Replicate hybrid zones suggest a limited role of plumage in reproductive isolation among subspecies of the Variable Seedeater (Sporophila corvina)

Diego Ocampo[1], Kevin Winker[2], Matthew Miller[3], Luis Sandoval[4], and J Albert Uy[5]

[1]University of Miami
[2]University of Alaska Museum
[3]Smithsonian Tropical Research Institute
[4]Universidad de Costa Rica
[5]University of Rochester

September 14, 2022

Abstract

After establishing secondary contact, recently diverged populations may remain reproductively isolated or hybridize to a varying extent depending on factors such as hybrid fitness and the strength of assortative mating. Replicated contact zones between hybridizing taxa offer a unique opportunity to explore how different factors interact to shape patterns of hybridization. Here, we used genomic and phenotypic data from three independent contact zones between subspecies of the Variable Seedeater (Sporophila corvina), to examine how coloration and genetic divergence shape patterns of hybridization. We found that plumage coloration has limited introgression across contact zones, but the degree of plumage divergence does not explain overall patterns of introgression. Across two parallel contact zones between populations with divergent phenotypes (entirely black vs. pied plumage) populations hybridized extensively across one contact zone but not the other, suggesting that plumage divergence is not sufficient to maintain reproductive isolation. Where subspecies hybridized, hybrid zones were wide and formed by later-generation hybrids, suggesting that hybrids present similar or higher fitness than parental subspecies. Moreover, contemporary gene flow has played an important role in shaping patterns of genetic diversity between populations. Overall, our results demonstrate that divergence in plumage coloration is important in reducing gene flow but insufficient in maintaining reproductive isolation in this clade, and that other factors such as divergence in song and time since secondary contact may also play an important role in driving patterns of reduced hybridization and gene flow.

Replicate hybrid zones suggest a limited role of plumage in reproductive isolation among subspecies of the Variable Seedeater (*Sporophila corvina*)

Running title: Limited role of plumage in hybridization

Diego Ocampo[1,2,3*], Kevin Winker[4], Matthew J. Miller[3,4,5], Luis Sandoval[6], J. Albert C. Uy[2]

1. Department of Biology, University of Miami, Coral Gables FL 33146, USA
2. Department of Biology, University of Rochester, Rochester, NY 14627, USA
3. Smithsonian Tropical Research Institute, 0843-03092 Balboa, Ancon, Panama

1. University of Alaska Museum, 907 Yukon Drive, Fairbanks, AK 99775, USA
2. Reneco International Wildlife Consultants, Abu Dhabi, UAE
3. Laboratorio de Ecología Urbana y Comunicación Animal, Escuela de Biología, Universidad de Costa Rica, 11502 San José, Costa Rica.

* Corresponding author: ocampov.diego@gmail.com

ABSTRACT

After establishing secondary contact, recently diverged populations may remain reproductively isolated or hybridize to a varying extent depending on factors such as hybrid fitness and the strength of assortative mating. Replicated contact zones between hybridizing taxa offer a unique opportunity to explore how different factors interact to shape patterns of hybridization. Here, we used genomic and phenotypic data from three independent contact zones between subspecies of the Variable Seedeater (*Sporophila corvina*), to examine how coloration and genetic divergence shape patterns of hybridization. We found that plumage coloration has limited introgression across contact zones, but the degree of plumage divergence does not explain overall patterns of introgression. Across two parallel contact zones between populations with divergent phenotypes (entirely black vs. pied plumage) populations hybridized extensively across one contact zone but not the other, suggesting that plumage divergence is not sufficient to maintain reproductive isolation. Where subspecies hybridized, hybrid zones were wide and formed by later-generation hybrids, suggesting that hybrids present similar or higher fitness than parental subspecies. Moreover, contemporary gene flow has played an important role in shaping patterns of genetic diversity between populations. Overall, our results demonstrate that divergence in plumage coloration is important in reducing gene flow but insufficient in maintaining reproductive isolation in this clade, and that other factors such as divergence in song and time since secondary contact may also play an important role in driving patterns of reduced hybridization and gene flow.

KEYWORDS: genetic divergence, hybridization, plumage coloration, replicated contact zones, secondary contact.

Contact zones between hybridizing taxa offer a window into the speciation process (Barton & Hewitt, 1985; Harrison, 1990; Price, 2008). For instance, contact zones shed light on which traits are important for assortative mating and reproductive isolation, as well as the genomic basis of such traits (Barton & Gale, 1993; Nadeau et al., 2014; Semenov et al., 2017). Hybridization between differentiated taxa may have different outcomes (Abbott et al., 2013), such as the merger of distinct lineages (Todesco et al., 2016), or even the rise of a new and independent lineage through homoploid hybrid speciation (Mavárez & Linares, 2008). However, the degree of the homogenization is dependent on the biology of the taxa involved (Gompert et al., 2017; Irwin & Schluter, 2021). Identifying the factors that promote or limit gene flow among divergent populations is key to uncovering the origin of reproductive barriers, and thus the drivers of speciation (Coyne & Orr, 2004; Jiggins & Mallet, 2000).

Characterizations of hybrid individuals and hybrid zones offer further insights into the dynamics shaping interactions among lineages. For instance, comparisons of hybrid indices, which estimate the proportion of an individual's ancestry from different lineages, and the level of heterozygosity at well-differentiated loci can classify individuals as first-generation hybrids, later-generation hybrids, or backcrosses (Fitzpatrick, 2012). Moreover, geographic clines provide insight into the role of deterministic processes in maintaining species boundaries, by fitting clinal models of how traits and genetic markers transition between distinctive populations (Endler, 1977). Here, the width of the cline provides a measure of the permeability of the reproductive barrier, and the concordance between cline widths of different traits can inform about the relative strength of selection on a given trait (Barton & Gale, 1993). Further, comparing cline centers may identify traits and genes that have undergone differential introgression (e.g., Baldassarre et al., 2014; Brumfield et al., 2001). Overall, these characterizations offer insights into which traits are important in causing reproductive isolation, the strength of selection on different traits, and even the relative fitness of individuals with mixed ancestry (e.g., Coster et al., 2018; Walsh et al., 2016).

Interpreting contact zone dynamics is often challenging, as the same pattern of phenotypic and genetic variation can result from different processes (Harrison, 1990). For example, an intergradation between two divergent populations might have occurred from ongoing parapatric divergence (i.e., primary contact) or from hybridization after differentiation in geographic isolation (i.e., secondary contact; Morales-Rozo et al., 2017). Comparing model support of alternative demographic scenarios based on coalescent theory may differentiate

primary versus secondary contact, thus providing better estimates of important demographic parameters, such as rates and timing of gene flow, that have created the observed patterns of genomic variation (Marchi et al., 2021). Characterizing the situation at replicated hybrid zones increases the power and resolution to infer the factors driving divergence and introgression, because parallel results increase the support and likelihood for a given interpretation (Nadeau et al., 2014) and different results can help to distinguish independent effects of different factors shaping hybrid zone dynamics (Scordato et al., 2020).

The Variable Seedeater (*Sporophila corvina*) is a small tanager species which consists of four subspecies demonstrating relatively low genetic differentiation yet substantial phenotypic divergence (Ocampo et al., 2022a). Three subspecies occur in relatively close proximity across Costa Rica and Panama. The nominate subspecies *S. c. corvina* has almost completely black plumage and is distributed along the Caribbean slope from northeastern Costa Rica through central Panama. The other two subspecies, *S. c. hoffmanni* and *S. c. hicksii,* have white collars, bellies, and rumps (a pied plumage), differing only on the extent of the white patch of the throat (Olson, 1981), and range along the Pacific slope from central Costa Rica to eastern Panama. The species is absent in the high-elevation mountain ranges that separate the Caribbean and Pacific lowlands of Costa Rica and western Panama, but the three subspecies have contact where their distributions overlap. The three contact zones are as follows. First, previous morphological studies addressing taxonomic limits and distributions in this species have characterized a hybrid swarm occurring between *S. c. corvina*and *S. c. hicksii* in central Panama (Hellmayr, 1938; Olson, 1981; Stiles, 1996; Figure 1A, no. 1), while the contact zone between *S. c. hoffmanni* and *S. c. hicksii* is less clearly understood as an intergradation of these subspecies at the Pacific slope in Veraguas province, based on a few specimen records of intermediate phenotypes (Olson, 1981; Figure 1A, no. 2). Finally, recent observations in the Central Valley of Costa Rica suggest the existence of a third contact zone between populations of *S. c. hoffmanni* and *S. c. corvina* (examples of observations available at eBird: www.ebird.org; Figure 1A, no. 3). This final contact zone might have been mediated by deforestation and urbanization in Costa Rica over the last few decades (Joyce, 2006), creating artificial corridors that could have facilitated interbreeding between previously isolated clades (Carantón-Ayala et al., 2018; Moulton et al., 2017). However, these contact zones and interactions between subspecies are poorly studied, and to date, little is known about the genetic consequences of these three independent contact zones.

Here, we take advantage of the replicate contact zones between subspecies pairs of *S. corvina* occurring in Costa Rica and Panama (Figure 1A), looking at the fine-scale genetic variation and structure between populations and subspecies, to evaluate the role of phenotypic divergence and ecological barriers in mediating gene flow. We evaluate how differences in morphology and plumage pattern covary with genomic divergence to test the hypothesis that differentiation in plumage color is an important signal for conspecific recognition and thus acts as a reproductive barrier. We expect lower genomic differentiation and higher rates of hybridization between phenotypically similar subspecies (i.e.,*S. c. hoffmanni* and *S. c. hicksii*) than more phenotypically divergent subspecies (e.g., *S. c. corvina* and*S. c. hicksii*). Moreover, we characterize genomic hybrids and hybrid zones, and use model-based demographic inference to infer the most likely evolutionary scenarios shaping current patterns of genomic admixture and differentiation between subspecies.

METHODS

DNA extraction and variant calling

We obtained tissue samples and phenotypic data of Costa Rican and Panamanian populations from natural history museums and from wild-caught individuals to study the genetic and morphological variation between subspecies of *S. corvina* from allopatric sites and across contact zones. Tissue samples from natural history collections were stored in ethanol or frozen, while blood samples were collected in the field from birds caught with mist nets, extracting blood from the brachial vein. Procedures to work with wild animals in the field were reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (IACUC, Permit number: 19-128-LF). We extracted genomic DNA from 257 individuals (Table S1), using Qiagen's DNeasy Blood and Tissue Extraction Kit (Qiagen, Hilden, Germany). We followed the manufacturer's protocol with the optional addition of 4 ul of RNase to remove potential RNA contaminants.

We then used a Genotype-by-Sequencing approach (GBS; Elshire et al., 2011), digesting the DNA samples with the ApeKI restriction enzyme, and sequencing short fragments across the genome. GBS library prep and sequencing were carried out by the Bioinformatics Resource Center of the University of Wisconsin-Madison on an Illumina NovaSeq 6000 2x150 Shared Sequencing. We called SNP variants using TASSEL version 5.2.65 (Glaubitz et al., 2014), following the GBSv2 SNP discovery and production pipeline. We ran this pipeline with mostly default parameters but increased the minimum quality score to 20 (default mnQS = 0). We aligned our short-read sequences to the reference genome of the Tawny-bellied Seedeater (*Sporophila hypoxantha,* GenBank: GCA_002167245.1; Campagna et al., 2017) using BOWTIE2 (Langmead & Salzberg, 2012) and the "very-sensitive" preset for high accuracy. Then, we filtered genome-wide single-nucleotide polymorphisms (SNPs) using VCFTOOLS version 0.1.16 (Danecek et al., 2011). Our filtered criteria consisted of including biallelic SNPs with no indels, minimum read depth [?] 3, minimum allele frequency [?] 0.05, a maximum proportion of missing data [?] 0.25, and SNPs that were [?] 100 bp apart to reduce the effect of highly linked markers. We further filtered the data file in TASSEL, keeping SNPs with a maximum heterozygosity [?] 0.8 to exclude potential paralogs, and removed individuals with [?]40% missing data. The resulting data set retained 255 individuals and 14,839 SNPs. For the demographic modeling (see below), we used different criteria, including biallelic SNPs with no indels, minimum read depth of [?] 3, maximum proportion of missing data of [?] 0.15, and SNPs that were [?] 1000 bp apart to obtain a complete site frequency spectrum (not truncated due to minimum allele frequency filtering) while keeping a manageable SNPs sample size, due to computer processing. This second data set retained 8,437 SNPs from 10 randomly selected genotypically pure individuals per subspecies (i.e., $q > 0.95$, see Results).

Morphometric and plumage color traits

We included phenotypic traits from adults only, excluding juveniles. We recorded geographic coordinates from sampling locations when available, or assigned approximate coordinates based on detailed localities from museum labels when explicit geographic coordinates were missing. We measured seven morphometric traits (i.e., body mass, total culmen length, beak depth and width at the nares, right wing chord, right tarsus length, and tail length) using a caliper (accuracy +- 0.1mm) from 193 males, 125 females, and nine individuals of unknown sex (Table S1). We combined beak measurements with a principal components analysis to obtain a single index that reflects beak size (PC1 explained 58.6% of variance), in which larger values of PC1 mean larger beak size. We measured plumage reflectance from museum skins from 86 males (Table S1). To reduce the effect of color degradation with specimen age, we included only specimens collected within the last 50 years (Armenta et al., 2008). We measured the spectral properties from the three body patches that differ between subspecies (i.e., rump, throat, and belly), collecting five reflectance measurements (from 300 to 700 nm) per patch. We used an Ocean Optics USB2000 spectrophotometer, and a PX-2 pulsed Xenon light source (Ocean Optics Inc., Dunedin, FL, USA), with the average reading set at 50, integrating time at 16 msec, boxcar smoothing at 10, and correction for electric dark noise activated. The tip of the fiber-optic probe was covered by a black anodized aluminum cap, angled at 45 degrees to reduce specular glare (as in Uy & Stein, 2007). To ensure consistency among measurements, we calibrated the spectrophotometer with a Spectralon(r) white diffuse standard and dark standard (Labsphere) after every five individuals measured. Spectral data were averaged between the five reflectance measurements per patch, we applied a Loess smoothing factor of 0.3, and corrected for any negative values. We estimated mean brightness (B2; Delhey et al., 2003) for each individual patch from the reflectance reads, using the R package PAVO2 (Maia et al., 2019). Morphometric measurements and plumage brightness values were used to compare phenotypic traits between individuals of the pure parental subspecies and fit geographic clines (see below).

Population structure of *S. corvina* subspecies

We assessed genetic structure between individuals of *S. corvina*using three complementary approaches. First, we estimate spatial genetic structure between samples using estimated effective migration surfaces (EEMS; Petkova et al., 2015). This software superposes a triangular grid of a predetermined number of demes (i.e., nodes) within the geographic area of interest and assigns the georeferenced samples to the closest deme. Then, EEMS estimates pairwise genetic distances between demes to identify paths of above- or below-average

genetic similarity, while correcting for geographic distance. Deviations from the average genetic distance for a given geographic distance are inferred to result from increased or reduced gene flow. Therefore, EEMS finds geographic regions that act as barriers or corridors to gene flow. We explored the spatial structure using an arbitrary number of 200, 300, and 400 demes, and completed three independent runs per number of demes with different seeds to assess consistency and the effect of demes density (Petkova et al., 2015).

Second, we used the program ADMIXTURE (Alexander et al., 2009) to test for the most likely number of genetically distinct population clusters (K) within Costa Rica and Panama, and we evaluated different population assignments and ancestry by identifying pure and admixed individuals. We ran ADMIXTURE for 100 iterations (-C), 200 bootstraps (-B), and 10 cross validations (-cv), with K values from 1 to 10. We found that $K = 3$ (see Results) was the most likely number of genetic populations and that they matched, in general, the three recognized subspecies. We therefore refer to pure individuals with$q$ values > 0.95 by their respective subspecies' name (i.e., *corvina* , *hoffmanni* , and *hicksii*). Then, we classified hybrids as those individuals with $q < 0.02$ from the third genetic cluster and in which q from the second genetic cluster was at least 2x the value from the third genetic cluster. Admixed individuals with $q > 0.1$ from the third cluster were considered three-way hybrids (as in Scordato et al., 2017) and were removed from further analyses, together with the remaining individuals in which q from the second and third populations were similar, because they cannot be confidently assigned for pairwise comparisons. We calculated average Weir and Cockerham'sF_{ST} per SNP between pure individuals (i.e.,*corvina vs hicksii* , *hoffmanni vs hicksii* , *corvina vs hoffmanni*) in VCFTOOLS to estimate genomic differentiation between subspecies pairs. Finally, we decomposed the genetic variation by a principal component analysis using the R package ADEGENET (Jombart, 2008), and visualized individuals based on the pure and hybrids categories described above based on q values from ADMIXTURE.

Characterization of hybrid individuals and contact zones

We characterized first-generation and later-generation hybrids to distinguish between current hybridization versus ancient gene flow. Our pairwise Weir and Cockerham's F_{ST} per SNP between pure parental subspecies allowed us to identify informative markers for subspecies ancestry ($F_{ST} > 0.60$). We then used these sets of informative markers to estimate the hybrid index and level of heterozygosity from parental subspecies and their corresponding hybrids on the R package INTROGRESS (Gompert & Buerkle, 2009). This method is commonly used to characterize hybrids, as it does not require fixed markers and broadly distinguishes ongoing hybridization (F1 hybrids with high heterozygosity) from historic hybridization and backcrosses (later-generation hybrids with low heterozygosity, e.g., Scordato et al., 2017).

We also characterized the contact zones between hybridizing subspecies (*corvina-hicksii* , *hicksii-hoffmanni* ; see Results) fitting geographic clines (Szymura & Barton, 1986). Geographic cline analyses were performed by fitting genetic and phenotypic clines using the R package HZAR (Derryberry et al., 2014), which allowed us to test how different traits covary along the geographic transition between two subspecies. Because contact zones can roughly align with the Caribbean (*corvina-hicksii* ; Figure 1A, no. 1) or Pacific slopes (*hoffmanni-hicksii* ; Figure 1A, no. 2), we set a line along the continental divide following some of the major highland peaks along the Costa Rica–Panama mountain ranges (Rincon de la Vieja, Poas, Chirripo, Baru), throughout the middle of Panama (Santiago, La Yeguada, Gamboa, El Llano, Higueronal, Meteti, Yaviza) to the Panamanian border with Colombia. Then, we assigned samples collected from nearby locations to populations, and extrapolated the average coordinates per population to the closest point on the line, using the "dist2line" function from the GEOSPHERE R package (Hijmans et al., 2017). We estimated geographic distances between each inferred population along this line, starting at Rincon de la Vieja volcano (km 0) using the "trackDistance" function from the R package TRIP (Sumner et al., 2009). This approach allowed us to convert the irregular topography of this region into a single linear axis against which both contact zones can be mapped (Figure S1).

For the phenotypic traits, we carried out cline analyses using only traits that differed between subspecies. First, we performed cline analyses on the male plumage traits that differ by changing from black to white between subspecies (i.e., *corvina-hicksii* : throat, belly, and rump, *hoffmanni-hicksii* : throat). For the

morphometric traits, we ran a single analysis of variance (ANOVA) per trait and per contact zone, but included sex as a covariable, and tested for its interaction with the subspecies factor. We performed the ANOVA between the most-distant locations (localities Arenal [ARE] and Sarapiqui [SAR] for *S. c. corvina* ; Garabito [GAR] and Quepos [QUE] for *S. c. hoffmanni* ; and Canazas [CAN] and Darien [DAR] for *S. c. hicksii* ; Table S1). Because we found no significant effect of sex for any of the morphometric traits, we estimated clines for the morphological traits including both males and females. For the phenotypic traits that differed between the most-distant populations, we converted each phenotypic trait into scores that ranged from 0 to 1. Scores values were calculated by applying the equation $(X_{\ i} - X_{\ min}) \ / \ (X_{\ max} - X_{\ min})$, in which$X_{\ i}$ corresponds to each trait's value per individual and $X_{\ min}$ and$X_{\ max}$ correspond to the minimum and maximum trait values in each cline comparison, respectively.

For the genetic clines, we modeled 15 possible clines on the average hybrid indices by fitting three different settings for scaling at the ends of the clines (fixed, free, and no-scaling), and five possible fits of the tails of the clines (none, right, left, both, and mirroring), in all possible combinations (per Derryberry et al., 2014). We replicated each model three times with different random seeds, ran three chains per model fit to assess convergence, and selected for the best model based on the Akaike Information Criterion (AIC). For the phenotypic clines, morphometric traits and brightness per body patch, we tested ten different models by applying five different fits to the cline's tails (none, right, left, both, and mirroring) and two settings for scaling (fixed and free), by fixing the scaling to the maximum and minimum scores. Likewise, for the hybrid index, we ran three chains and three replicates per model, and selected the best model based on the lowest AIC. We compared coincidence and concordance in clines (i.e., center and width) between different traits and hybrid index using the 95% confidence intervals (CI) for each cline parameter.

Testing possible demographic scenarios

We used model-based demographic inference to evaluate support for alternative scenarios describing the evolutionary dynamics of contact zones between subspecies. We built demographic models using the program MOMI2 (Kamm et al., 2019), which uses the joint Site Frequency Spectra (SFS) to evaluate different models that consider population size, divergence time, and gene flow for each clade. We ran the demographic inferences based on a mutation rate of 2.5 x 10^{-9}sites per generation (Nadachowska-Brzyska et al., 2015) and a generation time of 2.2 yr, which is a common generation time for Neotropical songbirds (Luzuriaga-Aveiga et al., 2021). We executed our models in two steps (as in Corbett et al., 2020). First, we built a model that estimated effective population size for the three lineages, based on the topology and time of divergence of the mitochondrial (ND2) phylogenetic reconstruction (Ocampo et al., 2022a), in which the two pied subspecies (i.e., *S. c. hicksii, S. c. hoffmanni*) are sister taxa to the black *S. c. corvina* (Figure 2A). Second, we built demographic models that added bidirectional bouts of gene flow and estimated the strength of the bouts.

Demographic models were built to test for two specific questions: 1) Is the apparent absence of *corvina-hoffmanni* hybrids at their contact zone consistent with a scenario of no contemporary gene flow between these subspecies? We tested whether gene flow (bouts of 10-25% of the population) occurred between *S. c. corvina* and *S. c. hoffmanni* 1000 years ago. 2) Did *S. c. corvina* diverge in isolation until recent secondary contact with *S. c. hicksii* or have these subspecies been in constant contact? We tested for the likelihood of constant gene flow by modeling temporal pulses of 5-10% of the population, occurring at 200, 400, and 600 Kyr ago to distinguish between a scenario of historical isolation or constant gene flow. Given that we found evidence for later-generation hybrids at both*corvina-hicksii* and *hicksii-hoffmanni* contact zones (see Results), we modeled bidirectional bouts of 10-25% of the population 1000 years ago and kept these gene flow events constant across all our models. We tested our two hypotheses in all possible combinations, for a total of four demographic models (Figure 2B-E), estimating the strength of the gene flow pulses. All models were optimized using the "L-BFGS-B" algorithm and a maximum of 100 iterations. With both steps, we carried out 100 runs per model, and selected the most likely set of parameters of each model based on log-likelihood values. We then compared between different demographic models using the AIC to select the best model. We did not fit more complex models (e.g., changes in effective population size or more complex scenarios of gene flow), as these simple models allowed us to test our two main questions and avoided overparameterization

(Bocalini et al., 2021).

RESULTS

Population structure within *Sporophila corvina*

We used three complementary methods to estimate patterns of genetic differentiation and population structure. The Estimated Effective Migration Surfaces (EEMS) approach found that the continental divide between Caribbean and Pacific slopes forms the most important barrier where gene flow is 100 times lower than the average despite relatively short geographic distance (Fig. 1B). This continental divide extends from the Central Valley of Costa Rica along the Talamanca mountain range throughout western Panama, reaching the Canal area in central Panama (map areas colored dark orange). Conversely, individuals on either side of both slopes showed high genetic similarity, likely due to strong connectivity. Our results were consistent among deme densities (200, 300, and 400 demes), and between runs (Figure S2).

Further, we found that *S. corvina* individuals from Costa Rica and Panama were best grouped into three genetic clusters (Figure 1C), as this clustering presented the lowest cross validation error (population assignments for $K = 2$–5 are available in Figure S3). The three genetic groups that we identified were largely consistent with the known distributions of the three subspecies present in the region (Figures 1A,C and S4). However, we also identified a large proportion of individuals with mixed ancestries, suggesting pervasive gene flow between subspecies. From the 255 individuals included, 107 were identified as pure parental subspecies (53 *corvina* , 37*hoffmanni* , and 17 *hicksii*) and 148 showed some evidence of admixture (79 *corvina-hicksii* , 16 *corvina-hoffmanni* , 46*hoffmanni-hicksii,* and 7 three-way hybrids; Figure 1C). We found relatively low genetic differentiation between subspecies pairs; the highest differentiation occurred between black and pied subspecies (*corvina vs hicksii* : $F_{ST} = 0.074$,*corvina vs hoffmanni* : $F_{ST} = 0.066$), while the two pied subspecies showed the lowest genetic differentiation (*hoffmanni vs hicksii* : $F_{ST} = 0.039$). Finally, the PCA analysis identified roughly three genetic clusters; the first axis of variation explained 4.44% of the genetic variation and primarily separated *corvina* individuals from the two pied subspecies and the second PC axis explained 2.10% of the genetic variation, and divided *hicksii* from the other two subspecies (Figures 3 and S5).

Characterization of hybrids and contact zones

Based on our test using only informative markers, we found that individuals of hybrid ancestry had generally low levels of heterozygosity across the three contact zones (Figure 4). Moreover, we also found a continuous distribution of hybrid indices between the two parental subspecies in the *corvina-hicksii* and*hoffmanni-hicksii* contact zones (Figure 4A,B). Interestingly, we found virtually no *corvina-hoffmanni* hybrids (Figure 4C), in this contact zone; all individuals with mixed ancestry can be confidently assigned to one of the parental subspecies based on the genetic makeup, contrary to the other two contact zones.

We fitted geographic clines along both contact zones in which subspecies hybridize, we fitted clines for the hybrid index, plumage traits, and significantly different morphometric traits. First, we identified morphometric differences between distant populations, testing for the effect of subspecies and sex, and found *corvina* and*hicksii* differed in beak size, wing length, and tail length, while *hoffmanni* and *hicksii* differed in beak size, tail length, and tarsus length (Table 1). For color patches, we focused on those patches that differed between subspecies. In both contact zones, the best fit model for the hybrid index had a fixed scaling from 0 to 1 and no tails fitted (Table 2). Plumage brightness of the different patches modeled showed a clinal distribution in which the brightness of the rump and belly in males for the *corvina-hicksii* contact zone, and the brightness of the throat for the *hoffmanni-hicksii*contact zone had cline centers that were coincident with their respective hybrid index clines. However, the center of the throat's brightness for the *corvina-hicksii* contact zone is displaced ˜60 km to the East, with no overlap of the 95% CI (Figure 5A,C; Table 2). Plumage brightness and genetic clines differ in cline widths, with plumage clines showing narrower widths than the hybrid index clines (Figure 5A,C; Table 2).

When the most distant populations differed in morphometric traits, we found a general pattern in which

hicksii is smaller than the other two subspecies, showing on average smaller beaks and shorter tails than *corvina* and *hoffmanni* , and shorter wings than*corvina* . However, *hoffmanni* had, on average, shorter tarsi than *hicksii* (Table 1). Morphometric traits along the*corvina-hicksii* contact zones (tail length and beak size) were not coincident with respect to the hybrid index, with tail length and beak size having displaced centers (Figure 5B; Table 2). Along the*hoffmanni-hicksii* contact zone, cline centers of beak size and tail length are coincident with the correspondent hybrid index based on the overlapping of the 95% CI (Figure 5D; Table 2). However, we found important intra-population variation, with none of the morphometric traits exhibiting a clear clinal distribution when modeling average trait values per population and, instead, showed a more linear, smoother transition from one parental population to the other, which result in low confidence of cline parameters (Figure 5B,D; Table 2). For example, the CI of the cline width of the tarsus length between*hoffmanni-hicksii* and wing length between *corvina-hicksii*were estimated to be between ˜20 km to 1200 km wide (not graphed, Table 2).

Demographic inference

We compared four different models to infer the evolutionary history of this group of *S. corvina* subspecies in Costa Rica and Panama. We found one model was preferred over the other three (Table 3). The best model included the default migration bouts between *S. c. hoffmanni* and *S. c. hicksii,* and between *S. c. corvina*and *S. c. hicksii* only (Model B; Figure 2). This model suggests a scenario of rapid divergence followed by isolation of the black and pied lineages until recent gene flow facilitated by secondary contact and is consistent with reduced hybridization between *S. c. corvina* and*S. c. hoffmanni* . Genomic homogenization due to gene flow in addition to rapid divergence inhibits accurate reconstruction of the phylogenetic relationship between subspecies, estimates of times of divergence, and rates of gene flow.

DISCUSSION

We compiled a dataset of 14,839 genome-wide SNPs from 255 individuals from Costa Rica and Panama to test whether phenotypic traits covary with population structure and hybridization patterns between *S. corvina* subspecies that differ in plumage coloration across three independent contact zones (Figure 1A). We found three genetic clusters of moderate genetic differentiation, consistent with subspecies distributions (Figures 1C and S4), that hybridized extensively across two of the three contact zones (Figures 3 and 4). Hybridization occurs despite their differences in plumage coloration, suggesting a limited role of plumage divergence in reproductive isolation. Across these two contact zones, subspecies differ in the plumage brightness of a few male color patches. For these color patches, we found coincident centers with the respective hybrid index/genetic clines but a sharper transition (i.e., narrower clines), suggesting limited introgression of these plumage traits (Figure 5; Table 2). We found no other clear morphometric variation among subspecies. Finally, our demographic inference supports our observations of no hybridization between *S. c. corvina* and*S. c. hoffmanni,* which recently established secondary contact in the Central Valley of Costa Rica (Table 3).

Genetic structure among subspecies

In a previous study, we found evidence for reduced gene flow near the contact zone between the all-black *S. c. corvina* and the pied*S. c. hicksii* and *S. c. hoffmanni* subspecies (Ocampo et al., 2022a). Here, we have extended the genomic aspect of our previous study by increasing sample size and spatial resolution of this region and adding phenotypic data. We found an important barrier to gene flow that is partially concordant with the Talamanca mountain range and with the range limits between the black and pied subspecies (Figure 1). Consistent with the Talamancas acting as a geographic barrier, high genetic divergence between Caribbean and southern Pacific populations have been found in other lowland taxa in this region (e.g., in frogs [Crawford et al., 2003, Robertson & Zamudio, 2009], snakes [Zamudio & Greene, 1997], caimans [Venegas-Anaya et al., 2008], beetles [Kohlmann & Wilkinson, 2007], and birds [Hackett, 1996; Marks et al., 2002]). Moreover, the South Pacific region of Costa Rica is considered an important climate refuge (Haffer, 1974), harboring high biological richness and endemism in plants and animals (Crain & Fernandez, 2020; Kohlmann et al., 2010; Pareira & Barrantes, 2009).*Sporophila corvina* subspecies established secondary

contact at both ends of the Talamanca mountain range (Figure 1A, no. 1 & 3), and we found significant gene flow between the Pacific and Caribbean populations at the Canal in central Panama (Figure 1B, blue shades). In contrast, there is reduced gene flow between *S. c. corvina* and*S. c. hoffmanni* in the Central Valley of Costa Rica. These observations suggest that levels of gene flow differed between the two contact zones despite similar degrees of phenotypic plumage divergence (black vs. pied plumage). Importantly, from the six individuals collected in Central Valley contact zone (San Ramon [SRA]; Table S1), two individuals showed the genetic makeup of the Pacific subspecies, while four individuals showed the genetic makeup of the Caribbean subspecies, which confirms that the two subspecies are in contact but are likely not hybridizing or doing so at levels we cannot detect in this study.

When determining genetic structure among individuals, our samples were best clustered into three genetic groups. Clusters are consistent, in general, with the subspecies assignments, suggesting that described subspecies correspond to recognizable lineages. However, we found evidence of admixture between clusters (Figure 1C), between pairs of clusters, and even a few individuals with signatures of ancestry from the three subspecies (Figure 3). We found that *corvina* is the most differentiated subspecies, with paired F_{ST}values that are twice the value between *hoffmanni* and*hicksii.* This pattern is consistent with our expectations of stronger differentiation between phenotypically differentiated subspecies and with the hierarchical population structure identified previously (Ocampo et al., 2022a).

Hybridization among *S. corvina* lineages

Two out of the three contact zones showed extensive hybridization. However, contact zones are formed by later-generation hybrids and backcrossed individuals, with no F1 hybrids, and the contact zones are wide suggesting that pure parental subspecies are not currently directly interbreeding. Classic tension zones are usually narrow and are maintained by low hybrid fitness and dispersion from parental populations (Barton & Hewitt, 1985). Our results suggest that these contact zones are hybrid zones (*sensu stricto* Barton & Hewitt, 1985), a "fairly continuous transitions between distinct forms", in which hybrids between *S. corvina* subspecies likely present relative fitness comparable to the parental subspecies but seem to be restricted to the contact zone perhaps due to environmental constraints and relatively low dispersal. For the third contact zone, which occurs in the Central Valley of Costa Rica where *S. c. corvina* and*S. c. hoffmanni* come into contact, we found no hybrids. The bimodal distribution of hybrid indices suggests low ancient hybridization with extensive backcrosses, where the introgressed genomic variation is highly diluted into the genetic pool of the local population. Given the extensive hybridization at the other two contact zones described above, it is very unlikely that postzygotic genetic incompatibilities (e.g., hybrid inviability), and low hybrid fitness (e.g., hybrid disadvantage) play an important role in reducing hybridization in this system. Instead, we hypothesize that premating reproductive isolation is the main driver maintaining isolation between*S. c. corvina* and *S. c. hoffmanni* subspecies in Costa Rica.

Reproductive isolation in secondary contact can be attained by conspecific recognition resulting in assortative mating. Given that we found the two genetically distinct populations (*S. c. corvina* and*S. c. hoffmanni*) within the same locality, we predict that assortative mating due to behavioral isolation will likely be found to play a major role in reproductive isolation. However, contrary to our expectations, we found two different outcomes in two contact zones with similar patterns of plumage divergence (i.e., black vs. pied plumage), suggesting that factors other than just plumage color may also shape the different patterns of hybridization. For instance, different patterns of local adaptation may result in asynchronous breeding phenology (Hau et al., 2008). For example, *S. c. corvina* at the Caribbean side of Costa Rica apparently have longer breeding seasons (almost year-round; pers. obs.), related with the lack of marked dry and rainy seasons, but*S. c. hoffmanni* from the Pacific side of Costa Rica appear to breed only two or three months after the beginning of the marked rainy season (Skutch, 1954). Additionally, divergence in male song characteristics is an important source of isolation in tropical birds (Freeman & Montgomery, 2007), and in this case both subspecies (*S. c. corvina* and *S. c. hoffmanni*) have different male songs (unpub. data), which may favor assortative mating.

Interestingly, where subspecies hybridize, plumage traits showed a sharper transition and reduced cline width

compared to the hybrid index clines (Figure 5; Table 2). The more abrupt change in phenotype than in hybrid index at the contact zones suggests these plumage traits are under divergent selection, causing limited introgression. In general, our results suggest that distinct plumage traits are under selection (likely as signals for conspecific mate recognition), but that they do not act as a strong barrier to gene flow by themselves. On the other hand, the striking intrapopulation variation in morphometric traits conceals a clear sigmoidal transition among contact zones, confounding the interpretation of the cline's centers and widths (Figure 5). However, the displaced clines of the beak size and tail length across the *corvina-hicksii* contact zone (Figure 5B) could result from directional selection favoring an asymmetrical introgression of some loci associated with selected phenotypes (Lipshutz et al., 2019; Stein & Uy, 2006; While et al., 2015).

Our models for demographic inference suggested rapid divergence paired with recent gene flow between lineages, but not contemporary gene flow between *S. c. corvina* and *S. c. hoffmanni.* This result suggests that the black *S. c. corvina* may have evolved in isolation from the pied subspecies and they subsequently established secondary contact and gene flow with *S. c. hicksii.* This is consistent with the hypothesis of the range of *S. c. corvina*expanding into the distribution of *S. c. hicksii,* mediated by periods of population expansions and contractions due to climatic fluctuations (Stiles, 1996). If the contact is not linked to an environmental gradient or ecotone, differential introgression may result in movement of the contact zone (Buggs, 2007). However, the contact zone that occurs between *S. c. corvina* and *S. c. hicksii* in Central Panama has been stable, at least for more than 100 years (Olson, 1981).

Biogeographic history of *S. corvina*

Based on our results and previous work on the genus *Sporophila* , we suggested a possible biogeographic scenario that explains the distribution, divergence, and patterns of gene flow between subspecies of *S. corvina* . The radiation of the genus of *Sporophila*seedeaters was initiated around the late Miocene to early Pliocene in South America, followed by multiple independent bouts of colonization of Middle America (Mason & Burns, 2013; Mason et al., 2018; Ocampo et al., 2022a). The ancestral population of the *S. corvina* likely diverged from the *S. intermedia* ancestor, in the late Pliocene, after a trans-Andes dispersion event (Ocampo et al., 2022a; Stiles, 1996), and dispersed northwards through the Panamanian isthmus (O'Dea et al., 2016). At this time, the Talamanca mountain range was already formed to its current elevation (Coates & Obando, 1996), thus imposing a barrier to lowland bird species. Therefore, the ancestral *S. corvina* population likely dispersed through the Pacific and Caribbean slopes, around the Talamanca mountain range. The population moving north of Talamanca likely passed through the wetter Caribbean slope reaching higher latitudes. This all-black lineage may have been isolated (Figure 2B) due to habitat fragmentation associated with forest expansion and contraction events, as well as wet and arid environmental conditions associated with climate oscillations during the Pleistocene (e.g., Garzon-Orduna et al., 2014). On the other hand, the population south of the Talamanca mountain range reached a boundary that prevented it from moving farther north – an ecological and geological boundary known as the "Tarcoles Line" (Kohlmann & Wilkinson, 2007). This ecotone separates the tropical wet forest of the Costa Rican South Pacific and Middle American dry forest, and constrains the distribution of many species (Kohlmann & Wilkinson, 2007), and is likewise largely consistent with the current limit of the subspecies range of *S. c. hoffmanni* .

Based on this biogeographic scenario, gene flow during secondary contact between clades, associated with most recent periods of population expansion, resulted in the currently continuous distribution of *S. c. hoffmanni* and *S. c. hicksii* and the introgression from*S. c. corvina* into *S. c. hicksii* at the Canal in central Panama. More recently, secondary contact was established in the Central Valley of Costa Rica, likely favored by deforestation due to human urban expansion (Biamonte et al., 2011; Joyce, 2006). We found no evidence of current hybridization between these two subspecies in the Central Valley region (Figures 3 and 4; Table 3). However, the strength of reproductive isolation is dynamic, and premating reproductive barriers vary with time since contact. Reproductive barriers can increase after secondary contact due to sexual characters displacement (e.g., Jaya et al., 2022), or the initial reduced gene flow after secondary contact can increase with time since contact, favoring hybridization and resulting in the merger of distinct populations (Bettles et al., 2005). Further behavioral studies in the Central Valley region could provide insights on the factors

maintaining reproductive isolation between subspecies at the early stages of secondary contact.

CONCLUSIONS

Sporophila corvina from Costa Rica and Panama form three genetically differentiated groups that are largely consistent with their current subspecies classifications and geographic distributions. The three subspecies have established three different contact zones and hybridize extensively across two of them, regardless of the differences in plumage patterns of the populations that came into contact (pied vs. pied or black vs. pied). However, even though plumage divergence does not act as a strong barrier to gene flow, we found that plumage traits are divergent and presumably under selection at the contact zones. Finally, model-based demographic inference suggests that the black subspecies *S. c. corvina* diverged in isolation until a recent secondary contact with *S. c. hicksii,* resulting in the hybridization pattern that we see today at one of these contact zones. Overall, our results suggest that divergence in plumage color is important in reducing gene flow between these populations, but not sufficient to stablish complete reproductive isolation. Other factors, such as reproductive timing or divergence in song, might explain why hybridization is reduced in one of the contact zones but not in the others, a pattern of isolation that is likely to change with time.

ACKNOWLEDGMENTS

We thank The University of Alaska Museum, Louisiana State University, Universidad de Costa Rica, and Smithsonian Tropical Research Institute collections and their personnel for providing tissue samples used in this study; SINAC in Costa Rica, and MIAMBIENTE in Panama for granting research permits; McMillan O., Amador S., Lopez O., STRI's personnel, Arce A., Biamonte E., Morrison O., Sanchez C., and Barrantes G. for assistance and logistical support in Costa Rica and Panama; CIRC (UR) for access to computing facilities; Searcy W., Campagna, L., the Mason lab (LSU), and the Uy lab (UR) for valuable comments that improved early versions of the manuscript; Lastly, we thank our funding sources, the University of Rochester's Global Visitor Program (College of Arts and Sciences), the Hesse student award from the American Ornithological Society, the Short-term fellowship from the Smithsonian Tropical Research Institute, and the Kushlan and Savage funds from the University of Miami (to D. Ocampo), and the Aresty Chair in Tropical Ecology from the University of Miami (to J.A.C. Uy).

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DATA AVAILABILITY STATEMENT

SNPs and phenotypic data are available on Dryad data repository (Ocampo et al., 2022b). "Scorvina_-SNPs_1.vcf" and "Scorvina_SNPs_2.vcf" contain the SNPs data sets according to our two filtering criteria. "Scorvina_B2.txt" including plumage brightness per patch per individual. "Scorvina_morph.txt" including morphometric data. Related metadata can be found in Table S1 (including georeferences in decimal degrees and date/month/year of sampling event).

BENEFIT-SHARING STATEMENT

Benefits Generated: A collaborative research was developed with scientists and institutions providing genetic samples, all collaborators are included as co-authors. Other contributors to the research are included in the ACKNOWLEDGEMENTS section. The results of the research have been shared with the scientific community.

AUTHOR CONTRIBUTIONS

D. Ocampo and A. Uy conceptualized the study; D. Ocampo, K. Winker, M. Miller, and L. Sandoval collected the samples; D. Ocampo collected phenotypic data and performed data analyses. D. Ocampo and A. Uy drafted the manuscript with significant input of all authors. All authors have read and approved the final manuscript.

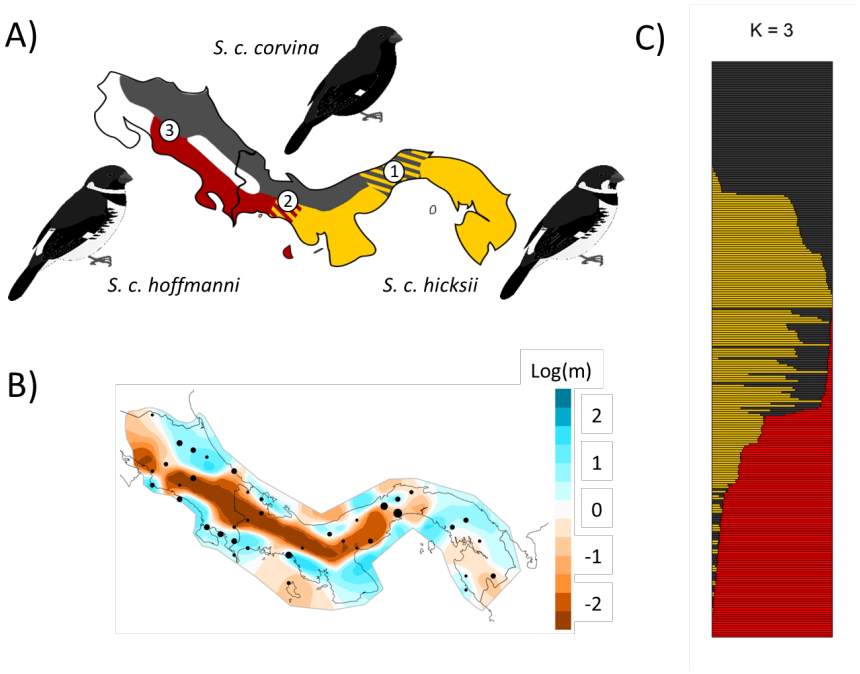

Figure 1. Distribution and genetic structure of the *Sporophila corvina*subspecies in Costa Rica and Panama. A) Typical male plumage per subspecies, subspecies distributions highlighting contact zones between subspecies (numbered), and known regions with intermediate individuals (striped pattern). Map based on the distributions from Olson (1981) and Stiles (1996). B) Estimated effective migration surface, dark orange region along the continental divide represents a barrier of 100 times reduced gene flow. C) Admixture proportion per individual, based on the best result from ADMIXURE ($K = 3$).

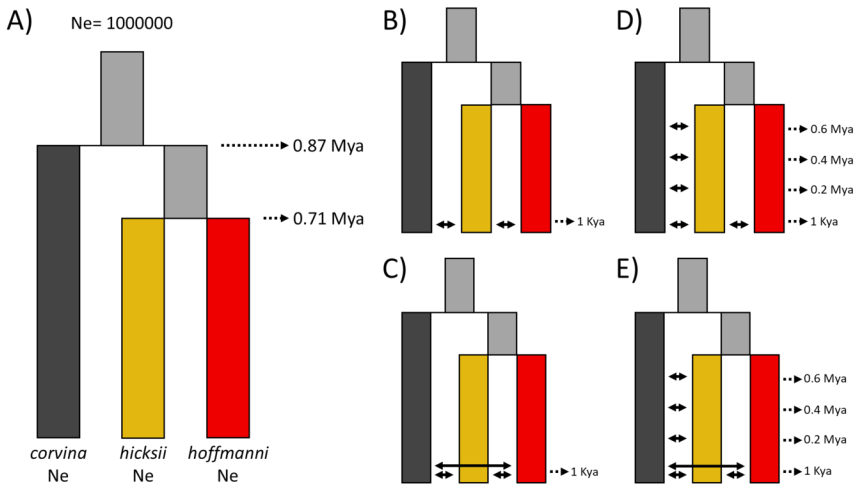

Figure 2. Demographic models tested in MOMI2; bold arrows represent gene flow bouts estimated for each model. Dark grey bar is *S. c. corvina,* yellow bar is *S. c. hicksii,* red bar is *S. c. hoffmanni,* and light grey bar is the ancestor. A) Model using the tree topology and time of divergence obtained from the mitochondrial marker ND2 (Ocampo et al., 2022a) to estimate effective population sizes. B-E) Models including different

bouts and pulses of gene flow to test for contemporary gene flow between *S. c. corvina* and *S. c. hoffmanni* , and to test for divergence in isolation of *S. c. corvina* .

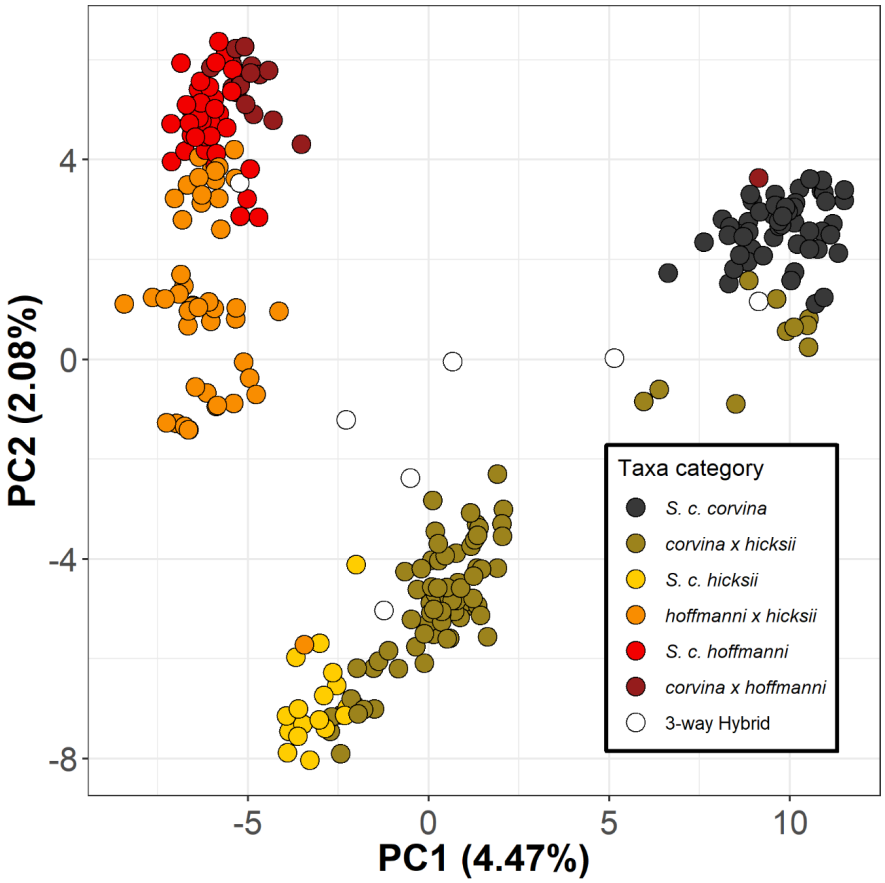

Figure 3. Principal Component Analysis on the genomic SNPs, colors represent subspecies and hybrid categories inferred from ADMIXTURE (K =3). Individuals with q values > 0.95 are considered genotypically pure individuals for each subspecies (i.e.,*S. c. corvina* , *S. c. hoffmanni* , and *S. c. hicksii*). Hybrids that have q values from the third genetic group < 0.02 and the q from the second genetic group was at least 2x the value from the third genetic group are labeled by their mixed ancestry (e.g., *corvina* x *hicksii*). Admixed individuals with $q > 0.1$ from the third population or if ancestries from the second and third genetic cluster where similar were considered three-way hybrids

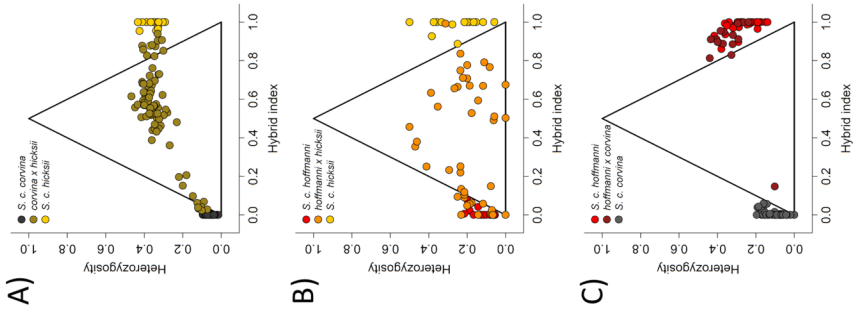

Figure 4. Pairwise characterization of the hybrid index and interclass heterozygosity among different parental subspecies of *Sporophila corvina* . A) between *S. c. corvina* and *S. c. hicksii* . B) between *S. c. hoffmanni* and *S. c. hicksii.* C) between*S. c. corvina* and *S. c. hoffmanni* .

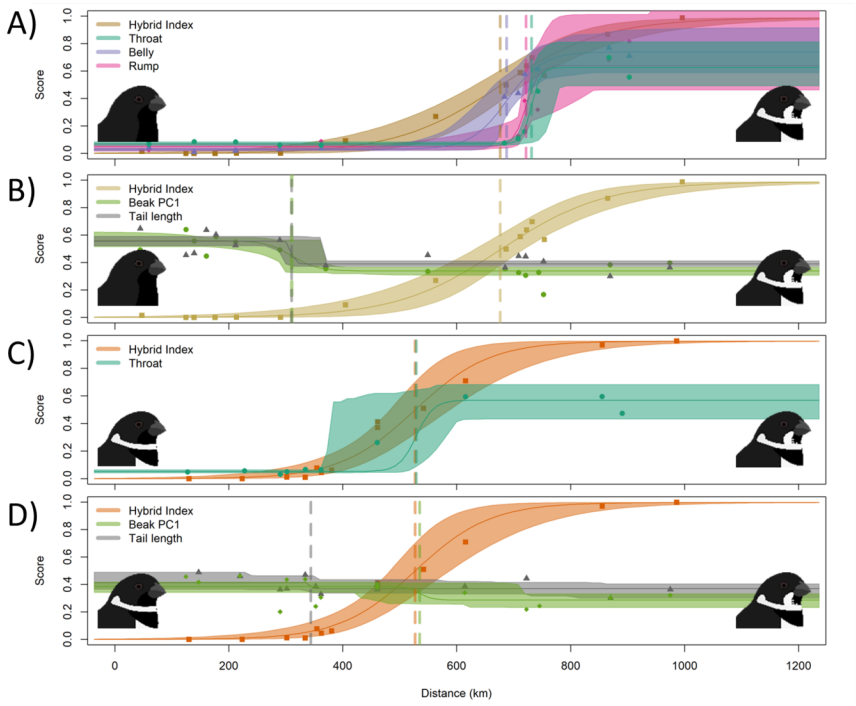

Figure 5. Geographic clines fitted for the hybrid index and divergent phenotypic traits. Dashed-vertical lines showing the cline centers, while the shaded area are the 95% confidence intervals for each cline. A) Hybrid index and plumage brightness clines fitted for the *S. c. corvina* and *S. c. hicksii* contact zone. B) Hybrid index and morphometric traits clines fitted for the *S. c. corvina* and*S. c. hicksii* contact zone. C) Hybrid index and plumage brightness clines fitted for the *S. c. hoffmanni* and *S. c. hicksii* contact zone. D) Hybrid index and morphometric traits clines fitted for the *S. c. hoffmanni* and *S. c. hicksii* contact zone.

Table 1. Analyses of variance among subspecies for each of the morphometric traits (Body mass, Beak size, Tarsus length, Wing length, Tail length).

Trait	**Effect**	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
corvina **vs** ***hicksii***	*corvina* **vs** ***hicksii***						
Mass	Subspecies	1	0.38	0.38	0.29	0.59	
	Sex	1	4.40	4.40	3.44	0.07	
	Subspecies:Sex	1	3.42	3.42	2.67	0.11	
	Residuals	33	42.18	1.28			
Beak size	Subspecies	1	16.09	16.09	13.38	<0.001	***
	Sex	1	1.29	1.29	1.07	0.31	
	Subspecies:Sex	1	3.47	3.47	2.88	0.10	
	Residuals	35	42.09	1.20			
Tarsus	Subspecies	1	0.12	0.12	0.43	0.51	
	Sex	1	0.09	0.09	0.30	0.58	
	Subspecies:Sex	1	0.57	0.57	1.93	0.17	
	Residuals	34	9.95	0.29			

Wing	Subspecies	1	8.30	8.30	5.12	0.03	*
	Sex	1	1.51	1.51	0.93	0.34	
	Subspecies:Sex	1	6.41	6.41	3.95	0.05	
	Residuals	35	56.70	1.62			
Tail	Subspecies	1	49.65	49.65	16.69	<0.001	***
	Sex	1	0.44	0.44	0.15	0.70	
	Subspecies:Sex	1	0.19	0.19	0.06	0.80	
	Residuals	35	104.08	2.97			
hoffmanni **vs** ***hicksii***	*hoffmanni* **vs** ***hicksii***						
Mass	Subspecies	1	0.46	0.46	0.74	0.40	
	Sex	1	0.15	0.15	0.24	0.62	
	Subspecies:Sex	1	0.33	0.33	0.53	0.47	
	Residuals	36	22.66	0.63			
Beak size	Subspecies	1	10.72	10.72	9.24	0.004	**
	Sex	1	0.05	0.05	0.04	0.83	
	Subspecies:Sex	1	0.72	0.72	0.62	0.44	
	Residuals	36	41.76	1.16			
Tarsus	Subspecies	1	1.45	1.45	5.54	0.02	*
	Sex	1	0.09	0.09	0.34	0.56	
	Subspecies:Sex	1	0.38	0.38	1.45	0.2	
	Residuals	36	9.44	0.26			
Wing	Subspecies	1	2.77	2.77	1.72	0.20	
	Sex	1	2.94	2.94	1.83	0.18	
	Subspecies:Sex	1	4.12	4.12	2.56	0.12	
	Residuals	36	57.77	1.60			
Tail	Subspecies	1	24.58	24.58	9.82	0.003	**
	Sex	1	1.36	1.36	0.54	0.46	
	Subspecies:Sex	1	0.00	0.00	0.00	0.98	
	Residuals	36	90.06	2.50			

Table 2. Best models for cline analyses of divergent phenotypic traits. The parameters included per cline are scaling and fitting of the tails and centers and width of the clines with their respective 95% confidence intervals.

Trait	**Scaling**	**Tails fitted**	**Center (95% CI)**	**Width (95% CI)**
corvina-hicksii				
Hybrid index	Fixed	None	676.3 (624.2-721.2)	366.5 (242.1-545.9)
Throat	Free	Right	734.8 (725.2-748.7)	28.3 (17.2-52.0)
Belly	Free	None	687.9 (655.9-701.1)	125.2 (55.3-196.3)
Rump	Free	None	722.0 (715.3-736.5)	43.4 (13.5-66.3)
Beak size (PC1)	Free	None	310.5 (278.3- 368.8)	90.6 (0.0- 187.0)
Wing	Free	None	605.0 (365.3-1175.2)	1150.1 (19.8-1200.0)
Tail	Free	None	310.5 (289.8-368.9)	8.8 (0.0-97.8)
hoffmanni-hicksii				
Hybrid index	Fixed	None	526.9 (478.2-596.0)	263.3 (165.6-431.5)
Throat	Free	None	529.3 (514.0-564.9)	59.4 (24.4-97.6)
Beak size (PC1)	Free	Left	535.2 (465.7-717.7)	42.6 (0.0-59.3)
Tail	Free	None	344.13 (337.6-609.2)	5.1 (0.1-36.5)
Tarsus	Free	None	620.4 (362.6-1174.6)	515.1 (19.8-1200)

Table 3. Results of the likelihood and fit comparisons for the four models tested using MOMI2. Models are described in the text and depicted in Figure 2.

Model	**Max ln (likelihood)**	**N parameters**	**AIC**	**Δ AIc**	**ωi**
B	-39869.48	4	79746.96	0	0
C	-39993.11	6	79998.22	251.26	0.22
D	-40011.21	6	80034.42	287.46	0.25
E	-40161.59	8	80339.18	592.22	0.52