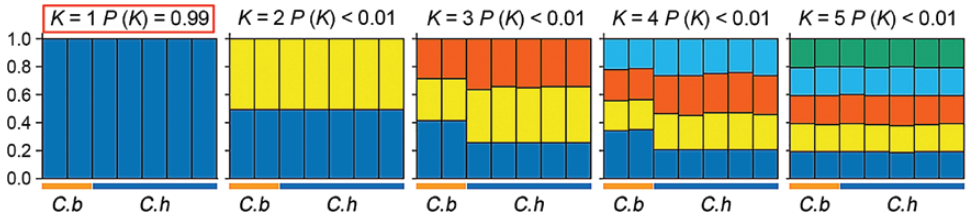
PCR mixes to amplify the MC1R gene were prepared to a final volume of 25 μL and contained 2 μL of DNA, 1.0X of Taq buffer, 3.0 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.48 μM of each primer 1corMSHR9 and 1corMHSR72 (Cheviron et al., 2006), 0.08% of BSA, and 1 U of Taq DNA polymerase recombinant (Invitrogen). Amplification conditions were as follows: 94°C for 5 min for initial denaturation, 35 cycles of 94°C for 30 s for denaturation, 64°C for 45 s for annealing and 72°C for 60 s for extension, and a final extension of 72°C for 5 min.

We used as outgroups for ND2 phylogenetic analyses sequences of *Coeligena lutetiae* (GenBank: FJ903516.1 and EU042542.1), and *Coeligena orina* (GenBank: KJ602225.1 and KJ602224.1), and as outgroups for MC1R analyses sequences of *Calypte anna* (GenBank: XM_008491753 partial sequence) and *Chaetura pelagica* (GenBank: XM_010008427).

Phylogenetic and population genetic analyses with UCEs. In addition to the concatenated analyses described in the main text, we inferred a species-tree considering gene-tree heterogeneity based on the UCE data. We initially used PhyML (Guindon et al. 2010) to generate gene trees from the 525 informative UCE loci employing the GTR substitution model for all loci, the NNI search method, and no bootstrapping while optimizing tree topology and branch lengths. We used these gene trees as input to build species trees in ASTRAL (Zhang et al. 2018, Rabiee et al. 2019) using default parameters. We conducted 3 separate ASTRAL analyses: (1) with no topological constraints, (2) enforcing the monophyly of species, and (3) enforcing the monophyly of subspecies. The topologies obtained in our concatenated analysis of UCE data (Figure 2) and in the species-tree analysis with no topological constraints were identical. The species-tree obtained enforcing the monophyly of species revealed a short branch separating *C. bonapartei* and *C. helianthea*,



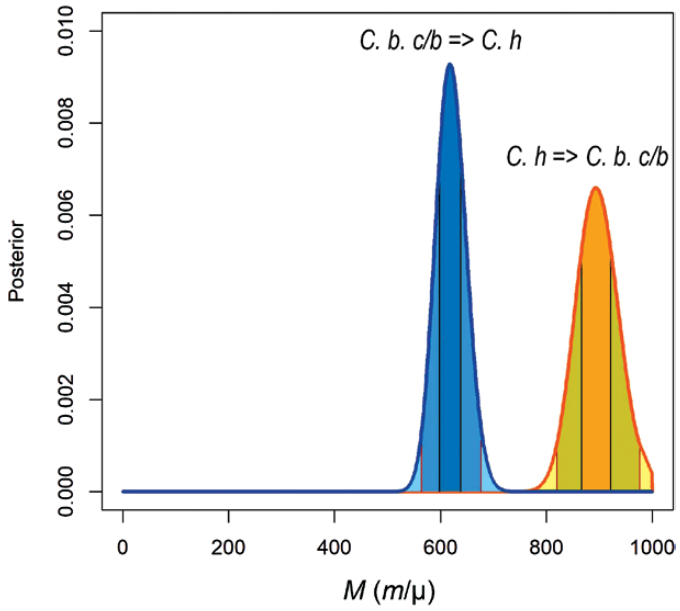
APPENDIX FIGURE 5. Bayesian phylogenetic reconstruction and divergence time estimates for lineages of *C. helianthea* and *C. bonapartei* inferred with ND2 sequences (1,041 bp) using a strict clock and a coalescent population constant prior. Node bars show the 95% credibility intervals of the node age.



APPENDIX FIGURE 6. Structure plots for $K = 1$ to $K = 5$ based on 293 SNPs from UCE data for *C. helianthea (C.h)* and *C. bonapartei (C.b)*. $K = 1$ has the highest probability (red box).

supporting their shallow genetic divergence. The species-tree obtained enforcing the monophyly of subspecies showed the same topology, with similar support values, as the unconstrained species-tree.

Of the 525 UCE loci with informative sites, 232 loci were informative at sites where data were missing for at least 1 individual; 293 loci were informative at a total of 368 sites for which we had information for all 7 individuals. To maximize independence among informative sites, we extracted a single site per each of these informative loci, resulting in a matrix of 293 SNPs, which we used for the following analyses. We used the program Structure 2.3.4 (Pritchard et al. 2000) to assess population structure using the 293 SNPs extracted from UCE data. We performed 10 runs for each value of K from $K = 1$ to $K = 5$, using a burn-in period of 10,000 steps and 100,000 repetitions. We



APPENDIX FIGURE 7. Posterior probability distributions of the migration parameter $M = m / \mu$ based on 293 SNPs from UCE data suggest gene flow between *C. helianthea* and *C. bonapartei* in both directions. Colors and lines correspond to the limits of the intervals accumulating 50% (darker colors) and 95% (medium colors) of the probability density.

followed Pritchard et al. (2000) to calculate the probability of different values of K using the mean ln likelihood value calculated over the 10 runs as $\text{prob}_{(K = n)} = (e^{\ln K = n}) / (e^{\ln K = 1} + \ldots + e^{\ln K = n})$. The most likely number of genetic clusters in the dataset was $K = 1$ ($\text{prob}_{(K = 1)} = 0.99$). Support for larger values of K was much lower and clusters defined assuming different values of K never corresponded to groups defined by species taxonomy (Appendix Figure 6).

Testing for divergence with gene flow With UCEs. We used our 293 SNP dataset for Migrate analyses after confirming that it met the assumption of neutrality by calculating Tajima's D using DNAsp 5.1 (Librado and Rozas 2009). We ran Migrate for the UCE data using the same priors than for the ND2 data after several test runs, and using a long chain of 1,000 million steps (sampling 1,000,000 steps recorded every 1,000 steps) with a burn-in of 500,000 steps.

Because sample sizes differed considerably between ND2 and UCE datasets, we also explored whether any potential discrepancies in the results of analyses of different markers could reflect sampling effects. We subsampled the ND2 matrix by randomly selecting the same number of individuals per taxon having UCE data and then estimated parameters in Migrate for several of these reduced datasets using the same priors as above. Because results were consistent among the complete dataset and the subsamples, we concluded that results from the UCE dataset are unlikely to be biased because of limited sampling.

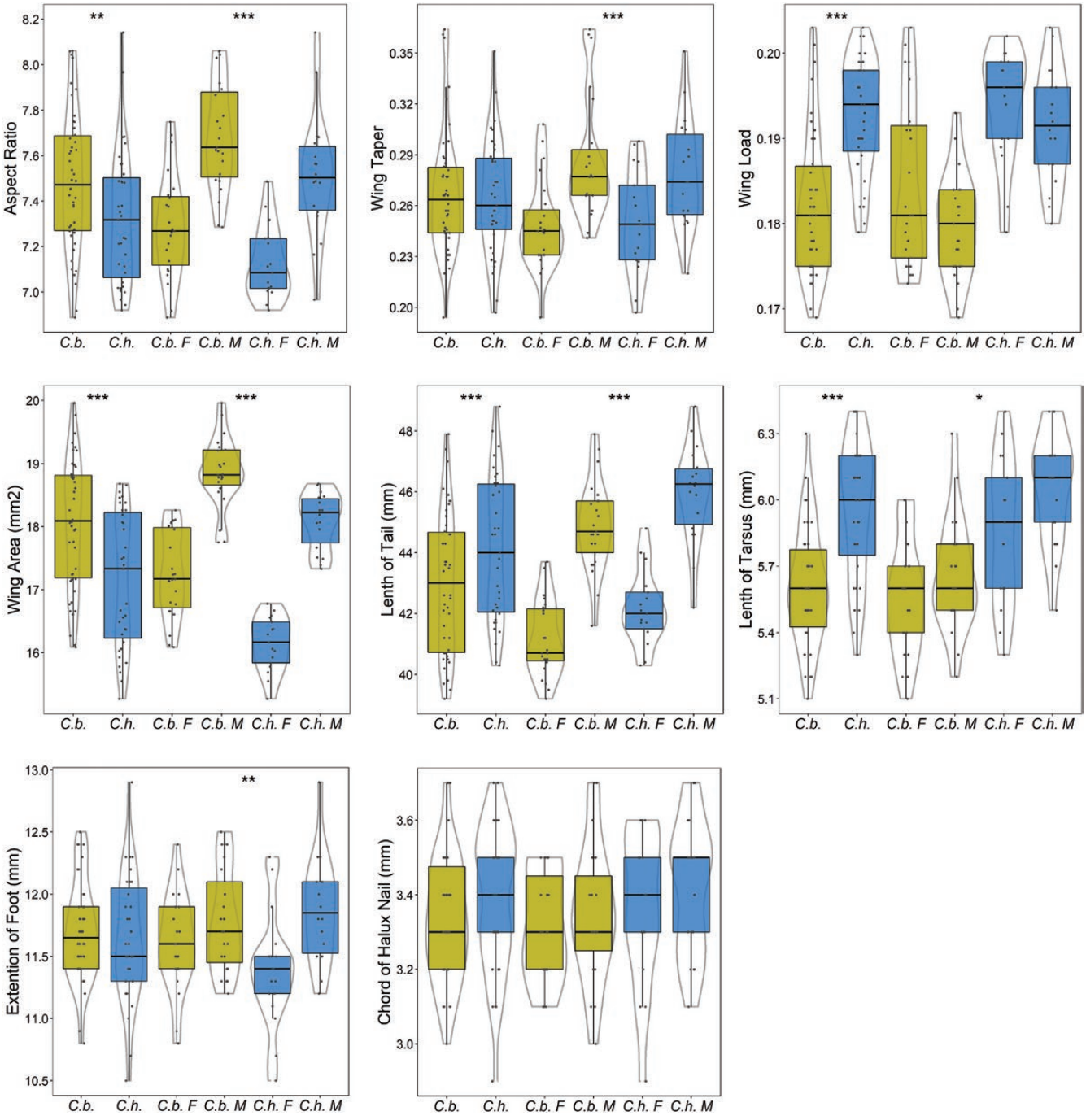
Our UCE dataset fit the assumption of neutrality (Tajima's D value was not significant: $D = 1.5$; $P > 0.1$). Whereas the magnitude of gene flow from *C. b. bonapartei/consita* to *C. helianthea* could not be estimated reliably in the ND2 data analyses, the UCE data suggested that there has been gene flow between *C. helianthea* and *C. b. bonapartei/consita* after their divergence in both directions. Mean estimates of migration ($M = m / \mu$) were different from zero: $M = 896.3$ from *C. helianthea* to *C. b. bonapartei/consita* and $M = 620.3$ from *C. b. bonapartei/consita* to *C. helianthea*. Also, the posterior probability distributions of M estimated from the ND2 data were wide, and in contrast to the UCE data they were narrowly concentrated around the mean: 95% CI: 819.3 to 976.0 from *C. helianthea* to *C. b. bonapartei/consita* and 95% CI: 564.0 to 676.7 from *C. b. bonapartei/consita* to *C. helianthea* (Appendix Figure 7), rejecting scenarios of no migration.

Migrate 3.2.1 (Beerli 2009) also offers the option to estimate parameter M for different time bins, allowing one to estimate migration at different moments through time. Given non-zero estimates of migration, divergence-with-gene-flow predicts higher values of M close to the time of divergence, whereas post-speciation gene flow (i.e. recent hybridization) predicts higher values of M near the present. We used our ND2 and UCE datasets and the previously described running conditions to estimate variation of M through time. Our results are consistent with the divergence-with-gene-flow model, but we found discrepancies in the

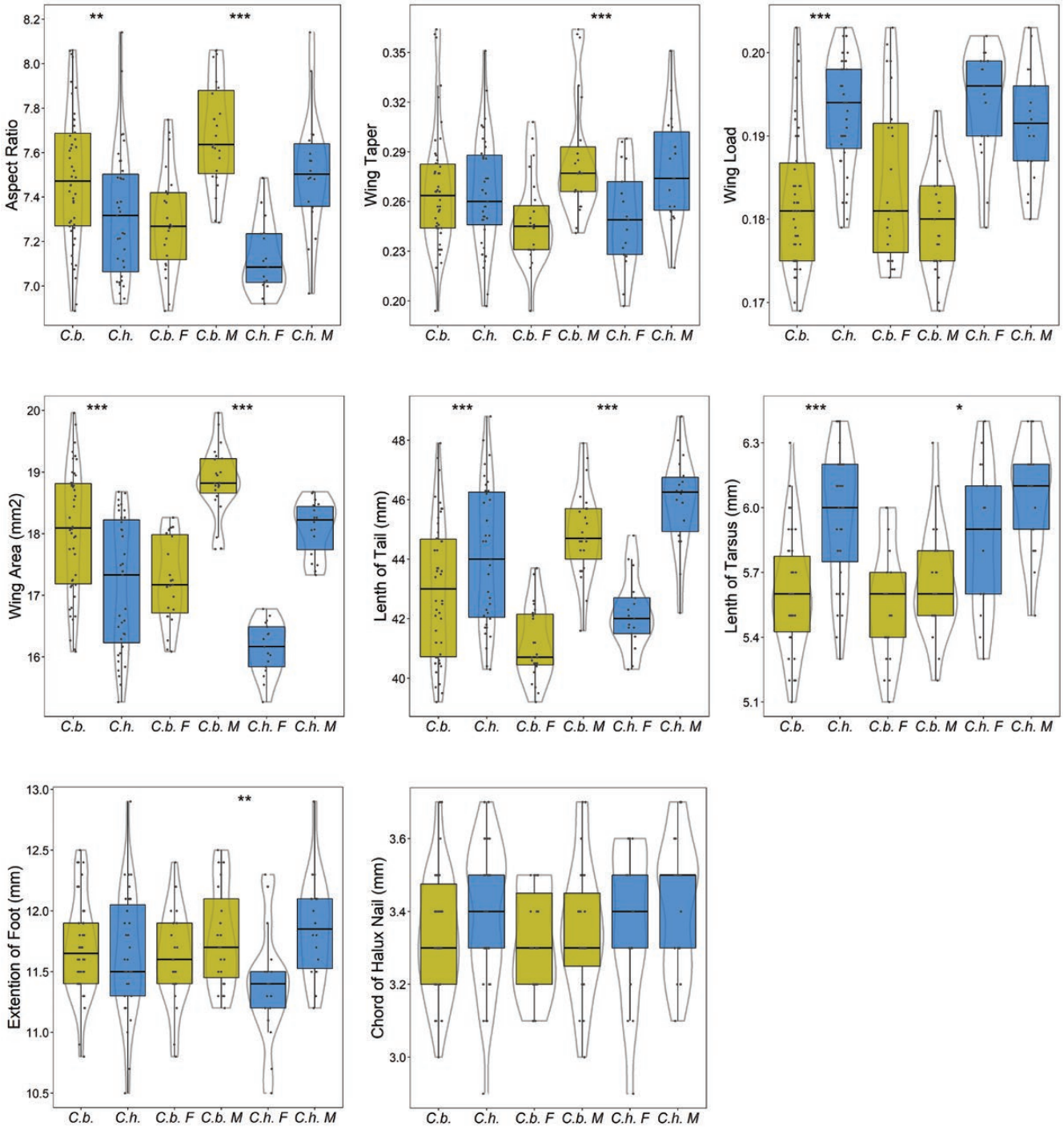
results obtained from these analyses. We found that despite a high posterior probability of no migration ($M = 0$) between *C. b. eos* and either *C. b. bonapartei/consita* or *C. helianthea*, the analysis through time shows high values of M for the same comparisons. Thus, we considered the results of M through time analyses unreliable. We do not know the details behind the estimation through time analysis option in Migrate, but it appears not to be working properly with our data, and we regarded the results from this analysis with caution.

ABBA-BABA test with UCEs. To evaluate whether low genetic differentition between species is more likely due to gene flow or incomplete lineage sorting, we used our 525 UCE informative loci to perform an ABBA-BABA test and to calculate the *D*-statistic and Z-score (Reich et al. 2009) using the ANGSD software (Korneliussen et al. 2014). We used as an outgroup UCE data from the genome of the Black-breasted Hillstar (*Oreotrochilus melanogaster*; C. C. Witt personal observation) and established the species topology as the one we obtained from the UCEs ([*C. helianthea*, *C. b. bonapartei*] *C. b. consita*) outgroup.

Despite the apparent evidence of migration found with Migrate for mitochondrial and UCE loci, the ABBA-BABA test performed on the UCE data revealed no significant differences in the number of ABBA and BABA sites ($|D\text{-statistics}| < 0.011$, $|\text{Z-scores}| < 1.1$; significance threshold = 3.0; Reich et al. 2009). This suggests that the low genetic divergence between *C. helianthea* and *C. bonapartei* is more likely due to incomplete lineage sorting than to introgression, a scenario requiring no role for selection if populations diverged in allopatry. We sought to implement other analyses with the goal of gauging support for scenarios of gene flow vs. incomplete lineage sorting (e.g., demographic model selection using PHRAPL; Morales et al. 2017) but results were inconclusive because of limitations in our sample size for UCE data.



APPENDIX FIGURE 8. Boxplots of 17 morphological variables showing variation between species and sexes within species. *C. b. bonapartei* (*C.b.*), *C. h. helianthea* (*C.h.*), females (*F*), and males (*M*). Asterisks at the top of the figures correspond to the values of significance in the ANOVA or Kruskal-Wallis tests, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.



APPENDIX FIGURE 8. *Continued*