Origin and cross-century dynamics of an avian hybrid zone

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Abstract

Background: Characterizations of the dynamics of hybrid zones in space and time can give insights about traits and processes important in population divergence and speciation. We characterized a hybrid zone between tanagers in the genus *Ramphocelus* (Aves, Thraupidae) located in southwestern Colombia. We tested whether this hybrid zone originated as a result of secondary contact or of primary differentiation, and described its dynamics across time using spatial analyses of molecular, morphological, and coloration data in combination with paleodistribution modeling.
Results: Models of potential historical distributions based on climatic data and genetic signatures of demographic expansion suggested that the hybrid zone originated following secondary contact between populations that expanded their ranges out of isolated areas in the Quaternary. Concordant patterns of variation in phenotypic characters across the hybrid zone and its narrow extent are suggestive of a tension zone, maintained by a balance between dispersal and selection against hybrids. Estimates of phenotypic cline parameters obtained using specimens collected over nearly a century revealed that, in recent decades, the zone has moved to the east and to higher elevations, and has become narrower. Genetic variation was not clearly structured along the hybrid zone, but comparisons between historical and contemporary specimens suggested that temporal changes in its genetic makeup may also have occurred.
Conclusions: Our data suggest that the hybrid zone resulted from secondary contact between populations. The observed changes in the hybrid zone may be a result of sexual selection, asymmetric gene flow, or environmental change.

Keywords: Andes, clines, Colombia, paleodistribution modeling, hybridization, moving hybrid zone, *Ramphocelus,* Thraupidae

Background

Characterizations of hybrid zones allow one to make inferences about traits and processes relevant to understanding the origin and maintenance of differences between populations and species [1, 2]. A classic question about hybrid zones is how are they formed, with previous studies proposing two main hypotheses (reviewed by [3]). The hypothesis of secondary contact posits that hybrid zones result from expansion of populations that were previously isolated geographically and which interbreed in contact zones because complete reproductive isolation between them was not reached during the allopatric phase [4]. An alternative hypothesis postulates that hybrid zones form in parapatry, by primary differentiation across ecological gradients [5]. Secondary contact is likely if environments that presently allow the distributions of hybridizing populations to overlap were disjunct in the past, a scenario that predicts one should observe genetic signatures of population expansions. Alternatively, primary differentiation along a gradient would occur if the extent of suitable environments for the hybridizing populations has been stable over time; this predicts that populations have not expanded their ranges historically, and that the position of the hybrid zone (as indicated by the position of clines in molecular and morphological traits) is coincident with an environmental transition [1, 6].

Inferring hybrid-zone origins from current patterns of variation is challenging because, with time, genetic signatures of secondary contact or primary intergradation tend to erode [3, 7]. Alternatively, then, tests of hypotheses posed to account for the origin of hybrid zones may be conducted by examining the historical distribution of hybridizing taxa using paleodistributional modeling, i.e. projecting ecological niche models, which characterize the current distribution of species in climatic space, onto models of historical climatic conditions to infer potential distributions in the past [8, 9]. Such models of historical distributions represent hypotheses one can further test using molecular data to evaluate their population-genetic predictions, such as signatures of population growth for presumably expanding populations and of constant population size and isolation by distance in populations occurring within climatically stable areas [10, 11]. This approach has revealed that several hybrid zones likely originated following range expansions leading to secondary contact [12-15].

Another focus of studies on hybrid zones is the analysis of their temporal dynamics, which can allow for a better understanding of the role played by different evolutionary forces in such scenarios. When hybrid genotypes are less fit than parental genotypes, ‘tension zones’ are formed, which are maintained by a balance between the homogenizing effect of dispersal into the hybrid zone and the diversifying effect of selection against hybrids [1]. If there is endogenous selection against hybrids, then there should be coincidence in location and concordance in width of clines describing the variation in different traits and loci across a hybrid zone, and such clines should remain stable over time [16, 17]. However, hybrid zones are often temporally dynamic (i.e. they may shift in location or

change in width) and because clines for different traits may change in different ways, one can make inferences about the action of particular processes (e.g., natural selection, sexual selection, competition, asymmetric hybridization, dominance drive) based on dynamics observed for different characters [18]. For example, discordant patterns of plumage and mitochondrial DNA variation across a hybrid zone between *Setophaga* warblers, coupled with behavioral experiments showing aggressive superiority of males of one species over the other, indicate that movement of this zone has likely been driven by competition-mediated asymmetric hybridization [19-21].

There is ample evidence of hybridization between members of the tanager genus *Ramphocelus* (Aves, Thraupidae; [22-25]), but detailed studies on hybrid zones involving species in this group are scant. Here, we characterize a hybrid zone between members of this genus located in western Colombia that has received little study although its existence was noted nearly a century ago [26] and was described in some detail more than five decades ago [23]. In the Cauca River Valley above c. 900 m elevation, one finds the larger form *flammigerus* (males are black with a scarlet rump), whereas the smaller and yellow-rumped form *icteronotus* occurs along the costal plains west of the Andes extending north into Costa Rica and south into northern Peru. Females and immatures are similar to their respective males, but are less strongly colored. Along a c. 140 km transect running approximately northwest from the city of Cali downslope along the western flank of the Cordillera Occidental, the two forms hybridize, forming a gradient in coloration and body mass ([23, 27]; Fig. 1 and 2). Currently, these forms are considered subspecies of *Ramphocelus flammigerus* [28] because gene exchange between them appears to be unrestricted, with apparently no selection against hybrids [23].

The *R. flammigerus* system is particularly well suited to studying the role of different evolutionary forces at work in hybrid zones owing to the existence of variation in characters with different modes of inheritance, and, presumably, under different forms of selection. Variation in rump coloration across the hybrid zone likely reflects variation in the concentration of a single carotenoid pigment and is influenced by the environment because carotenoids are obtained from the diet [23, 29, 30]. Furthermore, based on the strong sexual dichromatism in this species, and the likelihood that its mating system involves some degree of polygamy [31], plumage coloration is probably influenced by sexual selection. In contrast, morphometric variation [23, 27] likely has a strong genetic basis [32-34] and could be subject to natural selection [35-37]. The value of considering traits with different modes of inheritance and under different selective pressures to understand evolutionary forces at work in hybrid zones is illustrated by studies showing (1) that clines for traits involved in courtship are displaced with respect to clines for presumably neutral traits or loci, suggesting a role for sexual selection driving introgression [38, 39]; (2) that sex-linked molecular markers introgress over shorter distances than autosomal markers, suggesting a role for sex chromosomes in reproductive isolation

[40, 41]; or (3) that there is more limited introgression in organellar DNA than in nuclear genes, suggesting selection acts more strongly on hybrids of the heterogametic sex (Haldane's rule; [42]).

Here, we sought to evaluate whether the *Ramphocelus* hybrid zone in southwestern Colombia originated as a result of secondary contact or of primary differentiation, and to examine the zone's dynamics over nearly a century to make inferences about the action of different evolutionary processes. To accomplish this, we (1) reconstructed the biogeographic and demographic history of the hybridizing populations based on ecological niche modeling and coalescent analyses of mtDNA sequence data, (2) characterized genetic, morphological and plumage variation across the hybrid zone, and (3) compared spatial patterns of variation in morphometrics, plumage coloration, and genetic structure between specimens collected at different times to assess changes in the position and width of the hybrid zone.

Materials and methods

Samples

We characterized the *Ramphocelus* hybrid zone historically by examining specimens collected from 1894 to 1986 in the ornithological collections of the American Museum of Natural History, the Cornell University Museum of Vertebrates, Universidad del Valle, and the Instituto de Ciencias Naturales at Universidad Nacional de Colombia. To describe current patterns of variation, we collected 73 new specimens in 2007-2010: 65 of them are from localities ranging across the hybrid zone over a distance of 140 km by road connecting the cities of Cali and Buenaventura in department Valle del Cauca [23]; the remaining eight are from localities distant from the hybrid zone in the departments of Antioquia (3), Risaralda (3) and Cauca (2). Study skins and tissue samples are deposited in the Museo de Historia Natural de la Universidad de los Andes (ANDES, Table S1). All localities (historical and current) were plotted and their position along a transect line that best adjusted to points (estimated using a linear regression between latitude and longitude) was recorded. To construct character clines, we grouped sites into 12 sampling localities along our study transect (Fig. 2; Table S2).

Biogeographic history

To model potential distributions of the study taxa, we used 343 georeferenced localities obtained from museum specimens and reliable field observations from Costa Rica, Panama, Colombia, Ecuador, and Peru ([43]; GBIF Data Portal, C. Sánchez, pers. comm., our observations). We associated localities with GIS layers for 19 climate variables at a c. 1 km resolution developed for the present (WorldClim; [44]). With these data, we used a maximum entropy approach (Maxent; [45]) based on current climate layers to generate models of the ecological niche and potential distribution of *flammigerus* and

icteronotus at present (localities of both forms and presumed hybrids were considered together). Model performance in predicting present distributions (evaluated using receiver-operating-characteristic curves; [46]) was satisfactory (see below), validating the use of this approach to infer potential distributions in the past.

We projected models based on current climate data onto historical climate surfaces for 6,000 years ago and for the Last Glacial Maximum (LGM; 21,000 years ago) to determine whether the distributions of our study taxa were likely disjunct in the past as predicted by the secondary contact hypothesis, or have likely been continuous as predicted by the primary intergradation hypothesis. This approach requires assuming ecological niche conservatism and that climate represents a long-term stable constraint on potential distributions. Because ecological niche models are based only on climatic data, they tend to overpredict potential distributions into areas where the study species do not occur owing to historical limitations to dispersal (e.g., the Amazon region in *R. flammigerus*). To reduce overprediction, we cropped maps of current and historical distributions to include only areas within the Andes Ecoregion [47]. Although cropping potential distributions to this region probably did not remove all areas of model overprediction, it allowed for a semi-quantitative comparison of potential distributions across different time periods by calculating the extent of presence areas within the ecoregion. Maxent produces a continuous output ranging from zero to one describing the probability of the species potentially being present at different sites. We considered a threshold of 10% omission to categorize pixels as suitable or unsuitable for each of the time periods.

Genetic characterization and demographic history

We analyzed variation in DNA sequences of the cytochrome *b* mitochondrial gene for 58 of the *flammigerus/icteronotus* individuals collected in Colombia from 2007 to 2010. In addition, we obtained sequences for three individuals from Ecuador and two from Panama, and combined our data with two sequences of *flammigerus* available in GenBank: one from Ecuador (accession U15719.1; [48]) and one from Panamá (FJ799882.1; [49]; Table S1).

DNA was extracted from tissue samples or toepad samples taken from specimens using a Qiagen DNeasy Tissue Kit or a phenol-chloroform method [50]. PCRs used primers H16064 and L14996 [51] in 26 µl amplification reactions using the following conditions: 2 µl of template extract (~50 ng of DNA), 1 µl of 10 mM dNTPs, 1.2 µl of each primer (10 mM), 2.5 µl of 10X buffer with 1.5 µl of MgCl2, 0.125 µl Taq (5 units/ml AmpliTaq DNA polymerase), and 16.5 µl of sterile ddH2O. Reactions began with an initial denaturation at 94°C for two minutes, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for one minute, with a final extension phase at 72°C for 7 minutes. PCR products were purified with Affymetrix Exosap-IT and sequenced in both directions. Sequences were edited, assembled and aligned using Geneious Pro

3.6.1 (http://www.geneious.com). The mean length of these sequences was 988.8 bp (range 888-1008 bp).

We also analyzed mtDNA from historical toepad samples of 87 specimens collected by C. G. Sibley in 1956 and housed at the Cornell University Museum of Vertebrates [23]. To reduce the odds of contamination, DNA extraction and amplification of these samples was carried out in a historical DNA lab, following protocols described by Carling *et al*. [52]. For these specimens, we amplified and sequenced c. 210 bp of the cytochrome *b* gene (mean=209.9, range=203-210 bp).

We examined genealogical relationships among haplotypes observed in *flammigerus* and *icteronotus* at present using a maximum-likelihood (ML) phylogenetic analysis. The analysis employed the GTR+ Γ model. Nodal support was estimated using 1000 bootstrap replicates in RAxML, run from the RAxML BlackBox Web-Server [53]. We used as outgroups sequences of *Ramphocelus carbo* (AF310048.1; [54]) and *R. passerinii* (EF529965.1; [49]).

To assess potential changes in the genetic makeup of the hybrid zone over time, we examined population structure separately for the 1956 specimens and for our samples collected in 2007-2010 (hereafter 2010 specimens) employing procedures implemented in the program ARLEQUIN v3.5 [55]. Based on each data set, we conducted analyses of molecular variance (AMOVAs) by pooling localities based on their position along our sampling transect in three sectors: localities 1-6 (mostly yellow rump, *icteronotus* type), 7 and 8 (mixed/intermediate), and 9-12 (mostly red rump, *flammigerus* type). We calculated F-statistics to estimate differentiation among sectors (F_{CT}), among localities within sectors (F_{SC}), and among localities among sectors (F_{ST}). This analysis allowed us to examine whether there has been any change in the way in which genetic variation is distributed within and among sectors; if spatial genetic structure has become eroded (e.g., if introgressive hybridization has led to genetic homogenization across the transect), then one would expect an increase in genetic variation existing within sectors and a decrease in that existing among sectors over time (i.e. higher F_{CT} in the past than at present). To make data comparable across time periods, sequences for 2010 specimens were trimmed to match the 210 bp available for the 1956 specimens. As an additional way to visualize potential changes in genetic structure over time, we constructed median-joining haplotype networks for the 1956 and 2010 samples using the program PopART (http://popart.otago.ac.nz/index.shtml).

To determine whether populations of *flammigerus* and *icteronotus* have experienced demographic expansions or if these taxa have exhibited historically stable population sizes, we examined trends in effective population size through time using the Extended Bayesian Skyline Plot (EBSP) method implemented in Beast v1.7.4 [56] using sequence data for the 2010 specimens. Demographic expansions are expected if the hybrid zone originated following secondary contact and stable

population sizes are expected if the zone originated by primary differentiation. Analyses used the HKY+Γ model, which was selected as the best fit to the data according to the Bayesian Information Criterion (BIC) in JModelTest v 2.1.3 [57]. We ran the analysis for 25,000,000 iterations of which the first 10% were discarded as burn-in; genealogies and model parameters were sampled every 10,000 iterations. For time calibration we assumed a lognormal relaxed clock and a cytochrome-*b* substitution rate of 2.08% divergence per million years [58]. We used the mean of the distribution of population size as a prior (parameter "demographic.populationMean") calculated from a "Coalescent: constant time" tree prior, run with the same parameters as above. Because this analysis assumes no genetic structure within the sample, we only considered populations located between Cali and Buenaventura (i.e., from the hybrid-zone transect). The skyline plot was built in R [59] with code written by Valderrama *et al.* [60].

Phenotypic characterization

To characterize the hybrid zone phenotypically, we measured six morphological characters on specimens (n=139 males, 83 females) with dial calipers to the nearest 0.1 mm: wing length (chord of unflattened wing from bend of wing to longest primary), exposed culmen, bill depth (at the base), bill width (at the base), tail length (from point of insertion of central rectrices to tip of longest rectrix), and tarsus length (from the joint of tarsometatarsus and tibiotarsus to the lateral edge of last undivided scute). To describe morphological variation, we reduced variation in these characters using a principal components analysis (PCA).

We characterized plumage coloration based on reflectance spectra from 400 to 700 nm (the human visible spectrum) measured on the rump of adult museum specimens (n=144 males, 70 females) using an Ocean Optics USB4F00243 Spectrometer with the SpectraSuite software (Ocean Optics). Three color measurements were estimated for each reflectance spectrum based on segment classification analysis [61]: brightness, an index of how much light is reflected from the sample relative to a white standard; chroma, the saturation of color; and hue, which relates to the wavelength of maximum slope. These measurements were calculated using R code written by Parra [62].

Phenotypic clines and temporal dynamics

To compare patterns of morphometric and plumage color variation between adult specimens collected at different times in a geographical context, we defined three periods based on temporal sampling gaps: prior to 1911, 1956-1986, and 2010. For each of these time periods, we fitted equilibrium geographic cline models [63-66], and estimated cline center and width for morphometric and plumage-color data using the Metropolis-Hastings Markov chain Monte Carlo (MCMC) algorithm implemented in the HZAR package for R [67].

To model the shape of clines, we tested ten different models built based on three equations (Table S3; [67]). The first equation describes the central sigmoid-shaped transition zone and the other two describe the exponential tails of the constraining phenotypes at either side. We modified two parameters in the latter two equations to construct the different models. First, the fit in the extreme values of the exponential curves was estimated under two conditions (1) constraining the phenotypes in the extremes of the cline to the maximum and minimum values observed ("fixed"), and (2) letting the extreme values vary freely ("free") at both ends. Second, we tested the fit of five models of exponential decay of the tails on either side of the central sigmoid-shaped transition; a model without exponential fit ("none"), two models with exponential fit only in an extreme of the cline ("right" or "left"), a model with two tails of exponential fit symmetric in the center of the cline ("mirror") and a last model allowing exponential fit with different parameters on both tails ("both"). The combination of the fit in the extreme values ("fixed" and "free") and the five models of exponential decay ("none", "right", "left", "mirror" and "both") resulted in a total of ten models examined. We used three chains per model in MCMC searches; each chain was run for 100,000 iterations where the first 10% was discarded as burn-in. We started each chain with a separate seed in the MCMC process, and we randomized the initial values of parameters. We evaluated model support using the Akaike Information Criterion (AIC), and extracted cline center and width values from the best models. To describe uncertainty in the estimation of cline parameters, we considered the range of values within two log-likelihood units of the maximum-likelihood value [67].

The functions implemented in HZAR require that the data follow a nearly normal distribution. Because some of our data were not normally distributed, we used Box-Cox transformations to normalize variables. Because estimates of cline center and width based on transformed and untransformed data were very similar, we report results based on the untransformed variables, which are more intuitively interpreted. For cases where characters did not vary clinally or where the cline-fitting algorithm did not converge on parameter estimates, we described variation across the hybrid zone using scatter plots.

Results

Biogeographic history

A potential distribution model developed under current climatic conditions in Maxent accurately predicted the present-day distributions of *flammigerus+icteronotus,* with an area under the ROC-curve score of 0.987. Because this suggests that the assumption that climate limits distributions in these taxa is reasonable, we projected models onto past climatic conditions to estimate the potential historical distributions of *flammigerus+icteronotus* at 6,000 and 21,000 years ago.

Although our models evidently overpredict potential distributions, it is clear that the extent of suitable environments for *flammigerus+icteronotus* has not been stable over time. The modeled potential range of these taxa at present in the Northern Andes Ecoregion extends for c. 420,000 km^2. The predicted potential distribution based on climate for 6,000 years ago was of similar size, with c. 460,000 km^2 (Fig. 3a). This indicates that current climatic conditions and those from 6,000 years ago were similarly suitable for the presence of these taxa across the study region. Indeed, models suggest that the two forms could have been in contact at that time in the current location of the hybrid zone (Fig. 3a). In contrast, the predicted range during the LGM was considerably smaller than the predicted current range (c. 280,000 km^2; Fig. 3b). Moreover, suitable conditions for *flammigerus+icteronotus* 21,000 years before present were not continuous along the Pacific slope of the Cordillera Occidental, suggesting that these two forms were likely disjunct during the LGM. Thus, the hybrid zone may have originated following population expansions and secondary contact as a result of climatic change since the LGM, a possibility we address below based on patterns of genetic variation.

Genetic characterization

Overall, there was low genetic divergence between samples and genetic structure across the hybrid zone and among other localities was limited. Based on the recent samples for which we obtained long cytochrome *b* sequences, uncorrected mean sequence divergence within Colombia was only 0.3% (0-1.1%); samples from Ecuador and Colombia were 1.6% divergent, and samples from Panama and Colombia differed by only 0.4% on average. Except for a separation between samples from Ecuador and Colombia, relationships among haplotypes were not clearly resolved by the ML phylogenetic analysis, in which most nodes lacked bootstrap support and no clades associated with specific geographic regions or with plumage coloration were identified (Fig. 4). Among the complete sequences (989 bp), there were 18 haplotypes with a total of 15 segregating sites in populations along our hybrid-zone transect. Among the 87 individuals from 1956 analyzed (210 bp), there were six haplotypes, with a total of nine segregating sites; uncorrected mean sequence divergence was only 0.4% (0-3.7%) and most (69) individuals shared a common haplotype. Relationships among haplotypes were not consistent with position along the hybrid zone (Fig. 4). For the same 210-bp region, there were seven haplotypes with six segregating sites in the 2010 specimens; clear structure with respect to position along the transect was not observed in the haplotype network (Fig. 4).

AMOVAs suggested that patterns of genetic structure across our study transect differ between specimens from 1956 and 2010, with considerably greater genetic structure among sectors in the 1956 data (Table 1). For the historical data, F_{CT} values were marginally significant ($F_{CT} = 0.22$, $P = 0.047$), indicating that a significant fraction of genetic variation (92%) was apportioned among sectors of the study transect. This was not the case for the present-day data, in which no genetic structure across the transect was detected ($F_{CT} = -0.12491$, $P = 0.996$).

Although credibility intervals for population size in the Bayesian skyline plot were wide, this analysis suggested that populations show a genetic signature of demographic expansion (Fig. 5). Constant population size can be rejected because the median value of the parameter "demographic.populationSizeChanges" differed from zero (median value = 1, 95% highest posterior density 0-2). This result is consistent with the hypothesis that the hybrid zone originated as a result of secondary contact following expansion of populations from formerly disjunct areas.

Phenotypic characterization

Reduction of morphometric variation using PCA resulted in a first component (PC1) describing body size in both males and females. In both sexes, variables loading most heavily on this axis (which accounted for 24.3% of the variation in males and 34.7% in females) were tail length and wing chord. Thus, in the following we use PC1 as a general measurement of body size. We did not consider other principal component axes (e.g., PC2, on which bill dimensions loaded heavily) in additional analyses because they did not vary clinally across the hybrid zone.

Morphological data for historical and recently collected male specimens provide evidence of clinal variation in body size (i.e. PC1) along the hybrid zone, with birds from localities to the west (*icteronotus*-type) being smaller than those from the east (*flammigerus*-type; Fig. 6). For the three periods the best cline model according to AICc was model I (Table 2). This model estimated that the center of the morphometric cline is currently located at c. 84 km, near locality 9. In turn, the center of the cline in the pre-1911 and 1956-1986 samples was estimated at c. 73 and 78 km (closer to locality 8), respectively, suggesting the zone has moved some 10 km over the past century (Table 2, Fig. 6). We note, however, that likelihood-based estimates of uncertainty around the point estimates of cline centers overlap slightly. Estimates of cline width were substantially more uncertain, but also appear to differ between the present and past time periods: at present, the estimated width was only c. 2.8 km, but the width of the cline in the pre-1911 and 1956-1986 samples was estimated at c. 34.9 and 33.5 km, respectively (Table 2).

Patterns of variation in color in space and time were similar to those observed for morphology. Of the three measurements of plumage coloration, chroma showed the clearest clinal pattern of variation, ranging from the yellow *icteronotus* to the redder *flammigerus* (Fig. 6). The cline-fitting algorithm was unable to estimate cline parameters for the pre-1911 data set, possibly due to small sample size in this period (Fig. 6). Models III and I were the best fit for 1956 and 2010, respectively (Table 2), and estimated cline centers at 77 km for 1956 and 83 km for 2010 (Fig. 6). As with morphology, the cline was wider in the 1956 sample (width 16 km) than in 2010 (6 km; Table 2).

Morphological data for females, and measurements of hue and brightness for both males and females, did not show clear clinal trends (Figs. S1-S3). Therefore, we did not attempt to estimate cline parameters for these traits.

Discussion

Based on patterns of genetic variation, fossil pollen data, and ecological niche modeling, several studies in the north temperate zone indicate that the origin of many hybrid zones can be explained as a result of population expansions from isolated refugia during the Quaternary [12, 13, 68]. Although a similar hypothesis was proposed to account for the origin of contact zones in tropical rainforest organisms [69, 70], research on the origin of hybrid zones in the Neotropical region has been relatively limited [71, 72]. Our niche models indicate that potential distributions of *R. f. icteronotus* and *R. f. flammigerus* were likely disjunct at the LGM (21,000 ya), but were potentially in contact by 6,000 ya. This scenario is supported by the historical demography analysis based on mtDNA sequence data, which indicates that populations have experienced significant range expansions. This suggests that these forms likely diverged while isolated in each flank of the Cordillera Occidental (*icteronotus* in the Pacific lowlands and *flammigerus* in the Cauca Valley) and then expanded their distributions, presumably tracking the influence of Pleistocene climate change on vegetation [73, 74]. Our data also suggest that the divergence between the hybridizing *Ramphocelus* populations likely occurred in the Pleistocene, as indicated by low levels of mtDNA divergence suggesting recent differentiation. However, because Quaternary climatic oscillations started well before the LGM [75], it is possible that distribution ranges became disjunct and reconnected repeatedly at various times throughout the Pleistocene.

In contrast to our proposed scenario suggesting the origin of the *Ramphocelus* hybrid zone may date to at least 6,000 before present, Sibley [23] hypothesized that contact between *flammigerus* and *icteronotus* resulted from recent anthropogenic deforestation and expansion of crops creating scrub and second-growth habitats, which are favored by these tanagers over dense rain forest. Although our analyses suggest that climatic conditions were suitable for contact between these forms thousands of years prior to human alterations in the area, it is likely that anthropogenic activities have facilitated contact between them, possibly leading to an increased incidence of hybridization in recent times.

A recent study on a hybrid zone between *Heliconius* butterflies located in the same geographic region where we studied hybridization in *Ramphocelus* also provided evidence consistent with the hypothesis of origin via secondary contact [76]. Because there are additional documented cases of hybridization in the same general area of southwestern Colombia (e.g., other *Heliconius* butterfiles [77], *Oophaga* poison frogs [78]), work on the history of the region is necessary to better understand the origin and

maintenance of hybrid zones across taxa [12, 15].

Our analyses are consistent with Sibley's [23] overall characterization of the *Ramphocelus* hybrid zone: there is clinal variation in coloration and body size, with males exhibiting clearer trends than females (see also [27]). With the caveat that uncertainty around parameter estimates is broad, two main additional insights are provided by our cline analyses. First, our data consistently indicate that for each period, clines for morphology and chroma are coincident (i.e., they have equal or very similar centers). Second, variation in morphological PC1 and chroma followed the same trend over time: for both traits, clines moved to the east some 10 km and became narrower from the past to the present.

The coincidence of cline centers for different characters and their apparent concordance in width in each of the three periods is consistent with a tension-zone model. This is further supported by the width estimated for character clines. Assuming that the hybrid zone originated at least 6,000 ya according to climate-based models, that generation time in *Ramphocelus* is 1-2 years, and that dispersal distances per generation lie somewhere between 1 and 20 km, the expected width of clines under a neutral diffusion model (equations in [7, 65]) would be between c. 25 km and more than 500 km. This is much wider than what we observed (Table 2), which suggests some form of selection is acting. Alternatively, narrow clines may be a result of a more recent origin of the hybrid zone than implied by climate data. However, even if one assumes that the hybrid zone is as young as proposed by Sibley [23], the observed clines appear narrower than expected under neutral diffusion (c. 4-75 km). In sum, our results suggest that the *Ramphocelus* hybrid zone is likely a tension zone, maintained by a balance between dispersal and a genome-wide barrier to gene flow [1]. This interpretation contrasts with Sibley's [23] conclusions that "gene exchange appears to be unimpeded" and that there is "no evidence of selection against the hybrids". Future studies should examine survival and mating success of intermediate phenotypes resulting from hybridization relative to parental types.

That cline centers do not appear to be coincident across different periods for each of the characters suggests movement of the *Ramphocelus* hybrid zone, but this interpretation needs to be tempered because samples were not taken at exactly the same localities at each time period and because the centers we estimated in some cases had wide support limits. However, our field observations indicate that the current patterns of variation are, in fact, different from those described by Sibley [23]. Specifically, we frequently observed yellow-rumped individuals at Salado and Queremal (locality 8), where Sibley did not report any, and we also have occasional records of yellow-rumped individuals near Cali, the eastern extreme of the transect. Thus, our quantitative analyses and field observations suggest that the *icteronotus* phenotype (yellow rump and smaller body size) has indeed extended to the east. Several previous studies have also reported on moving tension zones [66, 79, 80] although others documented spatial stability [81, 82].

A possible explanation for the concurrent movement of clines for different characters in hybrid zones is competitive advantage of one phenotype mediated by aggression or by sexual selection [18]. Thus, movement of the *Ramphocelus* hybrid zone may have been driven by competition or sexual selection favoring the *icteronotus* phenotype. We believe it is unlikely that aggressive superiority of *icteronotus* explains this pattern as shown in other studies of hybrid zones owing to its smaller body size, although we note that in a *Manacus* (Pipridae) hybrid zone in Panama, the smaller *M. vitellinus* is more aggressive than the larger *M. candei* [83]. Thus, it would be of interest to study mating patterns in the field and to conduct mate-choice experiments to determine whether the *icteronotus* phenotype has increased reproductive success as a result of sexual selection via male-male dominance or female choice [84, 85].

If sexual selection is based on carotenoid plumage color, which presumably is strongly influenced by the environment [30], then it is somewhat puzzling that coloration seems to have moved across the hybrid zone in concert with morphometric variation, which presumably has high heritability and is not involved in mate choice. It is possible, however, that the ability to obtain, accumulate and metabolize carotenoids has a heritable genetic basis [86] and that the fitness advantages it confers may be linked to genes involved in reproductive isolation [87]. If this were the case in *R. flammigerus*, then it would represent a plausible explanation for the movement of coloration in concert with others traits.

An alternative explanation for zone movement was proposed by Sibley [23], who predicted that the *icteronotus* phenotype would introgress across the hybrid zone as a consequence of increased gene flow from coastal to interior populations resulting from larger populations sizes in the former. This could be studied in the future with multilocus estimates of effective population sizes and of the magnitude of gene flow in both directions [88]. In addition, if deforestation has indeed resulted in increases in population size as hypothesized by Sibley [23], then movement of the hybrid zone may also partly reflect anthropogenic influences. Because hybrid zones tend to become entrapped in areas of low population densities acting as sinks for migration [1, 82], any changes in population size related to habitat modification may have partly facilitated the observed movement of the *Ramphocelus* hybrid zone.

Finally, we note that the inferred movement of the hybrid zone involves not only a west-east displacement, but also a shift in elevation; at the estimated cline center for 1956 elevation is ca. 700-900 m, whereas elevation at the 2010 center is ca. 1000-1400 m. Because the elevational ranges of tropical birds may shift upslope in response to global warming [89], it is also possible that the movement of the hybrid zone is related to climatic change over the past few decades [18, 90], as recently documented for a contact zone between chickadee (Paridae) species in North America [91].

In contrast to multiple studies on hybridization in birds finding significant mtDNA divergence between populations located away from the center of hybrid zones and clinal variation in haplotype frequencies across them (e.g., [38, 42, 52, 71, 81, 92-96]) mtDNA variation was not geographically structured in our study system, a likely consequence of recent divergence of the hybridizing populations or of high levels of introgression. Because no clinal variation was observed across the *Ramphocelus* hybrid zone, we were unable estimate cline parameters for different time periods to examine zone movement as done in a few studies on hybrid zones examining genetic variation in specimens collected at different times [80, 94]. However, our analyses revealing more limited genetic structure across the zone as indicated by F-statistics in 2010 relative to 1956 provide some evidence that patterns of genetic variation have not been stable over time. We hypothesize that these results may reflect an increase in introgression of mtDNA across our study transect since Sibley's [23] time, but we acknowledge that because we only assayed a small fragment of mtDNA and because sampling was not spatially even between time periods, drawing any conclusion at this time would be premature. Nonetheless, our preliminary genetic data suggest that assessing temporal variation in spatial genetic structure using genome-wide markers (cf. [41]) would represent a fruitful avenue to better understand the ecological and evolutionary forces at work in this moving hybrid zone.

Competing Interests

The authors declare they have no competing interests.

Authors' Contributions

AMR and CDC conceived the study. AMR collected and measured specimens, conducted molecular genetic work, and performed initial analyses. MDC conducted molecular work on historical specimens. AMR and EAT analyzed data with supervision from CDC. AMR and CDC wrote the manuscript. All authors read, commented on, and approved the final manuscript.

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Fig. 1. Gradient of phenotypic variation in male specimens collected along the *Ramphocelus flammigerus* hybrid zone in southwestern Colombia. Individuals 1-7 correspond to *R. flammigerus icteronotus* (yellow-rumped form) from the plains of the Pacific coast (sector 1, localities 1-6; see Fig. 2). On the other extreme, individuals 11-14 correspond to *R. flammigerus flammigerus* (scarlet-rumped form) distributed towards the Cauca River Valley (sector 3, localities 9-12). Individuals 8-10 are intermediates collected near the center of the hybrid zone (sector 2, localities 7-8).

Fig. 2. Study transect encompassing the *Ramphocelus* hybrid zone from the Pacific lowlands to the eastern slope of the Cordillera Occidental of the Colombian Andes. Lowland areas are shown in grey and the higher elevations of the Cordillera Occidental in black. Blue dots indicate collection sites of historical specimens and purple dots indicate collection sites of current specimens sampled for phenotypic/genetic variation; black lines correspond to municipality limits, with text indicating the location of the larger cities of Cali and Buenaventura. Collection sites were grouped in 12 localities (white dots) along the transect for cline-fitting analyses as follows: (1) Ladrilleros, La Barra, Bahía Muerte, Bahía Málaga; (2) Bajo Calima; (3) Buenaventura, Río Raposo; (4) Anchicayá, Zabaletas; (5) San José; (6) El Placer, Bajo Anchicayá; (7) Juntas, Jiménez, Río Blanco, La Elsa, Loboguerrero, Zelandia; (8) El Salado, Queremal; (9) Calima, San Antonio, La Cumbre; (10) Cali, Navarro; (11) Palmira; (12) Florida. Sector 1 comprises localities 1-6, sector 2 localities 7-8, and sector 3 localities 9-12.

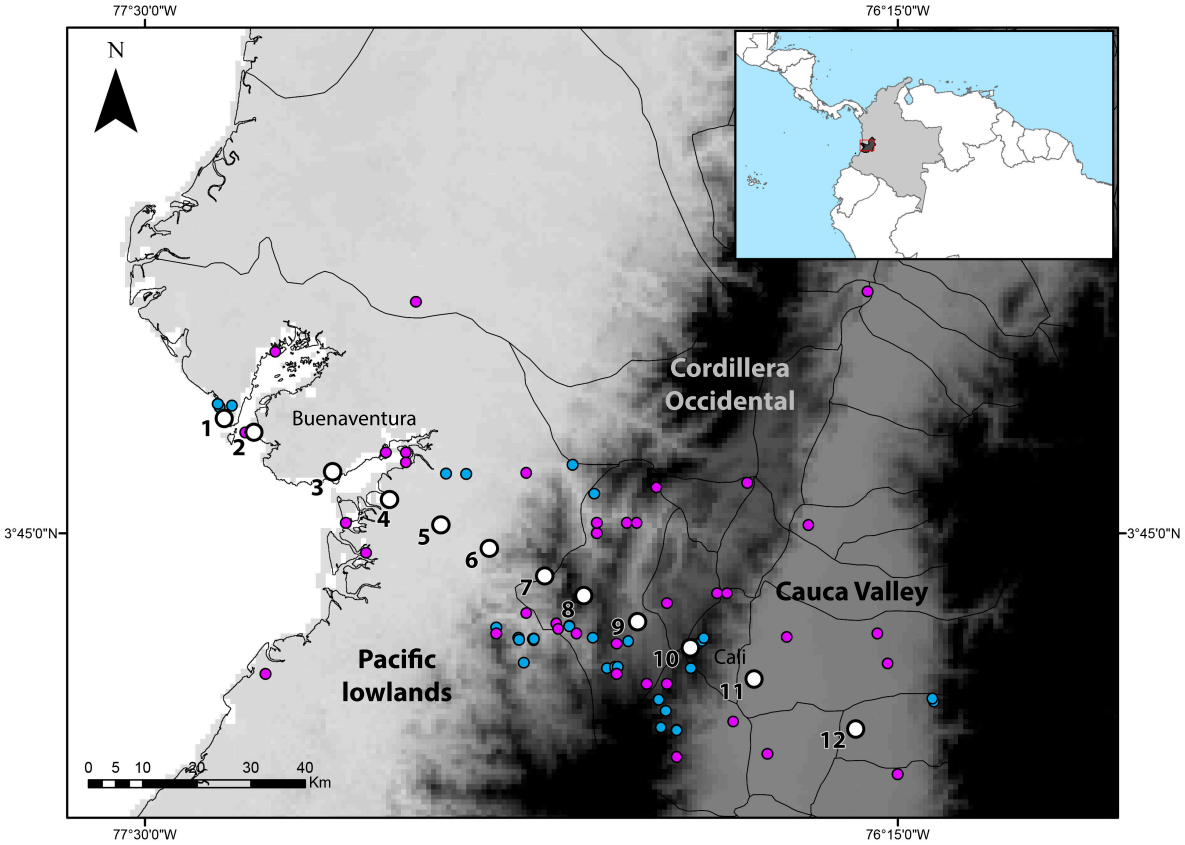

Fig. 3. Potential distributions of *R. flammigerus* predicted by MaxEnt using climatic data. Dark gray areas show suitable environmental conditions for the occurrence of *flammigerus* and *icteronotus* (A) 6,000 years ago and (B) 21,000 years ago (LGM). Light gray depicts climatically suitable areas for their occurrence at present. Note the smaller predicted range during the LGM and that the two forms likely did not exhibit a continuous range along the transect (dotted line) at that time, relative to the more extensive and continuous range modeled for 6,000 y.a. and under current conditions. Dots represent localities used to construct the models.

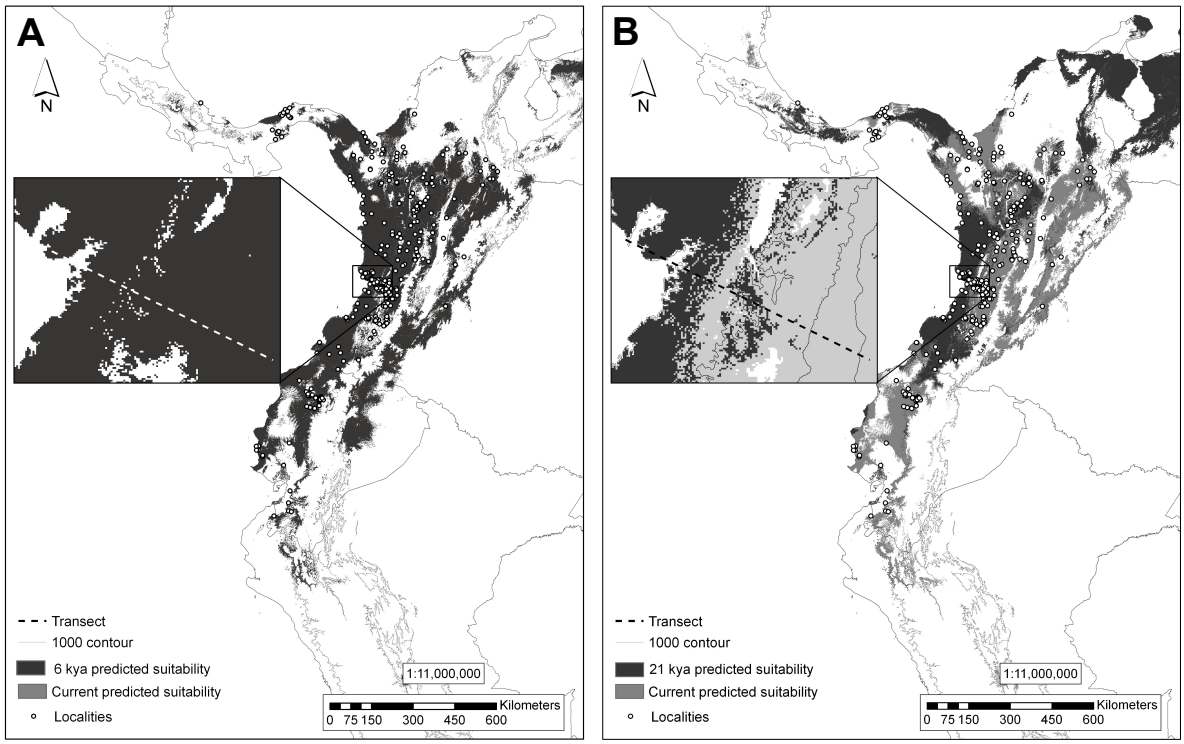

Fig. 4. Genealogical relationships of specimens of *R. flammigerus* showing limited geographic structuring and relatively low levels of sequence divergence among haplotypes. The phylogenetic tree on the left depicts relationships among nearly complete sequences of the cytochrome *b* gene obtained for individuals from the hybrid zone and other localitites inferred using maximum-likelihood (outgroups not shown); bootstrap values on nodes are shown when ≥ 50%. Colored circles indicate a qualitative assessment of the rump color (yellow, orange and red as in individuals 1-7, 8-10 and 11-14 in Fig. 1, respectively) and location in the hybrid zone (cyan, sector 1, localities 1-6; green, sector 2, localities 7-8; dark blue, sector 3, localities 9-12) of individuals from the study transect exhibiting each haplotype. The numbers correspond to specimen identifications in Table S1; all numbers refer to specimens from the hybrid-zone transect unless otherwise noted. Localities outside the transect in different provinces of Colombia (CO), Ecuador, and Panama are indicated with squares. Haplotype networks on the right focused on specimens from the hybrid zone show that genetic variation in 210 bp of the cytochrome *b* gene was not clearly consistent with position of individuals along the hybrid zone in 1956 (top) or in the present (bottom), although analyses of molecular variance suggest differences in patterns of genetic structure across time periods (see text); circle sizes are proportional to the number of individuals sharing each haplotype.

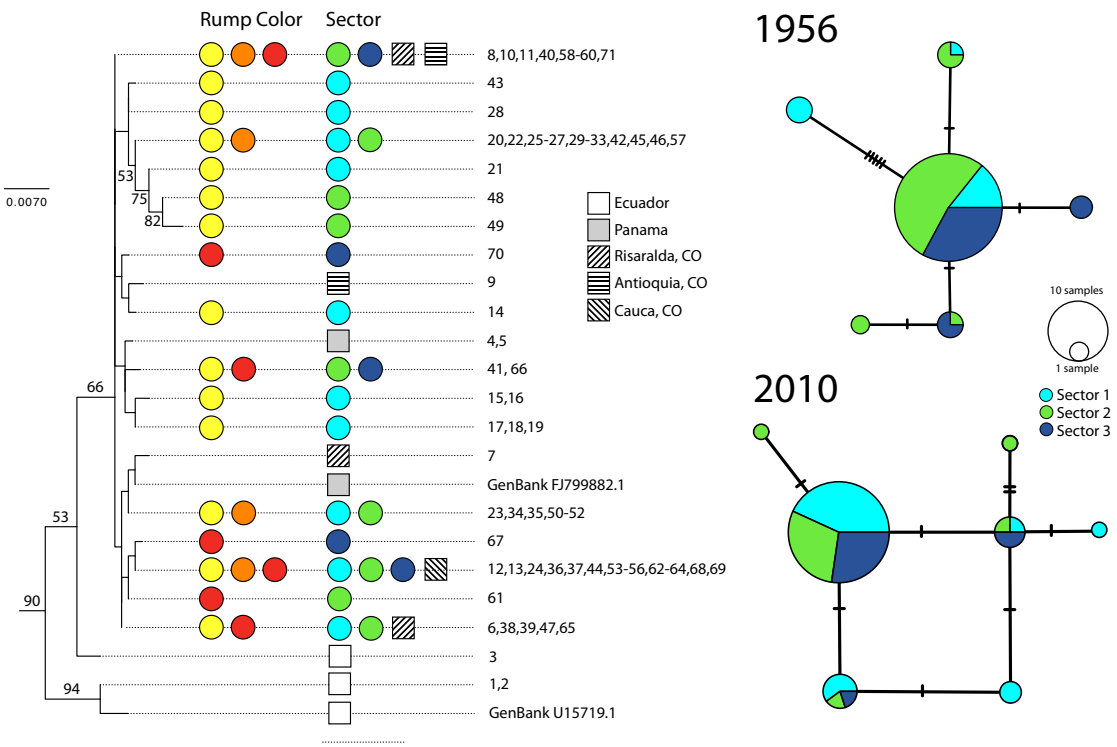

Fig. 5. Estimates of population sizes over the last 400,000 years obtained using the extended Bayesian skyline plot method applied to cytochrome *b* sequence data suggest demographic expansion towards the present in *R. flammigerus*. Median and credibility interval values are shown in black solid line and dashed lines, respectively. Blue lines correspond to 1000 genealogies used to estimate the 95% highest posterior density of population sizes. Bars in the histogramare proportional to the number of genealogies with values in the specific time interval.

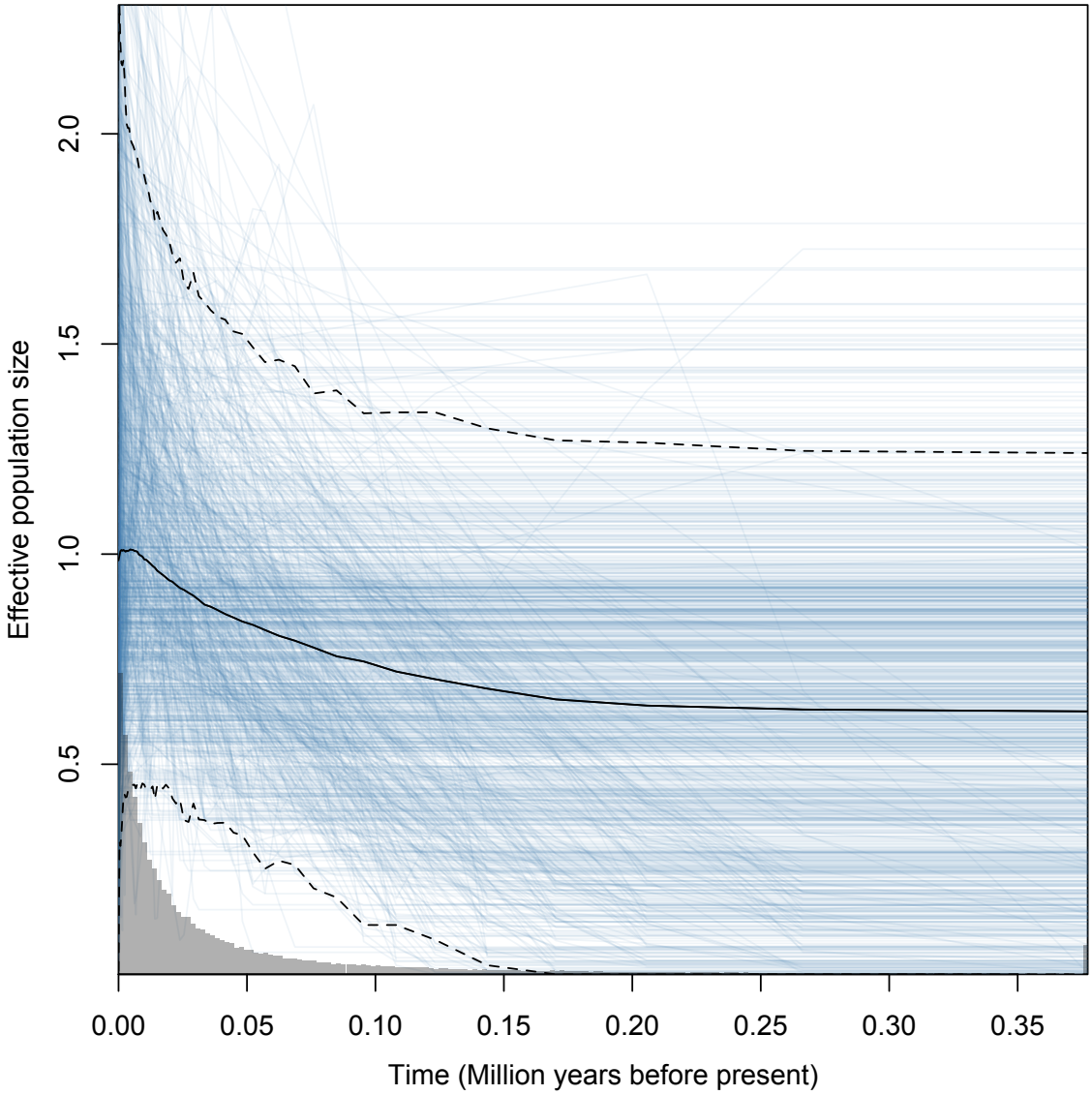

Fig. 6. Variation in morphology and coloration of males over c. 130 km across the *Ramphocelus* hybrid zone in historical and recent specimens. (A-F) Circles represent values observed at different sampling localities; for the traits and periods in which parameters could be estimated using HZAR, dark lines are maximum-likelihood clines for the best-fit models, with shading indicating confidence around cline estimates. **(G, H)** Maximum-likelihood estimates of cline centers at each time period and their confidence limits, revealing coincident centers for morphology and color at each time period and eastward movement of the hybrid zone over time as indicated by both traits (colors as in A-F); circles are the 11 sampling localities along the study transect.

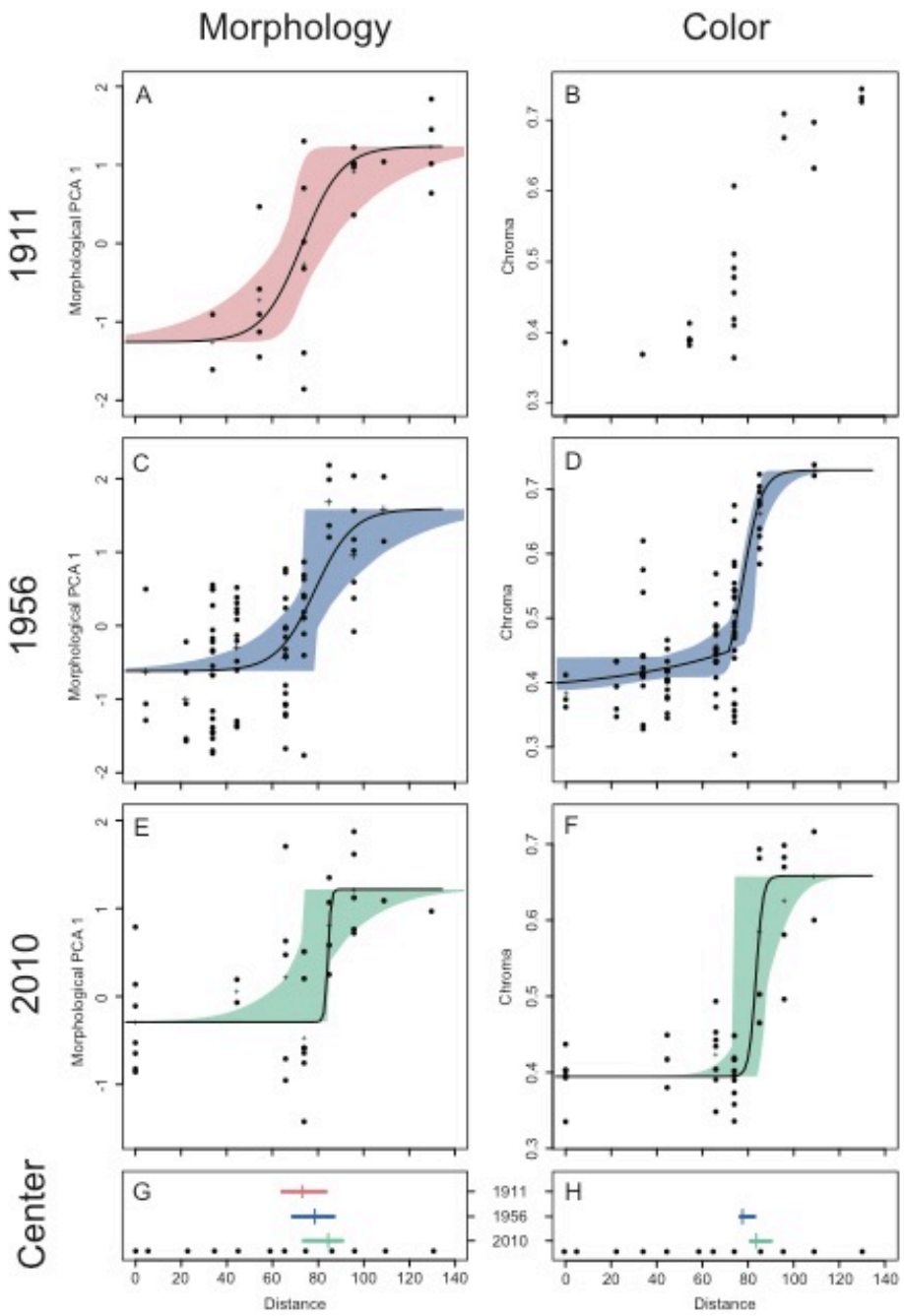

Table 1. Population genetic structure in historical and recent specimens. Results are shown for analyses of molecular variance (AMOVA) based on DNA sequences of the cytochrome *b* mitochondrial gene for 87 individuals collected in 1956 and 58 individuals collected in 2010.

	1956	**2010**
FST		
Among localities among sectors	0.15746	0.12917
P-value	0.05257	0.01099
Variance components	0.09225	-0.03696
FSC		
Among populations within sector	-0.08171	0.22586
P-value	0.88851	0.00257
Variance components	0.35154	0.07518
FCT		
Among sectors	0.22110	-0.12491
P-value	0.04693	0.99614
Variance components	0.09225	-0.03696

Table 2. Cline parameters estimated for morphological and coloration data in historical and recent specimens. Values are cline centers and widths (and their support limits based on two log-likelihood units) obtained by fitting curves for morphological variation (PC1) and plumage chroma for each time period based on data for male specimens. All values are given in kilometers, with cline centers measured as the distance along the transect from locality 1 to locality 12 (Fig. 2). Empty cells indicate that the model did not converge on parameter estimates.

		Males	
Variable		**Center**	**Width**
1911	PC1	73.10 (63.84-83.71)	34.88 (16.63-87.94)
	Chroma	-	-
1956	PC1	78.72 (73.25-87.32)	33.53 (1.30-68.16)
	Chroma	77.56 (76.12-82.60)	16.47 (5.72-25.07)
2010	PC1	84.37 (68.60-90.99)	2.74 (0.000025-78.98)
	Chroma	83.45 (79.56-88.63)	6.12 (0.079-25.99)

Additional Files (available upon request from ccadena@uniandes.edu.co)

Additional File 1: Table S1: Information on the samples of *Ramphocelus flammigerus* included in the study.

Additional File 2: Table S2: Sample sizes per population used for analyses of phenotypic and molecular variation across the hybrid zone between *R. f. icteronotus* and *R. f. flammigerus* in western Colombia.

Additional File 3: Table S3: Fit of different cline models to morphological and plumage color data for historical (1911, 1956) and recent (2010) specimens estimated using HZAR.

Additional File 4: Figure S1: Variation in morphology (morphological PC1) and plumage chroma in female specimens across the *Ramphocelus flammigerus* hybrid zone in southwestern Colombia.

Additional File 5: Figure S2: Variation in plumage brightness of male and female specimens across the *Ramphocelus flammigerus* hybrid zone in southwestern Colombia.

Additional File 6: Figure S3: Variation in plumage hue of male and female specimens across the *Ramphocelus flammigerus* hybrid zone in southwestern Colombia.