

Adaptive introgression of putative carotenoid pigment genes explains geographic variation in a sexually-selected plumage trait

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Abstract

Understanding the genetic architecture of sexually-selected traits is a fundamental goal in evolutionary biology because it can explain the constraints and processes that shape the production of these traits and emergent evolutionary processes, such as introgression. To address these topics, we leverage populations of hybridizing red-backed fairywrens (*Malurus melanocephalus*) that differ by plumage color (orange vs. red) across a well-classified hybrid zone with a priori evidence of strong female preference for males with redder plumage. We sequenced whole genomes of 36 individuals that vary in plumage hue and found that divergence between even the most phenotypically different individuals was very low, yet we identified several regions with high F_{ST} estimates relative to the background divergence. To determine whether loci in these elevated regions were linked to plumage variation across the species range, we sequenced top candidate genetic variants for color differentiation in 285 individuals from 16 populations and traced their frequencies across the range of the species. We found that 15% of these variants were concordant with the plumage cline, with some linked to putative carotenoid processing genes and exhibiting evidence of selection. Considered together, these findings suggest that geographic variation in the sexually-selected plumage color of male red-backed fairywrens is in part explained by adaptive introgression of genes involved in carotenoid coloration. This study highlights how genetic mechanisms underlying color variation can shape patterns of adaptive introgression via sexual selection and phenotypic differentiation in hybridizing taxa.

Keywords: plumage coloration, carotenoids, hybridization, adaptive introgression, sexual selection

Introduction

Showy male traits have long captured the imagination of naturalists and evolutionary biologists, inspiring the earliest theories of sexual selection (Darwin, 1871; Wallace, 1889). Color is often one of the most conspicuous traits that differs among closely related species (Delhey et al., 2017; Hofreiter & Schöneberg, 2010), and this spectacular diversity of ornamentation often evolves under strong sexual selection (Baldassarre & Webster, 2013; Hill, 1991). Understanding the genetic architecture of sexual selection is a fundamental goal in evolutionary biology, as it can explain evolutionary constraints and reveal how sexual selection mediates other evolutionary processes, such as trait introgression (Hooper et al., 2024; Kirschel et al., 2020; Lim et al., 2024; Long et al., 2024; Semenov et al., 2021). Though there has been recent progress in identifying genes important for color traits in many groups of organisms (e.g., Davoodi et al., 2022; Price-Waldman & Stoddard, 2021), their contri-

bution to the evolutionary history of species and the traits affecting fitness is poorly known. High throughput sequencing has facilitated the discovery of genes and pathways underlying color in birds (Funk & Taylor, 2019) and provides the opportunity to detect the signature of selection on genomic regions related to color evolution within and between species (Barrett et al., 2019).

Hybridization between species, subspecies, or phenotypically distinct populations provides a powerful opportunity to better understand evolutionary processes (Taylor & Larson, 2019), including the introgression of color traits between groups (Walsh et al., 2020). Adaptive introgression of traits via natural selection has been identified in a variety of taxa (Edelman et al., 2019; Orteu & Jiggins, 2020; Walsh et al., 2018); however, genomic evidence of adaptive introgression of a sexually-selected trait is challenging to show because systems are rarely sufficiently well studied to definitively link the pattern of introgression to the

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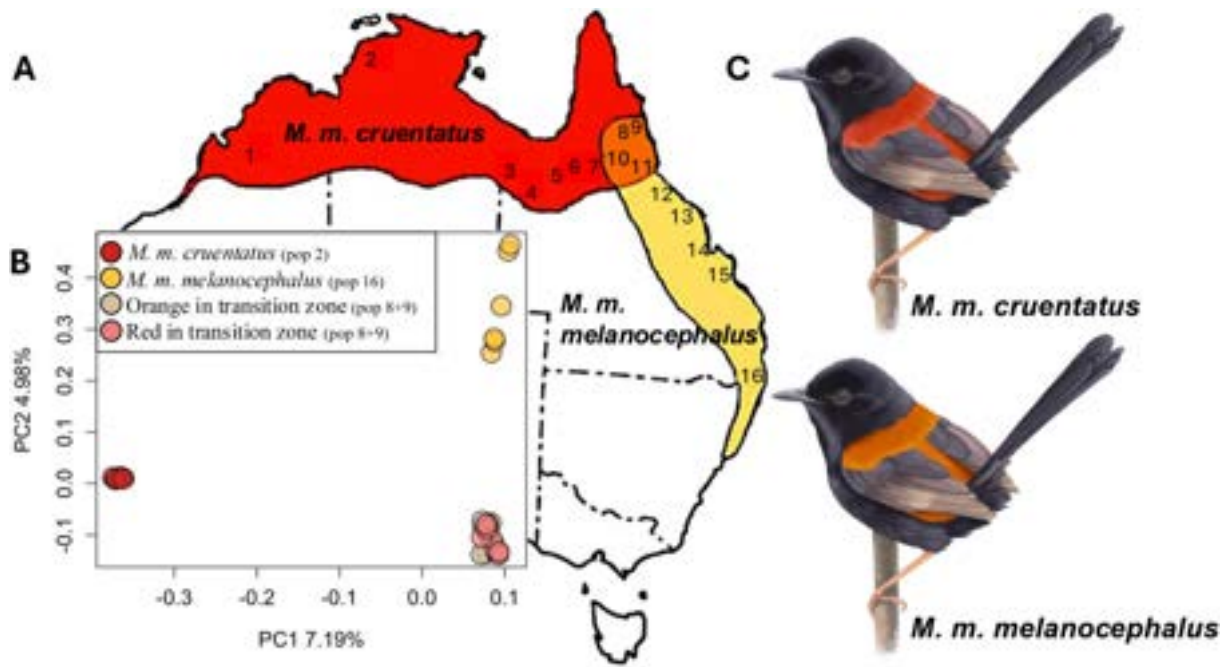


Figure 1. **A.** Subspecies distribution map of *M. m. cruentatus* in red and *M. m. melanocephalus* in yellow. The orange represents the plumage transition zone of the two subspecies. Numbers refer to sampling locations across the transect. **B.** Principal component analysis (PCA) of whole-genome sequencing SNP data. **C.** Illustrations of the two subspecies—subspecies differ predominantly by the color of their back plumage patch (red vs. orange). Illustrations by Jessica French.

underlying sexually-selected mechanism (e.g., female preference for a specific male phenotype). Accordingly, observing adaptive introgression of a sexually-selected trait requires a system in which (1) introgression of the trait has been established; (2) a likely selective mechanism driving introgression has been identified; and (3) the gene or genes underlying the trait have been identified and show the same pattern of introgression as the phenotypic trait itself.

The red-backed fairywren (*Malurus melanocephalus*) meets the first two of these three criteria, providing an opportunity to directly test for adaptive introgression of a sexually-selected trait in a wild population of passerine birds. First, it exhibits extensive natural variation in the color of the red plumage patch across its species range due to ongoing hybridization between two subspecies that vary in back color (red *M. m. cruentatus* and orange *M. m. melanocephalus*, Figure 1), forming a discrete plumage cline across the hybrid zone (Baldassarre et al., 2014). Second, there is experimental evidence for strong female preference for redder backs, with redder males having higher reproductive success than orange males (Baldassarre & Webster, 2013), indicating that back plumage color is a sexually-selected trait. Third, a previous study showed that plumage color variation is unrelated to environmental variation across the species range (Baldassarre et al., 2013), suggesting that color variation is instead more strongly controlled by genetic variation. Finally, discordance between the cline in plumage color and the cline in neutral genetic variation indicates asymmetric introgression of redder plumage across the hybrid zone and into the orange subspecies, likely driven by female preference for more red males (Baldassarre et al., 2014). As a consequence, the plumage transition zone is distinct from the genetic transition zone, whose centers are separated by 340 km (Baldassarre et al., 2014). Finding spe-

cific loci with clines that are concordant with the plumage color cline would allow us to identify the genes underlying this difference in color (i.e., criterion #3 above), as well as test for evidence of selection on those genomic regions.

To characterize the genomic mechanisms underlying plumage color differences in red-backed fairywrens, we first performed whole-genome sequencing on a subset of individuals from within and outside the plumage transition zone. We identified genomic regions associated with these color differences and tested those regions for signatures of selection and differential introgression, as we would expect to detect if those regions were under sexual selection and were responsible for variation in plumage color. We then performed targeted amplicon sequencing of these putative color loci on a much larger panel of individuals sampled from across the species range and ran geographic cline analyses to determine whether each of these genomic regions matches the known geographic pattern of plumage introgression. Under the hypothesis that male plumage color has adaptively introgressed via sexual selection, we predicted that genomic regions associated with color variation would show signatures of selection and show patterns consistent with introgression, including allele sharing among populations with similar color and clines concordant with the phenotypic cline in male plumage color.

Materials and methods

Sample collection and plumage color quantification

For whole-genome sequencing, we obtained blood samples from allopatric *M. m. cruentatus* from Darwin, Australia ($n = 6$), allopatric *M. m. melanocephalus* from Brisbane, Australia ($n = 6$), and individuals from the red-backed fairywren plumage transition zone ($n = 20$). Plumage transition

zone birds were chosen from a 10-year dataset from two long-term study sites (Donkey Farm and Moomin) of 332 unique males whose plumage hue was quantified by measuring reflectance of mounted feather samples using an Ocean Optics (Dunedin, FL) USB2000 UV-VIS spectrometer with an R200–7 UV-VIS probe and a PX2 pulsed xenon light source. Reflectance curves were analyzed using the program TetraColorSpace (Stoddard & Prum, 2008), following methods in Baldassarre et al. (2014). For this study, we focused on one measure of hue, theta (θ), because previous analyses have shown that it best captures the subtle variation in red to orange that differentiates these subspecies (Baldassarre & Webster, 2013; Baldassarre et al., 2013). Of the 332 plumage transition zone males we had plumage color hue data for, we chose to sequence the 10 most red (lowest θ) and 10 most orange (highest θ) individuals from this dataset. This sampling design allowed us to test our hypothesis that the genetic basis of color differentiation in individuals from the same hybrid population should be linked only to color loci, and not to fixation of neutral regions or the broader local adaptation that is common between allopatric populations.

For amplicon sequencing, we obtained blood samples from 285 red-backed fairywrens across 16 sampling locations (Table S1). Of these, 147 individuals had plumage hue measurements. These 16 sampling locations were the same as from Baldassarre et al. (2014), so for geographic cline analyses we arranged our sampling locations along a transect the same way, beginning with the westernmost location (Mornington), and ending at the location in the far south-east of the range (Brisbane). Each sampling location was assigned a distance along the transect, using the values used in Baldassarre et al. (2014). In short, each distance value corresponded to the shortest straight-line distance between it and Mornington, but in certain cases, this path would cross into unsuitable habitat where red-backed fairywrens do not occur. In these instances, we created a “pivot point” on the interior edge of the species range, and calculated the distance as the sum of the two straight-line distances passing through this point (described in Baldassarre et al., 2013; Greig & Webster, 2013). This resulted in a 3052 km transect spanning very nearly the entire species range. For this study, we mostly used the same individuals sampled for Baldassarre et al. (2014). However, for the two sampling locations within the plumage transition zone, Donkey Farm and Moomin, we sampled additional birds to ensure that we captured the full range of plumage color found within that zone.

Whole-genome resequencing and variant discovery

We sequenced whole genomes of 32 red-backed fairywrens (described in *Sample collection and plumage color quantification* above). Genomic DNA was extracted from each sample using DNeasy blood and tissue extraction kits (Qiagen, California, USA) and DNA concentrations were determined using a Qubit fluorometer (Life Technologies, California, USA). We used 200 ng of DNA from each sample to prepare individually barcoded libraries with a 550 bp insert size following the protocol for the TruSeq Nano DNA Library Prep kit (Illumina, California, USA). The libraries of the 20 plumage transition zone birds were pooled and then sequenced together on two lanes of an Illumina NextSeq500 (2×150 bp) at the Cornell University Biotechnology Resource Center. The remaining 12 libraries of the two al-

lopatric population birds were sequenced on a third lane of an Illumina NextSeq500 lane (2×150 bp). We concatenated the sequences for the plumage transition zone birds from the two lanes, and then assessed the quality of all samples using FastQC v. 0.11.5 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Unfortunately, a catastrophic data loss on a server resulted in the loss of one read pair for an orange individual from the transition zone (labeled or5). Therefore, this individual has the lowest average sequencing depth of all the individuals in this study. We subsequently performed trimming, adapter removal, and initial quality filtering using AdapterRemoval v. 2.1.1 (Schubert et al., 2016). We required a minimum Phred quality score of 10 and merged overlapping paired-end reads. Filtered sequences were aligned to the red-backed fairywren genome (Khalil et al., 2023) using Bowtie2 v. 2.2.8 (Langmead & Salzberg, 2012) with the very sensitive, local option.

All resulting SAM files were converted to BAM files, sorted and indexed using SAMtools v. 1.3 (Li et al., 2009), and PCR duplicates marked using Picard Tools v. 2.8.2 (<https://broadinstitute.github.io/picard/>). Variant discovery and genotyping for the 32 red-backed fairywrens were performed using the haplotypcaller module in GATK and GenotypeGVCFs tools (McKenna et al., 2010) using default parameters. We used the following hard filtering parameters to remove variants from the output file: $QD < 2.0$, $FS > 60.0$, $MQ < 30.0$, and $ReadPosRankSum < -8.0$. Subsequently, we filtered out variants that were not biallelic, had a minor allele frequency less than 5%, had a mean depth of coverage less than 2X or greater than 50X, or had more than 25% missing data across all individuals in the dataset using VCFtools version 0.1.16 (Danecek et al., 2011). This pipeline produced 26,659,515 single nucleotide polymorphisms (SNPs) genotyped across all 32 individuals.

Population genomic analysis

We visualized genetic clustering of the whole-genome dataset by performing a principal component analysis (PCA) using the “snpgdsPCA” function in the SNPRelate package (Zheng et al., 2012) in R v.4.2.3, calculating six principal components (PCs).

We characterized genome-wide patterns of divergence between allopatric red-backed fairywren subspecies, and between the red and orange plumage transition zone birds, by calculating Weir and Cockerham’s F_{ST} using VCFtools (Danecek et al., 2011) in 25 kb non-overlapping windows and at individual SNPs. We also calculated nucleotide diversity (π) and Tajima’s D for each of these four groups of birds across 25 kb windows. We used VCFtools to calculate Tajima’s D , and pixy (Korunes & Samuk, 2021) to calculate unbiased π by including invariant sites to account for missing data. To further reduce bias in our estimates, we only retained scaffolds with more than two windows and windows that contained more than 10 SNPs. We capped negative F_{ST} windows to zero when calculating weighted mean genome-wide F_{ST} .

In order to place red-backed fairywren scaffolds on chromosomes, we used SatsumaSynteny (Grabherr et al., 2010) to align all scaffolds to the *Taeniopygia guttata* genome (taeGut3.2.4; Warren et al., 2010) with the pseudochromosome command and default parameters. We visualized the results of the F_{ST} estimates on a chromosome

level by matching scaffold-level windows to the pseudo-chromosomes and plotting using the “manhattan” function in the qqman package in R v.4.2.3.

Characterizing the genomic landscape of introgression

To quantify which areas of the genome had introgressed from the *M. m. cruentatus* genome into the plumage transition zone, we computed the f^d statistic over 25 kb windows following Martin et al. (2015); this statistic ranges from 0 (no introgression) to 1 (complete replacement). f^d is a modified version of the D statistic that is more reliable when effective population size is low, can be used on windows (whereas D statistic should only be used to estimate genome-wide levels of introgression), and is overall a robust approach for identifying introgressed loci (Martin et al., 2015). We used the parseVCF.py script to prepare data for this analysis and the ABBABABAWindows.py script to estimate f^d across the genome. All scripts are from Martin et al. (2015) and are available at https://github.com/simonmartin/genomics_general.

We computed the f^d statistic using two combinations of populations: in both, we set allopatric *M. m. melanocephalus* as P1, allopatric *M. m. cruentatus* as P3, and a white-shouldered fairywren (*M. alboscapulatus*) individual as P4. For the first combination, we set all the plumage transition zone birds (20 individuals) as P2, and in our second combination we set only the red plumage transition zone birds (10 individuals) as P2. Both combinations were qualitatively similar, and moving forward, we report f^d statistics for the combination with only red plumage transition zone birds as P2. Significant peaks in f^d are representative of regions in red plumage transition zone birds that are more similar to allopatric *M. m. cruentatus* (red) birds than to allopatric *M. m. melanocephalus* (orange), suggesting directional introgression of genomic regions from *M. m. cruentatus* into plumage transition zone birds.

Identification and functional characterization of candidate genes

We first identified 25 kb windows in the genome that were elevated in the plumage transition zone comparison (orange vs. red individuals)—these included windows that were in the 99th F_{ST} percentile as well as the 25 kb window that was directly upstream and downstream of those windows (639 windows in total, Table S2). We identified areas of the genome as candidate color peaks if they had elevated F_{ST} in the plumage transition zone comparison, and were also elevated in the allopatric comparison (102 windows shared, Table S3). We compiled a list of genes using the annotation of our reference genome (Khalil et al., 2023) within these candidate peaks for color. To characterize putative candidate genes, we identified functional and ontology information from the Uniprot database (The UniProt Consortium et al., 2025). We additionally compared the identified list of genes to those known to be involved in carotenoid pigmentation and genes or gene families identified in recent analyses of pigmentation in other bird species (Price-Waldman & Stoddard, 2021; Toomey et al., 2022).

Amplicon sequencing, variant discovery, and principal component analysis

To test whether loci were linked to plumage variation across the species range, we designed primers to sequence one hundred and twenty 300-base pair amplicons that included both top candidate loci for color differentiation identified in the whole-genome resequencing comparisons, as well as other candidate loci based on highly differentiated regions near putative color genes. Top candidate amplicons contained at least one fixed SNP between our allopatric populations in or near a putative color gene (either in the 3'UTR, exon, or coding region of the gene based on the annotation of our reference genome). Primers were designed using the primer design tool in Geneious Prime 2020.2.3 to amplify a 300 bp region including the variant of interest within the first 150 base pairs (Table S4). Primers were designed to be approximately 25 bp long with a melting temperature of around 60 °C. Adapter overhang sequences were added to the forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]) and reverse (5'-GTCTCGTGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]) primer sequences. These allowed for addition of the Illumina Nextera barcodes in a second round of PCR. Primers were ordered from Integrated DNA Technologies, and primer sequences and which SNP they targeted can be found in Table S4. Primers were resuspended to a concentration of 250 μ M.

We sequenced amplicons for 285 red-backed fairywrens across 16 populations throughout the species range (Figure 1). Genomic DNA was extracted from each sample using DNeasy blood and tissue extraction kits (Qiagen, California, USA) and DNA concentrations were determined using a Qubit fluorometer (Life Technologies, California, USA). We amplified all the amplicons for each DNA sample in a single multiplex PCR by preparing a single primer mix containing all 240 primers each at a final concentration of 0.7 μ M. First round of PCR was performed using the Qiagen Multiplex PCR Plus Kit (206,152, Qiagen) with 0.3 μ l of the above primer mix and 20–40 ng of DNA in a final volume of 11 μ l. Initial denaturation at 95 °C for 5 min was followed with 35 cycles of 95 °C for 30 s, 62 °C for 90 s, and 72 °C for 30 s and a final extension step of 68 °C for 10 min. A 10-fold dilution of these products was used as the template for a second round of PCR to add the Nextera i5 and i7 index primers. This second round of PCR was performed in a total volume of 11 μ l with 2 μ l of the diluted PCR product, 0.5U Platinum™ Taq DNA Polymerase (ThermoFisher, 10966-034), 1 X PCR Buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 μ M of i5 and i7 primer. Reaction conditions are as follows: initial denaturation at 95 °C for 2 min, followed by seven cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. This was followed by 95 °C for 10 s, 55 °C for 3 min (with a 0.1 deg/s ramp), 72 °C for 30 s, and a final extension step of 72 °C for 5 min. PCR products were then pooled equally and cleaned using 1:1 ratio of SPRI beads (made using Sera-Mag Speed-Beads [Fisher Scientific, 09-981-123] following methods in Rohland & Reich, 2012). This library was then sequenced by the Cornell Institute of Biotechnology Resource Center on an Illumina MiSeq instrument (single end 150 bp).

We removed adapters from sequences using Cutadapt version 2.1 (<https://cutadapt.readthedocs.io/en/stable/#>), and then assessed the quality of all samples using

FastQC v. 0.11.5 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). We subsequently trimmed reads down to 300 bp using Cutadapt and performed quality filter using `fastq_quality_filter` (FASTX-Toolkit). For a sequence to be kept, we required that 100% of the bases in a sequence must have a Phred score of at least 20, and 95% of the bases in a sequence must have a Phred score of at least 30.

These filtered reads were then aligned to a reference consisting of the predicted amplified regions (based on the red-backed fairywren genome) using Bowtie2 version 2.2.8 with the very-sensitive-local option (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>). All resulting SAM files were converted to BAM files, sorted and indexed using SAMtools version 1.3 (Li et al., 2009). Variant discovery and genotyping for the 285 individuals were performed using the `mpileup` command from bcftools (SAMtools version 1.3). We filtered the VCF to only include the SNPs that were fixed in our whole-genome analysis (319 SNPs across 120 amplicons), and we found that there were 108 amplicons present in the VCF. Eight of the remaining amplicons never amplified; however, the remaining four had amplified, but did not have SNPs in the same position as the whole-genome data. To retain as many amplicons as possible, we added back those 4 amplicons to our dataset, which resulted in 267 SNPs across 112 amplicons in total. We removed individuals with a mean depth of less than 2, and filtered sites with more than 50% missingness, which resulted in retaining 192 SNPs across 85 amplicons and 281 individuals. Because SNPs from the same amplicon are likely linked, we retained only one SNP per amplicon, and kept the SNP with the least missingness, and then removed SNPs that were not biallelic, resulting in 78 amplicons in our final dataset (Table S7). We extracted allele frequencies of each SNP per population using `plink2` (<https://www.cog-genomics.org/plink/2.0/>).

We visualized genetic clustering of the ampli-typed SNP dataset by performing a PCA using the “`snpGdsPCA`” function in the `SNPRelate` package (Zheng et al., 2012) in R v.4.2.3, calculating six PCs. To assess whether there was a relationship between PC score of the amplicon data and individual plumage hue, we ran a linear model for the 147 individuals with hue data using the `lm` function in R, between the score for PC1 and the hue value (θ).

Geographic cline analysis

To identify SNPs that had introgressed alongside plumage color, we fit our amplicon SNP data to geographic cline models (Barton & Hewitt, 1985) using the Metropolis–Hastings Markov chain Monte Carlo algorithm in the package `HZAR` (Derryberry et al., 2014) R v.3.6.0. For each SNP, we evaluated nine cline models, each having one of three exponential decay curve (tail) parameters (neither tail, mirrored tails, or both tails), and one of three allele frequency estimates (none—no estimate, free—estimate, and fixed—fix them at 0 and 1). These nine models were then compared using AIC corrected for small sample size (AICc). For each trait, the model with the lowest AICc score was selected as the best-fitting model. All models estimated cline center (distance from sampling location 1, c) and width ($1/\text{maximum slope}$, w).

Patterns of asymmetric introgression are often visualized by comparing cline centers of a morphological trait cline to the neutral genetic transition between species, where if

a trait were under directional selection, it would be shifted from the neutral genetic cline (Brumfield et al., 2001). To assess whether specific SNPs have asymmetrically introgressed, we compared our amplicon SNP clines to both the neutral genetic cline and morphological plumage hue cline. We chose to compare our amplicon SNP clines to the Hybrid Index (Q) genetic cline from Baldassarre et al. (2014) because it was estimated using a neutral genotype-by-sequencing (GBS) dataset, whereas the amplicons we selected are unlikely to be evolving under a neutral model given their association with plumage color and divergence between fairywren subspecies. We therefore also compare our amplicon SNP clines to the morphological plumage cline from Baldassarre et al. (2014) to remain consistent in these comparisons. To assess whether any two given clines were coincident (i.e., overlap in their centers), we compared their range of two-log-likelihood support for estimated center values (hereafter CIs).

Genotype–hue correlations

We extracted genotype values for each SNP of our amplicon data for each individual using the `-O12` flag in `VCFtools`. This provided information about whether each individual was homozygous for the reference or alternative allele, or heterozygous. To test whether candidate SNPs were associated with plumage hue, we ran linear models comparing the genotype score (0, 1, or 2) to individual plumage hue (θ) using the “`lm`” function in R.

Results

Whole-genome sequencing of 32 red-backed fairywren individuals resulted in an average of 70 million reads per sample with an average of 91% mapped reads (range = 88.4%–93.3%), and a mean sequencing depth of 9.4X (range = 5.6X–12.8X, Figure S1). Full mapping statistics can be found in Table S5. Variant calling resulted in approximately 26.5 million SNPs distributed across the genome.

Genetic structure of the plumage transition zone

M. m. cruentatus, *M. m. melanocephalus*, and plumage transition zone individuals each clustered separately in a PCA of all SNP variants, and as expected all plumage transition zone individuals clustered together regardless of plumage color (Figure 1B, see also Baldassarre et al., 2014). Overall genomic divergence between the red and orange individuals within the plumage transition zone was extremely low (weighted genome-wide $F_{ST} = 0.003$, highest per-SNP $F_{ST} = 0.87$) as anticipated in this admixed population, but we identified several regions with high F_{ST} relative to the background (Figure 2). On a broader geographic scale, genomic divergence between the allopatric individuals (*M. m. cruentatus* vs. *M. m. melanocephalus*) was substantially higher (weighted genome-wide $F_{ST} = 0.106$, highest per-SNP $F_{ST} = 1$), with more regions identified with high F_{ST} estimates relative to the background (Figure 2).

We expected that highly divergent genomic regions that are shared between the phenotype contrast (red vs. orange within the plumage transition zone) and the allopatric population contrasts contain candidate loci linked to the color variation. To identify these windows of interest, we first

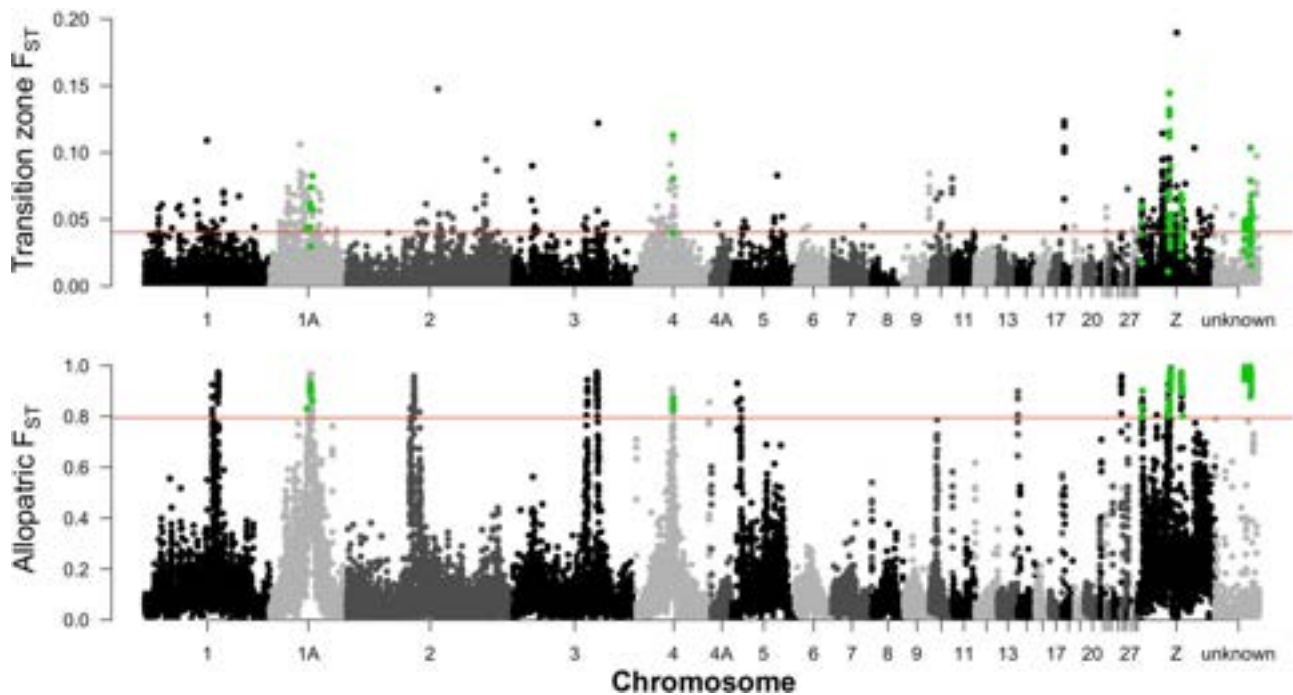


Figure 2. Genome-wide divergence between plumage transition zone orange and red individuals, and between allopatric red and orange populations. Manhattan plots depict the genomic landscape (F_{ST}) in non-overlapping 25 kb windows for each comparison. Red lines represent the 99th percentile of F_{ST} windows for each comparison. Green points represent the windows that are in the 99th percentile of the hybrid zone comparison (\pm the window directly up and downstream), which were also in the 99th percentile of the allopatric population.

identified the elevated F_{ST} windows in the plumage transition zone comparison, and then filtered this list to only include windows that were also elevated in the allopatric comparison. This resulted in 66 windows that mapped to three chromosomes (1A, 4, and Z), and 36 windows that mapped to unplaced scaffolds (Table S3). We identified 32 genes within these windows, 21 of which had orthology with other known proteins (Table S6).

Phenotype-genotype validation

To further validate whether loci in these elevated regions were linked to plumage variation across the species range, we sequenced amplicons that included the set of top candidate loci for color differentiation identified in the whole-genome resequencing comparisons in individuals from 16 populations across the species range. After sequencing and filtering, we retained 78 amplicons across 281 individuals with a mean sequencing depth of 84X (Table S8 and Figure S2), and we had plumage hue information for 147 of those individuals. We ran a PCA on these data (Figure 3A) and recovered a significant relationship between PC1 and individual hue ($p < 0.001$, adj $R^2 = 0.36$, Figure 3B), consistent with our sampling of loci associated with color differentiation.

To identify loci that underlie the introgressed plumage color across the species range, we ran geographic cline analyses for each SNP and compared these clines to the neutral genetic cline and plumage clines (Baldassarre et al., 2014). We found that 50% (39 SNPs) were concordant with the genetic cline, 18% (14 SNPs) had clines concordant with or extending further than the plumage cline, 6% (5 SNPs) did not match either cline, and 26% (20 SNPs) had SNPs that were not fixed in the two farthest allopatric populations

and could not be used in cline analysis (Figure S3). Though we used fixed SNPs identified in our whole-genome sequencing data to develop our amplicons for sequencing, the most western population used in our cline analysis was Mornington (population 1), while we sequenced individuals from Darwin (population 2) for our western allopatric population for the whole-genome sequencing. Therefore, it is possible that some SNPs that were fixed in population 2 were not also fixed in population 1, which would lead to the result of 20 unfixed SNPs appearing in the amplicon dataset. Cline and CI overlap statistics can be found in Table S7. We interpret concordant SNPs and SNPs that extend beyond the plumage cline center together, as the degree of concordance with the plumage cline will depend on interactions among loci and whether loci are dominant or additive. Of the 14 SNPs concordant with or past the plumage cline, 8 SNPs were in genes that had functions putatively associated with carotenoid coloration (fatty acid synthesis, Wnt signaling, and BMP signaling) or visual perception. These genes included *FASN* (*Fatty Acid Synthase*, 3 SNPs), *CSNK1D* (*Casein kinase I isoform delta*, 3 SNPs), *RGMB* (*Repulsive guidance molecule B*, 1 SNP), and *GRM8* (*Metabotropic glutamate receptor 8*, 1 SNP) (Figure 4A–D). We also compared the genotype of individuals to the plumage hue of those individuals, and found a significant association between genotype and hue score in all seven of those SNPs (Figure 4E–H, statistics reported in Table S7).

Testing for selection on putative color genes

To further assess whether *FASN*, *CSNK1D*, *RGMB*, or *GRM8* were associated with sexually-selected color variation, we searched for evidence of selection in the regions that contained these four genes using our whole-genome

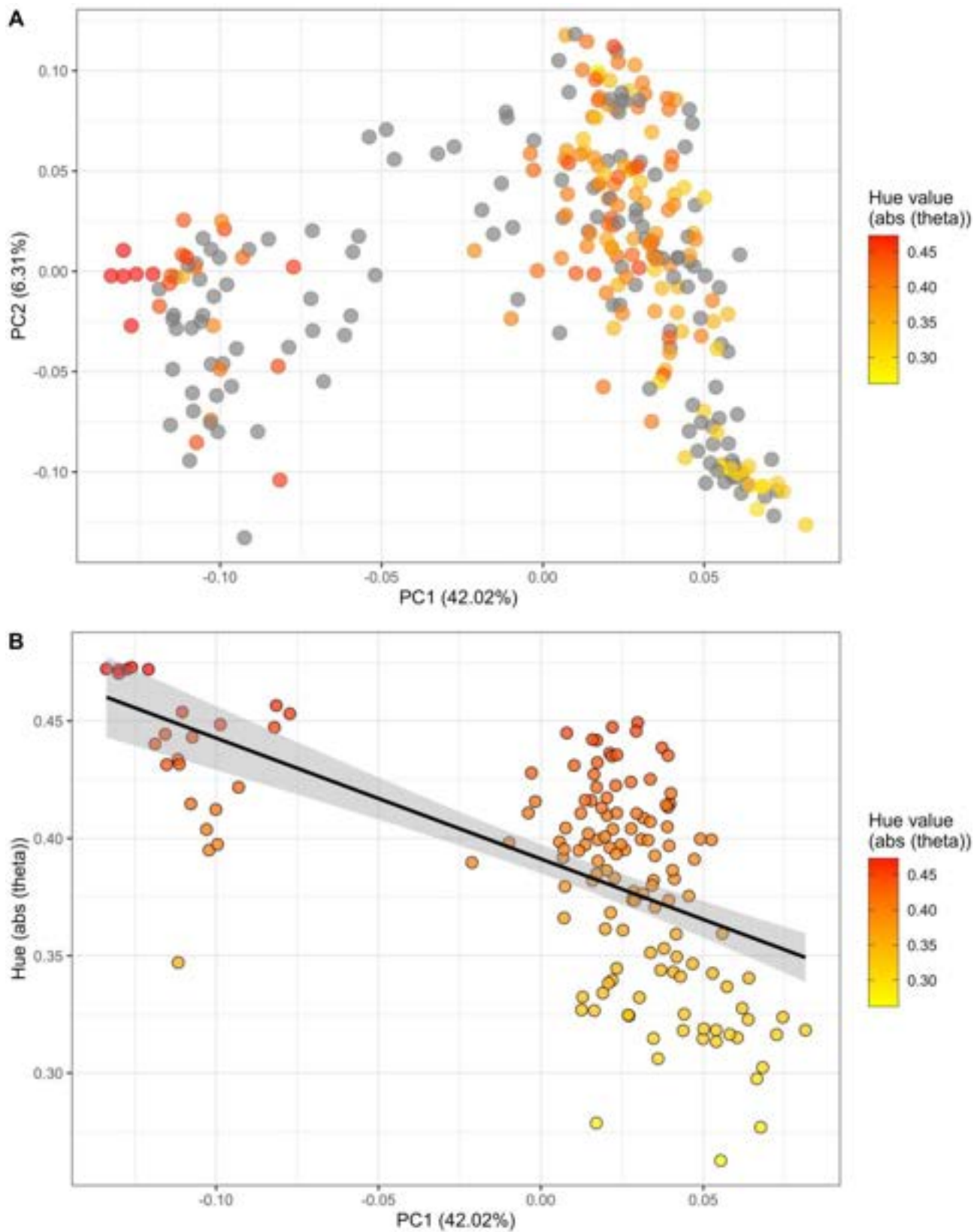


Figure 3. A. Principal component analysis (PCA) of genotype data of 78 amplicons, with 1 SNP per amplicon, for 281 individuals across the transect. Points are colored by back hue for the 147 individuals we have hue information for. Values for plumage hue have been converted to absolute values for visualization purposes. Gray points are individuals without individual hue information. **B.** Scatter plot of individual hue score of the 147 individuals as a function of PC1 score from above PCA, with the line of the linear model described in text.

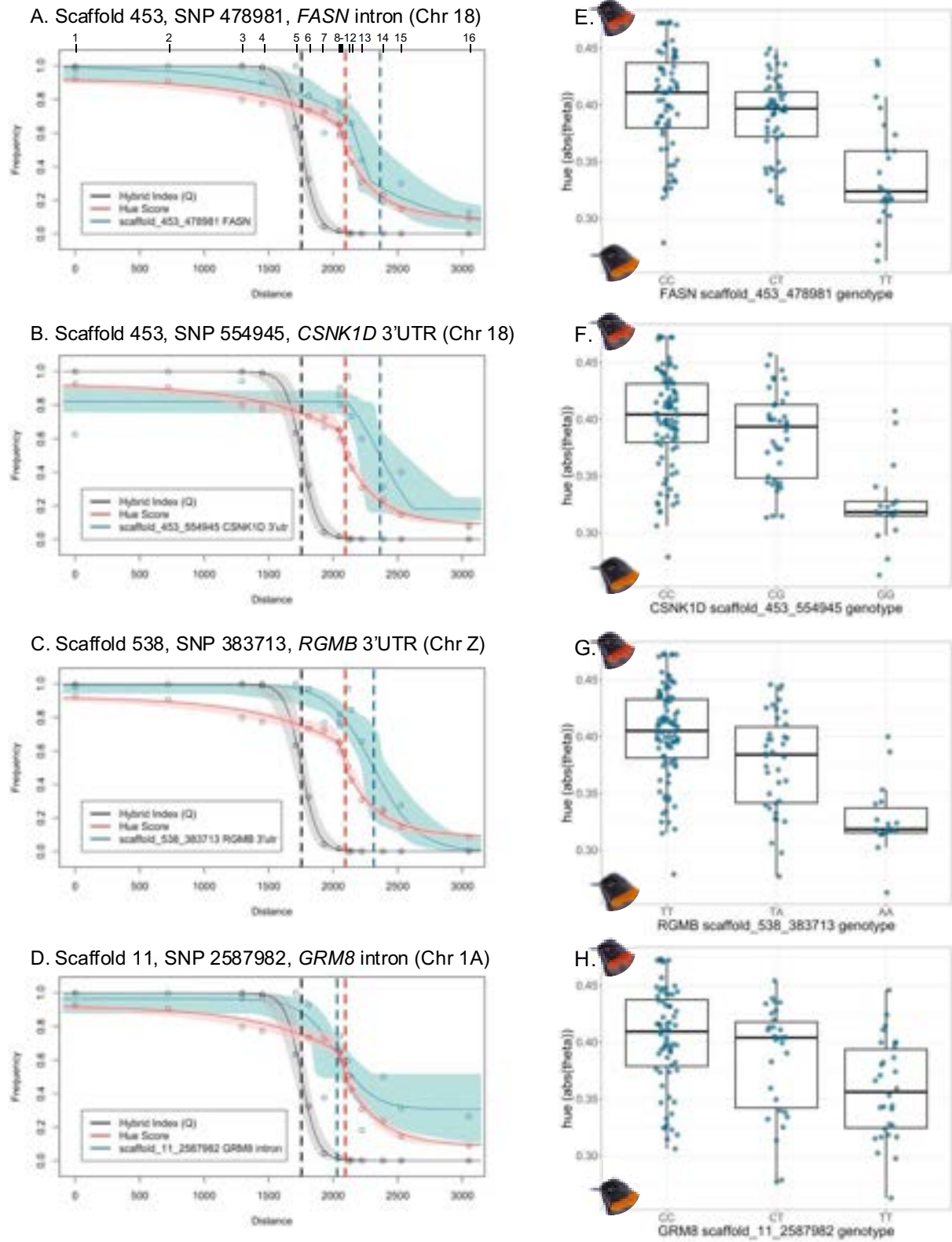


Figure 4. A–D. Maximum likelihood geographic clines and confidence intervals for the hybrid index (black), and plumage hue (red), and an amplicon SNP in the genes *FASN* (A), *CSNK1D* (B), *RGMB* (C), and *GRM8* (D). E–H. Individual hue value as a function of genotype for the same SNPs in A–D. Values for plumage hue have been converted to absolute values for visualization purposes, so higher values for hue correspond to redder plumage color. Fairywren illustrations by Jessica French.

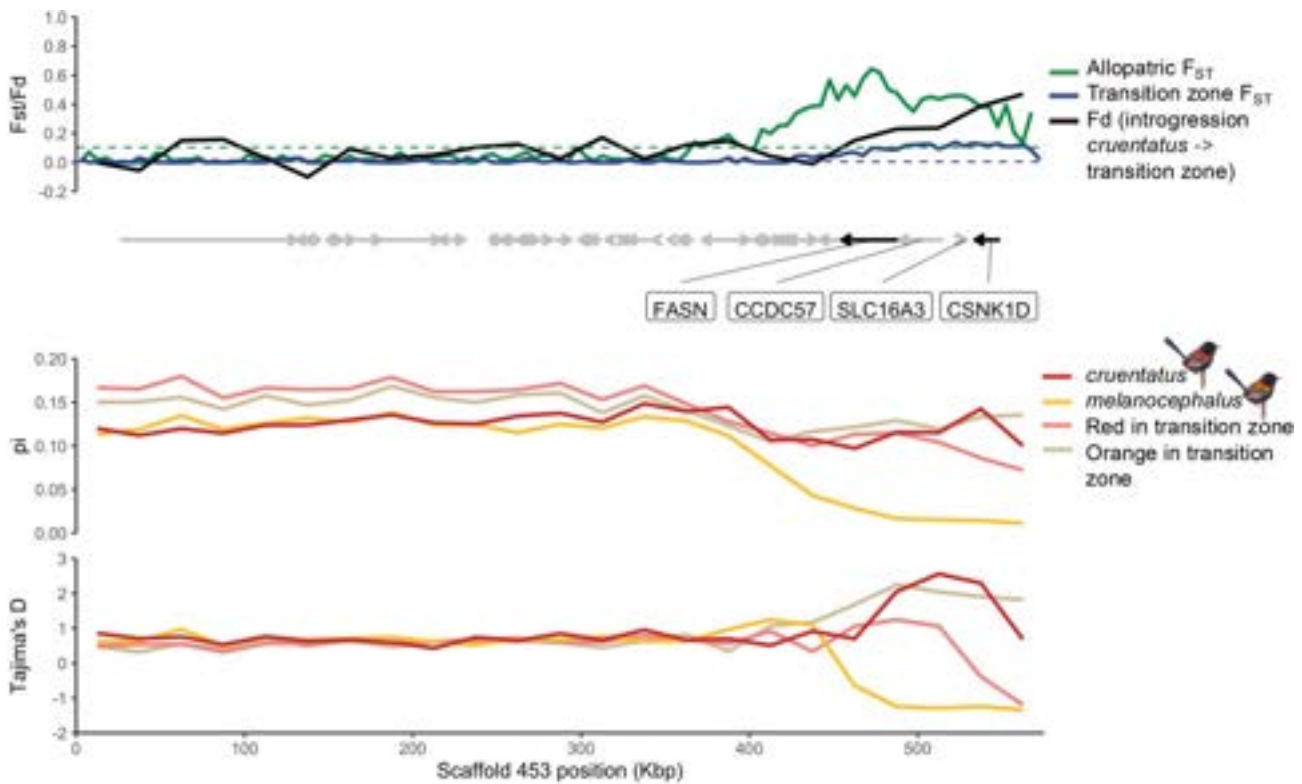


Figure 5. Windowed summary statistics along scaffold 453. Plot shows F_{ST} for the allopatric population comparison (green) and the plumage transition zone comparison (blue) in 5 kb windows, with the global background F_{ST} value represented by the green and blue dashed lines. F_d (black) represents the introgression statistic from *M. m. cruentatus* to the transition zone birds in 25 kb windows. All annotated genes are shown by the gray arrows, with the two genes of interest, *FASN* and *CSNK1D* in black. Nucleotide diversity (π) and Tajima's D in 25 kb windows are shown for each subspecies and for the red and orange plumage transition zone individuals separately at the bottom of the plot. Fairywren illustrations by Jessica French.

sequencing data. Because previous work has found experimental evidence for female preference of red males over orange males (Baldassarre & Webster, 2013), we predicted that loci responsible for color production should be under positive selection. We found that the region containing *FASN* and *CSNK1D* had elevated f^d (where higher values of f^d indicate higher levels of introgression) and F_{ST} in both the allopatric and plumage transition zone comparisons, with a paired reduction in nucleotide diversity in all groups and a negative Tajima's D value in *M. m. melanocephalus*, indicative of positive selection on this region in the orange population (Figure 5). In contrast, Tajima's D was positive in this same region in *M. m. cruentatus*, which can indicate either balancing selection or a recent population contraction. The region containing *GRM8* did not show any clear pattern in f^d ; however, nucleotide diversity was reduced in that region in the allopatric red *M. m. melanocephalus* population, as well as Tajima's D in the region just upstream of the gene (Figure S4). There were insufficient data to calculate nucleotide diversity or Tajima's D in the region containing *RGMB*.

Discussion

Understanding the genetic architecture of traits under sexual selection is a fundamental goal in evolutionary biology, as it can help explain the constraints and processes that shape the expression and evolution of these traits. Further, uncovering this genetic basis provides a clearer picture of emer-

gent evolutionary processes including, in this case, patterns of introgression that underlie trait dynamics in an avian hybrid zone. In this study, we provide evidence that selection has led to the adaptive introgression of specific genes underlying plumage color in the red-backed fairywren, driven at the behavioral level by female preference for redder plumage in this species (Baldassarre & Webster, 2013). Our whole-genome analyses indicate that while allopatric populations of the red-backed fairywren subspecies are genomically distinct from each other, individuals within the plumage transition zone are very similar to each other, even when they differ in plumage hue. This genome-wide similarity supports the scenario identified earlier using reduced-representation genomic data (Baldassarre et al., 2014) that there has been a substantial history of multi-generation hybridization within a recently established contact zone (Lee & Edwards, 2008), with little or no selection against hybrids. In the context of genomic exploration, this within-population homogenization in the hybrid zone allowed us to choose individuals with similar overall genotypes but with differences in hue approaching those seen among individuals from allopatric populations, thereby increasing our power to identify loci contributing to color differences.

Genomic divergence is low despite phenotypic differences

In contrast to the genomic similarity of individuals within the plumage transition zone, where red versus orange individuals had an extremely low global F_{ST} estimate,

genome-wide differentiation was much higher between the red and orange allopatric populations, with many regions of high divergence across the genome. We suspect that many of these divergent regions among allopatric populations were most likely driven to fixation by genetic drift or are associated with local adaptation not related to plumage color. If we had only the allopatric populations to compare, it would likely not be possible to discern which of these many differentiated regions were associated with the plumage color variation that is the likely target of female choice in the hybrid zone. Therefore, we predicted that regions showing high divergence in both the plumage transition zone comparison of red versus orange individuals, and between the allopatric populations that likewise differ in this color trait, would contain genes associated with plumage color differences.

Previous cline analyses using a neutral genetic dataset found that the plumage cline is discordant from the genetic cline (Baldassarre et al., 2014), suggesting that plumage color has introgressed from west to east across the hybrid zone (i.e., red plumage color is moving into populations with an orange genomic background). This geographic discordance between the cline for a sexually-selected trait from the genome-wide cline enhanced our ability to discern which loci are associated with plumage color, since the clines for associated loci should match the plumage cline but not the average genomic cline. Comparisons of our amplicon data identified 14 SNPs that were concordant with (or even farther east than) the plumage cline, suggesting that these SNPs may be associated with plumage color differences and introgression across the red-backed fairywren species range; 44 other amplicon loci were not concordant with the plumage cline, excluding them as candidates for contributing to observed feather color variation.

Putative color genes have introgressed alongside plumage hue

The red and orange feathers in male red-backed fairywrens are produced using carotenoid pigments (Khalil et al., 2023; Rowe & McGraw, 2008), and circulating levels of carotenoids are associated with flexible within-population phenotype differences, where ornamented males with red plumage have much higher levels of circulating carotenoids in their blood than do unornamented males with brown plumage (Khalil et al., 2020). Therefore, we predicted that many of the genes that have adaptively introgressed across the hybrid zone would have functions associated with carotenoid-based plumage production (gene ontology biological functions including fatty acid synthesis, Wnt signaling, and BMP signaling). We identified three clearly differentiated genes that matched these criteria: *FASN*, *CSNK1D*, and *RGMB*. As its name suggests, *FASN* has multiple biological functions related to fatty acid synthesis, including ether lipid biosynthetic processes, fatty acid biosynthesis, and fatty acid metabolism. These processes may be important for carotenoid synthesis since carotenoids are lipophilic and are structurally similar to lipids, and it has been suggested that lipid-associated genes may be co-opted for carotenoid production (Parker, 1996; Trams, 1969). For example, other genes involved in carotenoid processing in birds were initially described in humans as having lipid functions (Toomey et al., 2017). Furthermore, an experiment

where the liver transcriptome of the southern red bishop (*Euplectes orix*) was expressed in carotenoid-synthesizing bacteria found that the expression of *FASN* led to a color change in those bacteria colonies, suggesting a possible link between this gene and carotenoid-based color expression (Pointer et al., 2012). Taken together, these lines of evidence imply that *FASN* may function in mediating carotenoid coloration in red-backed fairywrens.

CSNK1D has multiple biological processes related to Wnt signaling, including the positive regulation of both the canonical and non-canonical Wnt signaling pathways. The Wnt signaling pathway appears to function in feather growth (Chang et al., 2004; Xie et al., 2020b), including specifically in carotenoid-based feathers (Xie et al., 2020a), and therefore *CSNK1D* may contribute to carotenoid-based feather production in red-backed fairywrens. Finally, *RGMB* is involved in the BMP signaling pathway, which, similar to the Wnt signaling pathway, has been shown to be important for feather development (Patel et al., 1999). In addition, *RGMB* has been found to be associated with red earlobe color in chickens, a carotenoid-based trait (Nie et al., 2016). Though none of these three genes have been tied to carotenoid production specifically, the known connections between their functions and pathways to color production in several other bird species suggest that they are similarly involved in feather pigmentation in red-backed fairywrens.

We also found a SNP in the gene *GRM8* to be highly concordant with the plumage cline. Though this gene serves many biological functions, its known connection to visual perception (Wu et al., 1998) may explain its tight correlation to plumage color in these fairywrens, as visual perception has been shown to have important evolutionary consequences for plumage traits. For example, zebra finch (*Taeniopygia guttata*) females perceive and assess male carotenoid-based traits categorically even though there is continuous variation in the trait within the species, which can have implications for signal evolution (Caves et al., 2018, 2020, 2021). If *GRM8* affects visual perception of red colors in female red-backed fairywrens, it may affect their ability to discern color differences and choose redder males to breed with, and therefore promote the introgression of red color across the species range.

Putative color genes are under selection

Because previous work has found experimental evidence for female preference of red red-backed fairywren males over orange ones (Baldassarre & Webster, 2013), we predicted that loci responsible for color production should be under positive selection and therefore searched for evidence of selection in the regions containing *FASN*, *CSNK1D*, *RGMB*, and *GRM8*. To test for signatures of selection, we estimated nucleotide diversity (π) and Tajima's D values, in addition to $f^{\prime}d$, a measure of genomic introgression, specifically looking at the level of introgression between the red plumage transition zone individuals and the allopatric *M. m. cruentatus* individuals.

We found evidence for positive selection in the region containing *FASN* and *CSNK1D*, which had elevated $f^{\prime}d$ and F_{ST} in both the allopatric and plumage transition zone comparisons, with a paired reduction in nucleotide diversity and a negative Tajima's D value in orange *M. m. melanocephalus*.

In contrast, Tajima's D is positive in this same region in red *M. m. cruentatus*, which can indicate either balancing selection or a recent population contraction. Two of the *FASN* SNPs whose clines were concordant with the plumage cline were in intronic regions, and one was in an exon (Table S7), though that SNP led to a synonymous change in the protein coding sequence. Although synonymous changes have traditionally been thought to be selectively neutral, recent work has suggested that many synonymous changes are under selection, particularly in genes with high expression levels (Perlmutter et al., 2021; Rahman et al., 2021). In addition, synonymous changes can also lead to different splice variants of mRNA, leading to a functional change in proteins (Mueller et al., 2015) and synonymous mutations have been associated with carotenoid pigments (Enbody et al., 2021). Alternatively, the SNPs selected for the amplicon panel may be in linkage disequilibrium with the causal loci for color variation. Balancing selection in the allopatric *M. m. cruentatus* population, as suggested by the positive Tajima's D , seems unlikely since the variation of hue in this population is limited, and we do not have the demographic data to suggest or rule out a change in population size. Either way, the opposing pattern of Tajima's D in the different subspecies provides an intriguing entry point to further investigate genomic signatures of selection putatively associated with female-choice-driven sexual selection of plumage. That f^d was also elevated in this region suggests directional introgression of this region from *M. m. cruentatus* into the plumage transition zone. Considered in concert, these lines of evidence suggest that variation in *FASN* contributes to, at least in part, the color variation in this species and that this locus may be a proximate genetic mechanism for the observed plumage introgression pattern. There were insufficient data to calculate nucleotide diversity or Tajima's D for *RGMB*, and we therefore have no evidence of selection on this region. The data for *GRM8* are potentially suggestive of selection on this region, but the signals (F_{ST} and π) are somewhat equivocal.

In summary, our genomic comparisons across a plumage transition zone show evidence for adaptive introgression of a sexually-selected trait, and identify at least part of the genetic architecture of this introgression. Impressively, recent genomic studies have shown that hybrid zone systems where color traits appear to be discordant with genetic clines are not rare (Hooper et al., 2024; Lim et al., 2024; Semenov et al., 2021), and leveraging these systems will continue to be important to identify the proximate mechanisms of trait introgression and selection dynamics around these traits (Jofre & Rosenthal, 2021). In this study, we have built upon previous work surrounding the evolution of color in red-backed fairywrens (Baldassarre & Webster, 2013; Baldassarre et al., 2014), identified specific SNPs and their associated genes that may underlie the plumage color introgression, and found evidence that these regions are under selection. We are particularly intrigued by the data for differentiation in *FASN*, a gene with now multiple lines of evidence for its putative function in carotenoid processing in this species and possibly other birds (Pointer et al., 2012). Identifying additional putative carotenoid processing genes like *FASN* furthers our understanding of how different evolutionary processes have led to the diversity in this important signaling modality. In addition, the color differences found

in the red-backed fairywren are continuous and subtle, unlike the discrete color or pattern differences studied in many other study systems used to identify genes underlying trait color (Uy et al., 2016), even in those focusing on carotenoid-based coloration (Brelsford et al., 2017; Enbody et al., 2021; Toews et al., 2016; Wang et al., 2020). Continuous phenotypic variation in color and other ornamental traits is common in natural systems, and this study demonstrates how traits with subtle variation can be shaped by sexual selection.

Supplementary material

Supplementary material is available online at [Evolution](#).

Data availability

The genomic raw data are available on NCBI under Project PRJNA1270078.

Author contributions

S.K., J.W., E.D.E., and J.K. contributed to conceptualization of the study. S.K., D.T.B., M.S.W., and J.K. contributed to data collection. S.K. conducted data analysis with input from J.W. and E.D.E. S.K. produced the first draft of the manuscript, and J.W., E.D.E., D.T.B., M.S.W., and J.K. contributed critically to drafts.

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Conflict of interest

The authors declare no conflict of interest.

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