Spread of yellow bill color alleles favored by selection in the long-tailed finch hybrid system

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SUMMARY

Carotenoid pigments produce the yellow and red colors of birds and other vertebrates. Despite their importance in social signaling and sexual selection, our understand of how carotenoid ornamentation evolves in nature remains limited. Here, we examine the long-tailed finch *Poephila acuticauda*, an Australian songbird with a yellow-billed western subspecies *acuticauda* and a red-billed eastern subspecies *hecki* that hybridize where their ranges overlap. We found that yellow bills can be explained by the loss of C(4)-oxidation, thus preventing yellow dietary carotenoids from being converted to red. Combining linked-read genomic sequencing and reflectance spectrophotometry measurements of bill color collected from wild-sampled finches and laboratory crosses, we identify four loci that together explain 53% of variance in this trait. The two loci of largest effect contain the genes *CYP2J19*, an essential enzyme for producing red carotenoids, and *TTC39B*, an enhancer of carotenoid metabolism. A paucity of protein-coding changes and an enrichment of associated upstream variants suggest that the loss of C(4)-oxidation results from *cis*-regulatory evolution. Evolutionary genealogy reconstruction indicates that the red-billed phenotype is ancestral and yellow alleles at *CYP2J19* and *TTC39B* first arose and fixed in *acuticauda* approximately 100 kya. Yellow alleles have subsequently introgressed into *hecki* less than 5 kya. Across all color loci, *acuticauda* derived variants show evidence of selective sweeps, implying that yellow bill coloration has been favored by natural selection. Our study illustrates how evolutionary transitions between yellow and red coloration can be achieved by successive selective events acting on regulatory changes at a few interacting genes.

Keywords: hybrid zone, selective sweep, carotenoids, population genetics, ancestral recombination graph

INTRODUCTION

Carotenoid coloration, and particularly coloration produced by metabolized carotenoids, serves as an important signal of individual condition and species recognition in many species of fish, reptiles, and birds[1-7]. Despite the evolutionary significance of carotenoid signaling, insight into the genetics of carotenoid color variation has only emerged recently[8-10] and we still know remarkably little about how these traits evolve in nature. The carotenoid pigments used to produce red and yellow ornaments cannot be synthesized by most vertebrates *de novo* but rather are sourced from their diets[6,11,12]. Crucially, most of the carotenoids ingested by vertebrates are yellow, so red carotenoid coloration requires a metabolic conversion. In the last decade, a combination of experimental and field-based studies has identified a small set of genes essential for carotenoid ornamentation. Key among these genes are 3-hydroxybutyrate dehydrogenase 1-like (*BDH1L*) and cytochrome P450 2J19 (*CYP2J19*), which together convert dietary yellow carotenoids to red ketocarotenoids[10,13,14].

Although significant advances have been made in identifying enzymes and cofactors that control the production of carotenoid coloration, understanding of the evolution of carotenoid ornamentation remains limited. Nearly all the genes known to be associated with carotenoid color variation were first detected from experimental work with color mutants from captive populations. These studies mostly identified key genes from large effect (e.g., loss-of-function) mutations that likely have pleiotropic organism-wide effects[10,13,15] that may not reflect how adaptive evolution proceeds under natural conditions. Moreover, while work on carotenoid color variation in natural systems have validated the importance of genes first described in experimental studies (e.g., *CYP2J19*[16-20] and *BCO2*[21-23]) – and identified other candidate genes – we still know remarkably little about how color traits evolve. Outstanding topics include the respective contribution of functional versus *cis*-regulatory changes, the repeatability and polygenicity of adaptation, and the selective forces driving color evolution[8,9]. Recent methodological innovations to retain haplotype information from population resequencing data (e.g., Meier et al.[24]) and analytical advances for estimating genome-wide genealogies (e.g., Speidel et al.[25]) hold tremendous promise for studying the genetics of adaptation but have yet to be utilized in the context of carotenoid color evolution.

The long-tailed finch *Poephila acuticauda* is a songbird endemic to the northern tropics of Australia and provides a unique opportunity to study the evolution of carotenoid-based ornamental coloration in a natural system. It consists of two subspecies that diverged approximately 0.43 million years ago[26] and differ prominently in bill coloration: yellow in western subspecies *acuticauda* and red in eastern subspecies *hecki* (Figure 1A and 1B). The two subspecies form a hybrid zone at the edge of the Kimberley Plateau in Western Australia[17]. Notably, the transition from yellow to red ornamentation is displaced ~350 km to the east of the genomic hybrid zone[17,27]. While a prior admixture analysis[17] reported an association between bill color variation and genes known (e.g., *CYP2J19*) and later shown (e.g., *TTC39B*) to be involved in carotenoid processing, these links remain poorly characterized. For example, it remains untested whether the displacement observed between the centers of genomic and bill color admixture results from introgression of *acuticauda* color alleles into an otherwise *hecki* genetic background. As the long-tailed finch is amenable to both field based and experimental study, it is well positioned for an investigation into how natural and sexual selection shape carotenoid ornamentation and the role of color displays as reproductive barriers between incipient species.

Our investigation involves four steps. First, we describe the distinct carotenoid composition in the bills of each subspecies and screen for differences in the oil droplets of retinal cone cells. Second, we examine the genetic architecture of bill color variation and the respective contribution of *cis*-regulatory versus protein-coding changes. Third, we utilize geographic and genomic cline analyses to evaluate

evidence of bill color alleles introgressing between subspecies. Fourth, we leverage haplotype information from population scale linked-read sequencing to model the strength and timing of selective sweeps on loci associated with carotenoid color variation.

RESULTS

Bill color distinguishes long-tailed finch subspecies

We used UV-vis reflectance spectrophotometry to examine bill color in 948 adult wild-caught and 550 captive-bred long-tailed finches (STAR Methods). In the wild, bill color variation is geographically structured into three distinct regions: *acuticauda*-yellow populations to the west of Timber Creek, Northern Territory (15.6° S, 130.5° E); *hecki*-red populations to the east of Katherine, Northern Territory (14.5° S, 132.3° E); and admixed-orange populations across the ~250 km wide region in between[17,27,28]. We evaluated bill color variation by quantifying H3, a colorimetric variable of hue representing the midpoint wavelength between 400 and 700 nm[29]. Bill hue effectively differentiated the yellow and red bills of the two subspecies under common garden conditions by 56.5 nm (H3: *acuticauda*: 526.1 $\pm$ 5 nm [mean $\pm$ standard deviation], N = 151; *hecki*: 582.6 $\pm$ 6 nm, N = 173; Figure 1C).

We next evaluated variation in bill hue in captive-crossed first-generation (F_1) hybrids to discern broadscale genetic dominance effects between alleles from each subspecies. As a group, F_1 hybrids had substantially redder bills than would be expected if the allelic contribution of each subspecies was entirely additive (all F_1 hybrids: 567.2 $\pm$ 8 nm, N = 226; hypothetical intermediate: 554.4 nm). However, we found that F_1 hybrid females differed significantly depending on the subspecies origin of their father (*acuticauda*: 561.1 $\pm$ 7 nm, N = 66; *hecki*: 571.3 $\pm$ 7 nm, N = 58; $P < 0.005$). In birds, as females are the heterogametic sex (i.e., ZW) and inherit their Z chromosome from their fathers, this suggests that the overall Z-linked contribution from *acuticauda* is recessive to *hecki*. Indeed, F_1 females with an *acuticauda* father had significantly yellower bills than both groups of F_1 hybrid males (*acuticauda* father: 567.5 $\pm$ 7 nm, N = 51; *hecki* father: 570.0 $\pm$ 7 nm, N = 51; $P < 0.005$ for both comparisons; Figure 1D).

To evaluate bill hue in the wild, we sampled finches across a ~1500 km transect (Figure 1E) that spanned the full spectrum of color variation observed in the species (Figure 1F; see STAR Methods). Only two out of 18 populations (pops. 24 and 25) exhibited a mean bill hue like that observed in our captive crossed F_1 hybrids (Figure 1D and 1F), suggesting recombination between bill color alleles has been substantial and provides an opportunity to map the underlying genetic basis in nature.

Each subspecies has a distinct carotenoid composition in the bill

We investigated bill tissue carotenoid composition using high-performance liquid chromatography (HPLC) analysis and found highly distinctive differences between subspecies (Figure 2 and S1). We detected five primary carotenoid pigments in the yellow bills of *acuticauda*: three dietary yellow-orange carotenoids (lutein, zeaxanthin, and β-cryptoxanthin), a metabolized yellow carotenoid (anhydrolutein), and a dietary red carotenoid (lycopene). The red bills of *hecki* were predominantly comprised of six carotenoid pigments: a dietary yellow carotenoid (lutein), a metabolized yellow carotenoid (anhydrolutein), a dietary red carotenoid (lycopene), and three metabolized red carotenoids (astaxanthin, α-doradexanthin, and adonirubin). The three dietary yellow-orange carotenoids that occur at highest abundance in *acuticauda* are each the direct antecedent of one of the three red ketocarotenoids found at highest abundance in *hecki* (Figure 2C). Critically, each of these ketocarotenoids is a byproduct of the same metabolic pathway: C(4)-oxidation[30]. Altogether, this suggests that a lack of C(4)-oxidation is responsible for producing the yellow bills of *acuticauda*.

Both subspecies have red oil droplets in their retinas

We next asked whether the loss of C(4)-oxidation observed in *acuticauda* is systemic by examining another tissue known to contain ketocarotenoids: the retina. Most birds possess six subtypes of retinal cone photoreceptor, five of which consistently contain oil droplets with distinctive autofluorescence signatures resulting from their carotenoid composition[31]. The oil droplets of long-wavelength-sensitive cone cells are red because they contain ketocarotenoids, often astaxanthin[31,32], a metabolic byproduct of C(4)-oxidation[30] (Figure 2C). Using brightfield microscopy, we compared the composition of retinal cone cell subtypes of each subspecies and observed no difference in the occurrence of red oil droplet-containing single cone cells between them (Figure 2D). As *acuticauda* appears to synthesize red ketocarotenoids within the retina at some level but does not do so in the bill integument, this suggests that the difference in bill color between subspecies is *cis*-regulatory rather than protein-coding.

Genomic differentiation is concentrated on the Z chromosome and largely unlinked with bill color

We used the haplotagging approach described in Meier et al.[24] to generate whole genome linked-read (LR) sequence data for the long-tailed finch and its allopatric sister species the black-throated finch *P. cincta* (diverged 1.75 million years ago[26]). We sequenced 1133 *P. acuticauda* (both subspecies) and 96 *P. cincta* samples to a median read coverage of 1.38× with samples both individually and molecularly barcoded; see STAR Methods). Across the 1204 samples that had high molecular weight DNA available for haplotagging, we recovered a mean molecule N50 of 12.2 kbp ($\pm$ 4.1 kbp) with maximum molecule sizes averaging 106.3 kbp ($\pm$ 22.1 kbp; Table S2). Following variant calling and imputation, we retained a set of 29.3 million SNPs and observed 3.9 million fixed differences in both *Poephila* species relative to the zebra finch reference genome.

Genomic differentiation between long-tailed finch subspecies is enriched ~40-fold on the Z chromosome relative to the autosomes (median allopatric F_{ST} autosomes: 0.016; chrZ: 0.709; Figure 3A) and is largely associated with a chromosome inversion[17]. Using a set of ancestry informative markers (see STAR Methods), we identified a hybrid zone 126.9 km wide (107.8 – 148.7 km, 95% highest posterior density interval) located along the edge of the Kimberley Plateau, Western Australia (see Figure S2). Remarkably, the average bill color of hybrid zone individuals was just as yellow as individuals from allopatric populations of *acuticauda* (H3: *acuticauda* 535.2 $\pm$ 10 nm, N = 265; hybrid zone 534.7 $\pm$ 10 nm, N = 158; $P = 0.9$, Tukey's HSD test). As bill color alleles from *hecki* (red) appear in aggregate to be dominant (Figure 1D), this suggests that *acuticauda* alleles (yellow) have introgressed eastward and onto otherwise *hecki* genomic backgrounds following secondary contact (see Figure S2).

Association mapping identifies key genes linked with the biosynthesis of ketocarotenoids

We performed genome-wide association studies (GWAS), taking genetic structure into account, to identify loci underlying variation in bill hue (see STAR Methods). We found eleven association peaks that together explained 93.8% of the variance in hue (Table S5) and focus below on the top four association peaks that together accounted for 53% of variance (Figure 3B).

The SNP most significantly associated with bill hue (chr8:3144828, $P = 2.00e^{-33}$; Figure 3D) explained 21.6% of variance (β = 12.0 nm) and was located 21.9 kbp upstream of the oxidative ketolation gene *CYP2J19* within a long non-coding RNA (lncRNA). In vertebrates, *CYP2J19* is critical for the metabolic conversion of dietary yellow-orange carotenoids to red ketocarotenoids via C(4)-oxidation[10]. This variant, and another 420 bp away, are the only two autosomal SNPs with $F_{ST} > 0.95$ between subspecies and in both SNPs the derived allele originated in *acuticauda* (see STAR Methods). We mapped a further 10 SNPs above genome-wide significance within the genic domain of *CYP2J19*, all within introns, and 13 SNPs were located less than 20 kbp upstream.

The second most strongly associated SNP (chrZ:67547840, P = 1.72e^{-26}; Figure 3D) explained 18.9% of variance (β = 11.6 nm) and was located within the first intron of the oxidative ketolation enhancer gene *TTC39B*. When co-expressed with *CYP2J19* and *BDH1L*, *TTC39B* has been shown to greatly enhance the production of red ketocarotenoids[10]. This Z-linked SNP represents a fixed difference (i.e., F_{ST} = 1.0) between subspecies and the derived allele originated in *acuticauda*. A total of 53 SNPs above genome-wide significance were located within the genic domain of *TTC39B*, seven of which are 3' UTR variants and one of which is a synonymous substitution. An additional 12 SNPs above genome-wide significance were located less than 20 kbp upstream of the gene.

In the third association peak, also situated on chromosome 8, the most significant SNP (chr8:21738547, P = 5.08e^{-14}; Figure 3C) explained 6.5% of variance (β = 7.3 nm) and was located within an intron of the gene *PTPRC*. This large transmembrane glycoprotein is found on the cell surface of most hematopoietic cells and contributes to innate immune response[33]. Eleven of the 57 SNPs above genome-wide significance were located within exons, but only two encode non-synonymous substitutions (R401G and A864G), two are synonymous substitutions, and seven are in the 3' UTR. To our knowledge, there have been no direct links between this gene and carotenoids or color variation in other systems.

In the fourth association peak, situated on chromosome 2, the most significant SNP (chr2:21479975, P = 1.03e^{-13}; Figure 3D) explained 6.1% of variance (β = 8.1 nm) and was located 11.7 kbp downstream of ATP-binding cassette transporter gene *ABCB1*. Members of its gene family (*ABCA1* and *ABCG1*) have been associated with the deposition of carotenoids in the retinas of chickens[34] and the red feathers of red-backed fairywrens[20], respectively. Three SNPs above genome-wide significance were located within the third intron of *ABCB1*. A second gene of interest within this association peak, *CROT*, is involved in mitochondrial fatty acid β-oxidation. While this gene has no prior association with carotenoid color variation, its role in lipid metabolism within the mitochondria is noteworthy as carotenoid ketolation and ornamentation may be functionally linked to mitochondrial performance[35-37].

Geographic and genomic clines suggest that bill color alleles have introgressed from *acuticauda* into *hecki*

The geographic cline centers for the two SNPs most strongly associated with bill hue are located 220.4 km (*CYP2J19*: chr8:3144828, center = 868.0 km from most western population [849.7 – 885.3 km 95% HPDI]) and 373.5 km (*TTC39B*: chrZ:67547840, center = 1021 km [993.7 – 1051.0 km 95% HPDI]) to the east of the center of genomic admixture between subspecies (hybrid index, center = 647.6 km [642.4 – 652.8 km 95% HPDI]), respectively (Figure 4). While the introgressing alleles at both color loci originated within *acuticauda*, the center of the *TTC39B* cline is located a further 153 km east of the *CYP2J19* cline center (Figure 4D). Moreover, while the *acuticauda* allele of *TTC39B* was fixed or nearly fixed (from 0.86 to 1.00) within color-admixed populations, the frequency of the *acuticauda* allele of *CYP2J19* was at intermediate allele frequencies (from 0.16 to 0.65) within these same populations (Figure 4B and 4C).

Genomic cline analysis further bolsters the evidence for introgression of *acuticauda* alleles at *CYP2J19* and *TTC39B* (Figure 4E). Of the 649 ancestry informative markers used to calculate a hybrid index (STAR Methods), the three SNPs with the strongest support for introgression were all located within 38 kbp of *CYP2J19* and the most significant was the top GWAS SNP chr8:3144828 (c = 0.994, P = 5.90e^{-201}). The SNP with the next strongest support was located within an intron of *TTC39B* (chrZ:67498406, c = 1, P = 2.14e^{-71}) and significantly associated with bill hue (GWAS P = 5.92e^{-23}). Results of geographic and genomic clines showcase the introgression of yellow bill color alleles from *acuticauda* into a genomic background that is otherwise that of red-billed *hecki* (Figure 4E).

Evidence of epistasis between *CYP2J19* and *TTC39B*

A biochemical relationship between the products of *CYP2J19* and *TTC39B* has been established experimentally, which identified the latter as a potent enhancer of ketocarotenoid biosynthesis[10]. To investigate epistasis between *CYP2J19* and *TTC39B* in the long-tailed finch, we evaluated variation in bill hue (H3) for individuals carrying zero, one, or two copies of the *acuticauda* allele at chr8:3144828 (*CYP2J19*) and chrZ:67547840 (*TTC39B*). We found significant additive effects at both color loci (additive effect a = 14.1 $\pm$ 0.8 nm and 10.3 nm $\pm$ 0.9 nm, s.e.m. at *CYP2J19* and *TTC39B*, respectively, both $p < 2e^{-16}$) and at *CYP2J19*, that the yellow *acuticauda* allele is recessive (d = -5.0 $\pm$ 1.3 nm, $p = 1.4e^{-4}$). For *TTC39B hecki* homozygotes, the recessive effect at *CYP2J19* is most obvious: *CYP2J19 hecki* heterozygotes were indistinguishable in hue from homozygotes (H3 = 579.4 vs. 584.6, N = 13 and 99 for heterozygotes and homozygotes, respectively, Figure 4F). Statistical models containing an interaction term between loci provided a significantly better fit than ones without (ΔAICc $\geq$ 2.8, N = 508), indicative of a genetic interaction, or epistasis, and is consistent with *TTC39B*'s role as an enhancer. However, if we limit ourselves to only those samples with a *hecki* genomic background, the support for epistasis becomes non-significant (ΔAICc < 2.0, N = 234; see STAR Methods). Altogether, the recessive nature of the yellow *acuticauda* allele of *CYP2J19* is consistent with the role of this enzyme as an oxidative ketolase while the additive contribution of each *TTC39B* allele matches expectations for its role as an enhancer of carotenoid metabolism[10].

Signatures of selection on *acuticauda* bill color alleles introgressed into *hecki*

The introgression dynamics of bill color alleles from *acuticauda* into *hecki* are suggestive of an adaptive benefit for individuals with yellower bills. To explore this possibility more directly, we scanned for signatures of selective sweeps within each of the four regions most strongly associated with bill color variation by reconstructing local gene trees, to approximate the ancestral recombination graph (ARG) (see STAR Methods). Selective sweeps are expected to result in an increase in haplotype homozygosity around a target of selection[38] and a decrease in the time to coalescence for haplotypes carrying a favored allele[39,40].

We found clear evidence of selective sweeps on SNPs associated with bill color variation in the long-tailed finch. Within the genomic window on chromosome 8 containing *CYP2J19*, the *acuticauda* derived variant at bill color associated SNP chr8:3094115 (GWAS P = $5.45e^{-19}$) exhibited evidence of strong selection in *hecki* (Figure 5, s = 0.0059, logLR = 32.4). Nearly fixed within *acuticauda* and the hybrid zone (pops. 1 – 19, ρ = 0.99), this variant appears to now be approaching fixation within color-admixed *hecki* (pops. 20 – 27, ρ = 0.83; Figure 5B). Consistent with introgression following secondary contact, the sweeping variant first appeared in *acuticauda* approximately 40 kya (based on a two-year generation time, see STAR Methods) and has subsequently undergone a rapid increase in frequency within *hecki* between 5 kya and the present day (Figure 5C). The variant of the SNP most strongly associated with bill hue, chr8:3144828, arose in *acuticauda* 100 kya but exhibited weaker support for selection in *hecki*, possibly due to the haplotype diversity on which it is found (Figure S4; see STAR Methods). Variants that originated in *acuticauda* associated with *CROT* and *PTPRC* (see Figure 3) also exhibited recent increases in frequency consistent with selective sweeps less than 5 kya (Figure S5). Evidence from haplotype homozygosity summary statistics (see STAR Methods) provided further support for selection on *acuticauda* variants within each association peak (Figure S6).

Compared to autosomal loci, evidence of selective sweeps was more difficult to interpret on the Z chromosome (see STAR Methods). Notably, however, a variant of the bill color associated SNP chrZ:67480260 (GWAS P = $1.8e^{-15}$) linked with *TTC39B* shows evidence of selection within *hecki* (s = 0.0046, logLR = 10.7; Figure 6). This variant is nearly fixed within color-admixed *hecki* (ρ = 0.90) and at intermediate frequency within the hybrid zone (ρ = 0.41) but – strikingly – is at low frequency within *acuticauda* and red-billed *hecki* (ρ = 0.03 in both). The genetic background on which this variant is found

is definitively *acuticauda* and its present geographic distribution suggests that it originated either in the hybrid zone or populations of what are now color-admixed *hecki* (Figure 6A and 6B). Allele trajectories indicate that this variant has rapidly increased in frequency within *hecki* between 3 kya and the present day (Figure 6C). We hypothesize that it arose on – and is currently hitchhiking with – the much older *acuticauda* haplotype of *TTC39B* (as represented by chrZ:67547840 – see Figure S4) that is the actual target of selection.

DISCUSSION

Despite the significance of animal color in evolution[41], researchers have only recently gained insight into the key genes underlying the diversity of carotenoid color traits[8-10]. In this study, we report the molecular composition, genetic architecture, and evolutionary history of naturally occurring carotenoid color variation between hybridizing subspecies of the long-tailed finch. We find that red bill color is an ancestral state and that the gain of yellow bill coloration in subspecies *acuticauda* has been achieved via a *cis*-regulatory change in C(4)-oxidation that precludes production of red ketocarotenoid pigments in the bill integument. Variation in bill hue is most strongly associated with a set of genes that include *CYP2J19*, an enzyme required for C(4)-oxidation of dietary yellow-orange carotenoids into red ketocarotenoids, and *TTC39B*, a known enhancer of carotenoid metabolism. While yellow bill color alleles at *CYP2J19* and *TTC39B* first arose in *acuticauda* ~100 kya, during a period of allopatric divergence that began approximately half a million years ago[26,42], both genes exhibit signatures of recent adaptive introgression from *acuticauda* into *hecki* between 5 kya and the present day. Importantly, eastward introgression of yellow alleles across multiple loci argues against some form of frequency-dependent selection or assortative mating by color scenario where the most frequent local morph is preferred, strengthening the argument for their adaptive introgression[43]. We show that evolutionary transitions between yellow and red color ornamentation, which occur frequently in birds[44,45], can be achieved by natural selection acting upon regulatory mutations of large effect on a small set of genes involved in carotenoid metabolism.

The presence of red cone oil droplets in the retinas of both subspecies suggests that *acuticauda* evolved a yellow bill by suppressing C(4)-oxidation specifically within the bill integument. While nearly all diurnal birds have red cone oil droplets in their retinas, and all red cone oil droplets examined contain ketocarotenoids[10,31,32,46,47], comparatively few species have red bills or feathers. In groups of birds with carotenoid ornaments, transitions between yellow and red coloration can evolve dynamically[44,45] but the underlying genetic mechanisms responsible have rarely been characterized[16]. One simple hypothesis to explain how such evolutionary transitions can be achieved without compromising color vision is through *cis*-regulatory changes to where and when *CYP2J19* is expressed[10,14]. Consistent with this prediction, we observed a lack of protein-coding differences between *acuticauda* and *hecki* alleles and an abundance of strongly associated variants upstream of *CYP2J19*. We posit that the evolutionary transition from red to yellow bill coloration in the long-tailed finch has arisen largely through a regulatory change to where expression of *CYP2J19* – and thus C(4)-oxidation – occurs.

Recent experimental work shows that *CYP2J19* cannot convert dietary yellow carotenoids to red ketocarotenoids alone: oxidative ketolation requires both *CYP2J19* and the gene *BDH1L,* and this process is enhanced by *TTC39B*[10]. While we found no evidence that *BDH1L* was associated with color variation in the long-tailed finch we did observe a significant contribution of *TTC39B* suggestive of an epistatic interaction with *CYP2J19* (see Figure 4). Yellow alleles at both genes are of similar ages (Figure S4), are derived in *acuticauda*, have introgressed into *hecki* while exhibiting the hallmark signatures of selective sweeps, and better explained variation in bill hue together than either locus did alone. As with *CYP2J19*, an absence of missense mutations between subspecies suggests that the adaptive significance of the *acuticauda* allele of *TTC39B* is *cis*-regulatory. In contrast to the highly pleiotropic and largely loss-of-

function mutations that dominate the literature on carotenoid color variation in captivity[10,13,14], our work adds to a growing body of research finding that color variation observed in nature is predominately associated with *cis*-regulatory changes[16,20,21,23,48-50].

What fitness advantages might have propelled the spread of yellow alleles? One hypothesis to explain their adaptive introgression is that both long-tailed finch subspecies prefer partners with yellower bills. This argument for a central role of sexual selection has little empirical support. First, while carotenoid plumage ornamentation is strongly associated with the intensity of sexual selection at a broad level[51,52], carotenoid bill coloration is better explained as a social signal in passerines[53,54]. Second, there is no evidence in the wild (in *acuticauda*) for either assortative mating or higher reproductive output in relation to bill color[55]. Third, a behavioral assay performed on long-tailed finches in controlled captive conditions found that females did not exhibit assortative mate preferences for bill color after controlling for behavioral traits like song and courtship[56]. Another possibility is that sustaining C(4)-oxidation over prolonged periods of time is costly[57,58], favoring selection on yellow bills, and reducing that physiological cost. In contrast to carotenoid-colored plumage, which involves carotenoid metabolism only during the molt, bare part coloration is dynamic and requires a constant production of pigments.

Color ornamentation differences – and sometimes little else – can be key components of reproductive isolation between closely related bird species[21,59,60]. In the long-tailed finch, bill coloration does not appear to play such a role. Rather than acting as a trait that drives reproductive isolation to restrict gene flow, introgression of yellow alleles appears to have been favored by natural selection. Indeed, the recent increase in frequency of yellow alleles within *hecki* may well be replicating today the very selective sweeps that drove these alleles to fixation within *acuticauda* long ago. Evidence of introgression of genes associated with ornamentation differences between allopatric populations is not without precedent and many cases have been associated with sexual selection. In birds, the asymmetrical movement of color alleles between hybridizing taxa due to mate preference for the introgressing phenotype has been reported in fairywrens[61], mannikins[62-64], tinkerbirds[18], and wagtails[65]. A particularly striking example of this in deeper time comes from new world warblers, where alleles of the carotenoid-cleaving gene *BCO2* conferring yellow plumage show evidence of repeated exchange between distantly related species[22]. Determining the fitness effects of divergent metabolic programs for the processing of carotenoids, which ultimately manifests as yellow or red coloration, will be key to understanding the putatively adaptive evolution of carotenoid coloration in the long-tailed finch and other vertebrates. The increasing accessibility of linked-read population-scale genomic data provides evolutionary biologists with a better toolkit than ever before with which to examine the genes, evolutionary history, and role of selection underlying the diversity of colorful ornamentation of birds and other animals.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Daniel M. Hooper (dhooper@amnh.org).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Data generated during this study are available through the links provided in the key resources table. Linked-read and short-read genomic sequence data for *Poephila acuticauda* and *P. cincta* have been

deposited in the Sequence Read Archive (BioProject PRJNA1147746 and PRJNA1101033). This paper does not report original code, but custom analytical scripts have been deposited in GitHub, https://github.com/dhooper1/Long-tailed-Finch. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

DMH and SCG designed research; DMH, CSM, MJP, NMJ, MK, NH, and SCG performed research; DMH analyzed genomic data; MJP and NMJ analyzed carotenoid data; DMH contributed tables and figures; YFC, GEH, PA, and SCG provided critical resources; and DMH wrote the paper with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Bill color differentiation between subspecies of the long-tailed finch

(A) Approximate geographic distribution of bill color variation in the long-tailed finch. Western subspecies *acuticauda* have yellow bills, eastern subspecies *hecki* have red bills, and a region of phenotypic admixture where individuals have orange bills is located in between them. The source populations used to establish research colonies at Macquarie University in Sydney, NSW, are represented as black circles and a black star, respectively.

(B) Representative photograph of both subspecies from our research colony: *acuticauda* (yellow bill) on left and *hecki* (red bill) on right.

(C) Reflectance spectra for bills of *acuticauda* (N = 71) and *hecki* (N = 80) are shown as mean (solid line) and standard error (yellow and red shading, respectively). Bill hue (colorimetric variable H3 or λ_{R50} is the wavelength midway between maximum and minimum reflectance between 400 and 700 nm) reported for each subspecies as mean and standard deviation. Vertical dashed lines represent mean H3 for each subspecies.

(D) Variation in H3 observed for each subspecies and their first generation (F_1) hybrids. Hybrids are grouped by the direction of hybrid cross and sex (ZZ: males; ZW: females). Values for each group are plotted as a density distribution with mean value presented as a vertical line.

(E) Geographic distribution of 34 populations sampled across the range of the long-tailed finch (shown in blue).

(F) Natural variation in H3 across populations sampled in this study. A total of 948 individuals were measured in the wild. Values for each population are plotted as a density distribution with mean value presented as a vertical line.

Figure 2. Carotenoid composition of long-tailed finch bills and retinal cone oil droplets

(A and B) Representative high performance liquid chromatography (HPLC) chromatogram of carotenoids isolated from the bill integument of *acuticauda* (A) and *hecki* (B). Each unique carotenoid is annotated numerically from left to right first in *acuticauda* and then in *hecki*. Each is labeled by source [dietary or metabolized] and color [yellow-orange or red]. A group of metabolized ε,ε-carotenoids only found in the bills of *acuticauda* (i.e., canary xanthophylls a and b) are annotated with an * within a solid yellow circle in (A) (see Figure S1 and STAR Methods).

(C) Metabolic conversions utilized to produce the three red ketocarotenoids found in *hecki* from the three dietary yellow-orange carotenoids observed in *acuticauda*.

(D) Retinal wholemounts of *acuticauda* (left) and *hecki* (right). Example red cone photoreceptor oil droplets are indicated with arrows. These red oil droplets likely contain the ketocarotenoid astaxanthin (see STAR Methods).

Figure 3. Genome wide association studies identify four regions underlying most variation of bill color in the long-tailed finch

(A) Genomic differentiation (F_{ST}) between allopatric *acuticauda* (pops. 1-7) and allopatric *hecki* (pops. 28-34) is concentrated on the Z chromosome. F_{ST} calculated in 20 kb windows with 10 kb step size after excluding singleton sites. F_{ST} on the Z chromosome was calculated only using male samples (STAR Methods).

(B) Manhattan plot for GWAS of bill hue (H3) variation identified eleven association peaks (refer to Table S5). Association support plotted as -log10(Pwald). Dashed red line denotes the genome-wide significance threshold as determined by permutation.

(C) Zoom-in to the top four association peaks in (B). Genes within each window are annotated and the most strongly associated SNP in each window is represented with a red circle.

(D) Genotype by phenotype (H3) boxplots for the most strongly associated SNP in each window in (C). Genotypes are given with alleles polarized ancestral or derived from left to right with bill hue (H3) given in nanometers on the y-axis. The GWAS p-value (P) is shown for each SNP.

Figure 4. Geographic and genomic cline evidence of introgression at *CYP2J19* and *TTC39B*

(A) Geographic variation in bill hue (H3). Populations between dashed lines 1 and 2 represent color-admixed *hecki*. Immediately west of dashed line 1 are yellow-billed hybrid zone populations. East of dashed line 2 are populations from red-billed *hecki* (see Figure S2).

(B and C) Population allele frequencies of the two SNPs most strongly associated with H3 variation. Derived and ancestral alleles are color-coded blue and red, respectively. F_{ST} and GWAS significance given in top right inset of each panel.

(D) Geographic clines for genome-wide hybrid index (grey), *CYP2J19* (orange), and *TTC39B* (blue). Best fit clines shown as solid lines with 95% highest posterior density interval (HPDI) shading. Cline centers presented as vertical dashed lines with distance from the western-most population given at top in kilometers.

(E) Genomic cline analysis identified variants associated with *CYP2J19* (orange) and *TTC39B* (blue) as significant introgression outliers relative to SNPs from the genomic background (grey).

(F) Allelic dominance and epistasis between *CYP2J19* and *TTC39B*. Each grid cell represents a genotype combination for the two SNPs most strongly associated with bill hue. The mean H3 of each genotype is given within each cell and represented as an open circle along a 55 nm gradient between *acuticauda* and *hecki* (see STAR Methods).

Figure 5. Evidence of selection on *CYP2J19* from ancestral recombination graph (ARG) inference

(A) Relate marginal tree for SNP chr8:3094115 (2N = 1928 haplotypes). Branches colored light red represent haplotypes carrying the derived allele. Evidence for selection over the lifetime of the mutation (P_R) shown for *acuticauda* (*ACU*: pops. 1-7, 2N = 282) and *hecki* (HEC: pops. 20-34, 2N = 522). Vertical hash marks beneath tips of marginal tree represent haplotypes observed in red-billed *hecki* (dark red, pops. 28-34), color-admixed hecki (orange, pops. 20-27), hybrid zone (purple, pops. 8-19), and yellow-billed *acuticauda* (pops. 1-7). Horizontal dashed lines 1 and 2, as seen in panel (B), represent geographic breaks between the hybrid zone and color-admixed *hecki* (line 1) and between color-admixed and red-billed *hecki* (line 2).

(B) Population allele frequencies with derived and ancestral alleles color-coded blue and red, respectively. F_{ST} and GWAS significance given in top right inset of panel.

(C) Historical allele trajectory of the *acuticauda*-derived allele in subspecies *hecki*. s, selection coefficient. See Figure S4 for more details.

Figure 6. Evidence of selection on *TTC39B* from ancestral recombination graph (ARG) inference

(A) Relate marginal tree for SNP chrZ:67480260 (2N = 1206 haplotypes observed in males, see STAR Methods and Figure 5 for more details).

(B) Population allele frequencies with derived and ancestral alleles color-coded blue and red, respectively.

(C) Historical allele trajectory of the derived allele in subspecies *hecki*. s, selection coefficient. See Figure S4 for more details.

STAR METHODS

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Blood was collected from wild-caught and captive-reared finches housed at Macquarie University, Australia. All experimental procedures described in this study were approved by the applicable ethics committee or authorities at Macquarie University. Blood samples were stored in 99% ethanol or on EDTA-soaked filter paper in Drierite to preserve red blood cells for DNA extraction later. The study and sampling of finches has been approved by the Australian government. Long-tailed finch *Poephila acuticauda* individuals of each subspecies were bred in captivity from wild-derived stocks established less than ten generations prior. The population of yellow-billed subspecies *acuticauda* was descended from individuals collected from two locations in Western Australia in 2009: Mt. House (17°02'S, 125°35'E) and Nelson's Hole (15°49'S, 127°30'E). The population of red-billed subspecies hecki was descended from individuals collected from October Creek (16°37'S, 134°51'E) in the Northern Territory in 2010 (Figure 1A). Populations of each subspecies were kept reproductively isolated from one another save for the purpose of experimentally producing F_1 hybrids.

METHODS DETAILS

Bill color phenotyping

Bill color was measured via UV-vis reflectance spectrophotometry for wild-caught and captive-bred adult long-tailed finches following protocols published in prior studies[27,56]. In brief, spectral reflectance of the upper mandible from three consecutive scans per individual were averaged and smoothed using the R package Pavo 2[29]. Spectra were then normalized by their maximum and minimum reflectance values, and the colorimetric variable H3 was calculated for all samples. H3 is a measure of bill hue that represents the wavelength midway between the minimum and maximum reflectance of a surface, bounded between 400 and 700 nm[29]. This metric has previously been shown to effectively differentiate the bill colors of the two long-tailed finch subspecies and their hybrids[56]. To supplement reflectance data first examined by Griffith and Hooper[27] and McDiarmid et al.[56] we measured bill color for an additional 165 wild-caught and 137 captive-bred samples in this study for a total of 948 wild and 550 captive samples with reflectance data (N = 1498 total). Our sampling transect extended across ~1500 km from west to east and included phenotypically "pure" populations from each subspecies (i.e., >80% of member individuals were within two standard deviations of the mean bill hue of each subspecies measured in captive common garden conditions).

Evaluation of bill carotenoid composition with HPLC

Carotenoids were isolated from the bill tissue of five individuals of each subspecies following procedures adapted from McGraw et al.[83] and McGraw and Toomey[84]. Thin slices of integument (0.002-0.01 g) were shaved from the outer bill using a razor. Carotenoids were extracted from the shavings in the presence of solvent (5–6 mL hexane: tert butyl methyl ether, 1:1, v/v) using a mortar and pestle. Ground tissue and solvent were centrifuged, and the supernatant recovered for saponification to remove esterification that impedes elution and accurate quantification of carotenoids via HPLC[84]. Importantly, while this method increases recovery of ketocarotenoids and dietary carotenoids, it does result in the loss of canary xanthophylls a and b[85]. To saponify carotenoid samples, the supernatant was evaporated to dryness and the carotenoids resuspended in 100 µL of 100% ETOH. Next, we added 100 µL of 0.02M KOH in MeOH, vortexed for 30 seconds, capped with N_2 gas, and incubated the extract at RT in the dark overnight. After incubation, 250 µL H_20, 500 µL TBME (100%), and 250 µL hexane (100%) were added sequentially, with vortexing after the addition of the H_20 and hexane. Finally, esters were precipitated from the extract with the addition of 100 µL saturated saltwater (pure NaCl) and 30 seconds of vortexing. The saponified

carotenoids were moved to a new tube and evaporated to dryness before resuspension in 200 µL of acetone for immediate HPLC injection.

Carotenoid samples were analyzed via HPLC using a Shimadzu Prominence UPLC system. Extracts were injected in 10 µL volumes into a Sonoma C18 column (10 µm, 250 x 4.6 mm, ES Technologies, New Jersey, USA) fitted with a C18 guard cartridge. Separation and elution of carotenoids was done using an adapted tertiary mobile phase (adapted from Wright et al.[86]). The mobile phases used here were: A) 80:20 methanol: 0.5M ammonium acetate; B) 90:10 acetonitrile: H2O; and C) ethyl acetate. We used a tertiary linear gradient with a flow rate of 1mL min^{-1} that consisted of 100% A to 100% B over 4 min, then 80% C: 20% B over 14 minutes, then 100% B over 3 minutes, ending with 100% A over 11 minutes to re-equilibrate the column. Samples were run consecutively with an autosampler fitted with an internal cleaning port with 1:1 v/v MeOH:H_2O to remove cross-contamination. Carotenoids were detected using a Prominence UV/Vis detector set to 450 nm.

Carotenoids were identified based on comparisons to pure standards, an internal system database of retention times, and published accounts. We classified carotenoids as dietary yellow-orange, dietary red, metabolized yellow or metabolized red (see Figure 2 and S1). Dietary yellow-orange carotenoids are metabolized into red ketocarotenoids via C(4)-oxidation, a process in which a carbonyl group is added to the C(4) position of a β-ring end group[30]. The low concentration of ε,ε-carotenoids (i.e., canary xanthophylls a and b) detected in the yellow bills of subspecies *acuticauda* (Figure S1) is likely to be an artefact, the result of a necessary saponification step used during carotenoid pigment extraction[85]. We therefore refrain from formally comparing total carotenoid content in yellow bills to that in red bills.

Retinal imaging and cone photoreceptor subtype classification

Eyes were obtained from frozen and/or freshly deceased long-tailed finches that had been culled for a complementary project. Three yellow-billed *acuticauda* and three red-billed *hecki* birds were examined. The left eye of each finch was removed and hemisected at the equator using a scalpel blade. The posterior segment of the eye was placed into phosphate buffered saline (PBS; pH 7.4; 340 mOsmol kg^{-1}) to facilitate dissection of the retina. Small pieces (~2×2 mm) of retina from the dorsal or ventral retinal periphery were dissected away and mounted on a glass microscope slide in a drop of PBS, and a top coverslip applied and sealed with clear nail varnish. The retina was viewed under bright-field illumination using an Olympus ×100/NA 1.4 oil immersion objective on an Olympus BX-53 microscope fitted with DIC optics. Images were captured using an Olympus DP74 digital camera and cellSens software. We then conducted a visual examination of cone cell subtypes in the retinas of all individuals, which are distinguishable due to the distinct collection of carotenoids found in their associated oil droplets. Specifically, we compared whether there was a difference in the occurrence of long-wavelength-sensitive (i.e., red) cone cell oil droplets between *acuticauda* and *hecki*. In birds and other vertebrates, these red oil droplets have been found to contain the ketocarotenoid astaxanthin[10,31,32], a metabolic byproduct via C(4)-oxidation of the dietary carotenoid zeaxanthin[30] (Figure 2C). As such, observing red oil droplets in each subspecies would be parsimoniously consistent with both *acuticauda* and *hecki* using C(4)-oxidation to convert yellow-orange dietary carotenoids to red ketocarotenoids in the retina.

DNA extraction and quantification

Genomic DNA was extracted from blood and tissue samples stored in ethanol using a DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol and concentrations were then quantified using the Qubit dsDNA Broad Range Quantification Assay Kit (Thermo Fisher Scientific) on a Qubit fluorometer (Life Technologies).

Enrichment for high-molecular weight DNA

We performed high-molecular weight (HMW) DNA enrichment to compensate for the high level of degradation in most of the gDNA samples. Here, we chose a cut-off of >8 kbp. The full protocol is detailed on https://www.protocols.io/. Briefly, 50 µl of gDNA in 10mM Tris buffer pH=8, was gently mixed with exactly 64 µl of size selection bead buffer (20 mM Tris pH=8, 6% PEG8000, 833 mM NaCl, 70 mM MgCl2) containing 1 mg/ml of Sera-Mag Speedbeads (Cytiva). Each size selection reaction was incubated for 25 min at 65°C. Beads were then washed on magnetic stand twice with 80% ethanol and the enriched HMW gDNA was eluted with TE buffer at 45°C for 30 minutes.

Sequencing library construction

Linked-read (LR) genomic libraries were prepared for 1229 samples following published protocols from Meier et al.[24]. For clarity, we will repeat the protocol used here and provide the full protocol on https://www.protocols.io/. One main modification was the use of a higher-throughput NovaSeq flow cell for multiplexed sequencing (hence pooling of more than 96 samples together). To do so, we used alternative ligation overhangs between barcode segments (original: "A/C" overhang as in Meier et al.[24]; here we added "T/G" and "C/T" panels, resulting in 3 x 96 panels for a total 288 individual-level barcodes), thus expanded the original set of haplotagging beads for the purpose of individual barcoding to make up 3 different set of "stock 96-well plates" (Table S1).

For constructing libraries, 1.25 µl of haplotagging beads (~0.8 million beads, each carrying one of 885K well-specific barcodes) were transferred into twelve 8-tube-PCR-strips and closed with strip lids. On magnetic stand, the storage buffer was removed and replaced with 110 µl of WASH buffer (20 mM Tris pH8, 50 mM NaCl, 0.1% Triton X-100). Next, 10 µl of 0.15 ng/µl DNA was transferred with a multichannel pipette and 200 µl wide-orifice pipette tips strip-by-strip, and mixed 5 to -10 times to re-suspend the beads. Next, 30 µl of 5x tagmentation buffer (50 mM TAPS pH 8.5 with NaOH, 25 mM $MgCl_2$, 50% N,N-dimethylformamide) was pipetted into each strip, closed with the lid, mixed by inverting the tubes 3 to 5 times, and incubated at 55°C for 10 min.

During the incubation, strip-tubes were mixed by inverting 3 to 5 times every 3 minutes. After the incubation, samples were placed on ice for 1 minute, pulse spun-down, and 8 µl of 6% SDS was pipetted into each sample to inactivate and strip Tn5 from the DNA. Samples were incubated at 55°C for 10 min, then pulse spun-down, and placed on magnetic stand. All liquid was removed and beads were washed twice with 150 µl of WASH buffer, while not disturbing the beads. Beads were kept in the second wash until further use.

Next, beads were pooled and subsampled at 1:5.6 ratio before pooling. Thus, $5/28^{th}$ of each sample's beads was transferred with a multichannel pipette, strip-by-strip, into one 8-tube-PCR-strip. This corresponds to approximately 1.6M beads (barcodes) and ~67 pg DNA per sample, or ~135 haploid copies of the long-tailed finch genome. Pooled and subsampled beads of each of the plates, carrying a total of 6.4 ng DNA per library (96 x 67 pg), were split into two equal samples and incubated at 50°C for 25 minutes with exonuclease 1 (New England Biolabs) to remove unintegrated barcoded transposon adapters. Samples were incubated at 37 °C for 30 minutes, and then washed twice for 5 minutes with 150 µl of WASH buffer.

Each plate's DNA library was then amplified in two 50 µl Q5 High-Fidelity DNA Polymerase reactions (New England Biolabs) in a 50 µl PCR reaction according to manufacturer's instructions, using 4 µl of 10 µM custom TruSeq-F and TruSeq-R primers, with the following cycling conditions: 10 min at 72°C followed by 30 sec 98°C and 11 cycles of: 98°C for 15 sec, 65°C for 30 sec and 72°C for 60 sec. Libraries were pooled after PCR into a single library pool, size selected with Sera-Mag Speedbeads at 0.45× followed by 0.85× bead:sample ratios to remove >1 kbp library and <300 bp fragments, respectively.

After that the library concentrations were quantified using Qubit, and adjusted with 10 mM Tris, pH8, 0.1 mM EDTA to 2.5 nM concentration for sequencing.

The set of samples with LR data (Table S2) included 1133 long-tailed finches: 982 from across the geographic distribution of the species in the wild and 171 captive reared individuals descended from wild-caught individuals of each subspecies held in aviaries at Macquarie University in Sydney, Australia. We also prepared LR libraries for 96 black-throated finches *Poephila cincta* (48 *P. c. cincta* and 48 *P. c. atropygialis*). This allopatric sister species to the long-tailed finch was used as a closely related outgroup in population genetic analyses. Bill color phenotype data was available for 508 of the sequenced samples.

Short-read (SR) genomic libraries were prepared for 22 samples (5 *P. a. acuticauda*, 15 *P. a. hecki*, 1 *P. c. cincta*, and 1 *P. c. atropygialis*) using an Illumina TruSeq DNA Nano Library Preparation Kit (Illumina). The 20 long-tailed finch samples with SR libraries were used as technical replicates for evaluating genotype imputation accuracy and phasing performance with LR data (Table S3).

Sequencing and demultiplexing

LR libraries were sequenced on a NovaSeq 6000 2x150bp S4 flow cell (Illumina) from a commercial service provider (MedGenome) with a 151+13+13+151 cycle run setting for a total of 3.3 TBases of sequencing data. Raw CBCL sequencing data was converted to fastq using bcl2fastq (Illumina) without sample sheet and with parameters --use-bases-mask=Y151,I13,I13,Y151 --minimum-trimmed-read-length=1 --masked-short-adapter-reads=1 --create-fastq-for-index-reads. beadTag demultiplexing was performed using a demultiplexing c++ script to decode each of the four barcodes and saved as BX:Z tag information in modified fastq files. Reads were subsequently split into plates based on the combination of ligation overhangs. Demultiplexing information is available at https://github.com/evolgenomics/haplotagging. Reads were trimmed of adapter sequences and low-quality bases using cutadapt v4.8[67] and then mapped to the zebra finch reference genome v1.4 (GCF_003957565.2) using BWA mem[68]. PCR duplicates were removed under the barcode-aware mode Picard's MarkDuplicates module (http://broadinstitute.github.io/picard/). We recovered an average of 8.4 million paired end reads and a median depth of coverage of 1.38× for the 1229 samples with LR data.

Our long-tailed finch SR libraries were sequenced on five lanes of a HiSeq 4000 2x150bp (Illumina) operated by a commercial service provider (Novogene) and our black-throated finch SR libraries were sequenced on five lanes of a HiSeq 4000 2x150bp operated by the University of Chicago Genomics Facility. We included data from 20 additional wild-caught long-tailed finch samples generated in a previous study (10 *P. a. acuticauda* and 10 *P. a. hecki*; standard SR genomic sequence from ENA Study: PRJEB10586[66]). Reads from all SR data samples were processed as above, except without barcode-aware duplicate marking. The 42 samples with SR sequence data had an average of 150 million paired end reads and a mean depth of coverage of 26.1×.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using the R software, v4.2.1. The details of specific statistical analyses are detailed in each section below.

Bill color variation

We compared bill hue (H3) between captive-crossed F_1 hybrids to evaluate a recessive sex-linked contribution to bill color first noted by McDiarmid et al.[55]. In birds, as females are the heterogametic sex (i.e., ZW) and males the homogametic sex (i.e., ZZ), a recessive sex-linked locus affecting bill color will

result in F_1 females having a bill color more like their father's than F_1 males or F_1 females from the reciprocal cross direction. Specifically, we tested whether F_1 hybrid females with an *acuticauda*-yellow or *hecki*-red father differed in bill hue from each other and from F_1 hybrid males of either cross direction using Tukey's HSD test following an ANOVA using functions from the 'lm' and 'stats' packages in R.

Variant discovery and genotyping

We generated an initial set of genomic variants found in our set of 1249 samples with WGS data using the mpileup function of bcftools[69]. A subset of biallelic SNVs were then selected after bcftools filtering for proximity to indels (i.e., -g3) and based on variant quality and sequencing error artefacts (%QUAL<500 || AC<2 %QUAL<50 || %MAX(AD)/%MAX(DP)<=0.3 || RPB<0.1 && %QUAL<50). This quality-filtered subset of variants was then pruned of sites that overlapped with annotated repetitive regions of the zebra finch genome using RepeatMasker[70]. A total of 37.69 million SNVs remained after initial quality filtering and repeat masking.

Genotype calling and imputation with this set of SNVs was performed using read data in BAM format from all 1249 samples using STITCH v1.6.6[71]. We ran STITCH in 1 Mb windows in pseudohaploid mode with the following parameters: K=100, nGen=1000, shuffle_bin_radius=100, niterations=40, switchModelIterations=25, buffer=50000. Based on the significant negative relationship between chromosome size and per bp recombination rate in birds[66], STITCH was run across three chromosome classes using the following recombination rate tuning (i.e., >25 Mb, expRate = 1.0; <25 Mb, expRate = 5.0, and < 10 Mb, expRate = 10.0). Microchromosomes smaller than 2.6 Mb (a set of 9 chromosomes encompassing <1.5% of the genome) were excluded from further analysis due to poor imputation performance likely resulting from their high per bp rate of recombination. A final set of 33.23 million SNVs with high information content (INFO_SCORE > 0.4) were retained for downstream analyses (88.2% of the initial set of variants identified).

Population genetic analyses

We calculated F_{ST} between allopatric populations of each subspecies (i.e., 110 *acuticauda* from pops. 1 – 7 and 110 *hecki* from pops. 28 – 34 in Figure 1) for all SNPs with a minor allele count of at least 2 and in 20 kb windows along each chromosome with a step size of 10 kb using vcftools v0.1.16[72]. We quantified F_{ST} on the Z chromosome using a subset of allopatric males (70 *acuticauda* and 69 *hecki* samples) to circumvent any effects of female hemizygosity.

We performed hybrid index estimation using a set of linkage-disequilibrium (LD) pruned ancestry informative SNP markers. We defined a set of ancestry informative markers as the 755 autosomal SNPs above an F_{ST} threshold of 0.7 and the 42668 Z chromosome SNPs above an F_{ST} threshold of 0.95 between allopatric populations. We used PLINK v.1.9[73] to LD-prune SNPs in this marker set above an r^2 of 0.2 within 100 kb windows and a 10 kb step size (--indep-pairwise 100 10 0.2) and with any amount of missing data across samples (--geno 0.0). Of the initial 43423 ancestry informative SNP markers, 1137 SNPs remained after LD pruning. We used the R package gghybrid[74] to perform Bayesian MCMC hybrid index estimation on a set of 649 ancestry informative SNPs remaining after further filtering for an allele frequency difference of at least 0.8 between allopatric populations and a MAF of less than 0.05 in one of the two allopatric populations. This set of markers included 99 autosomal and 550 Z-linked SNPs. We modeled the ancestry proportion (i.e., hybrid index) of all 1153 wild and captive long-tailed finch samples as the h posterior mode from gghybrid function hindlabel (Figure S2 and Table S4). We inferred the geographic center and width of admixture between subspecies using hybrid index information from all wild-caught samples.

GWAS for bill color variation

We performed a genome-wide association study (GWAS) for bill color using a measure of bill hue: H3. As described above, the colorimetric variable H3 was quantified from reflectance spectrophotometer readings as the wavelength midway between the minimum and maximum reflectance of light reflected off a bird's upper mandible. We retained a total of 508 individuals with bill color phenotype data for association mapping. We investigated the genetic basis of bill color using the Wald test implemented in Gemma v0.98.5[75]. We fit a univariate linear mixed model to test for an association between phenotype and each SNP genotype. We included a relatedness matrix to correct for population structure and because hybrid sex[56] and genome-wide hybrid index are significantly correlated with bill hue (Spearman's $r = 0.66$, $p < 2.2\times10^{-16}$) we also included these as covariates during modeling. Keeping only variants with a minor allele frequency above 2%, genome-wide data from a total of 17,566,649 variants were analyzed (mean of 17.3 SNPs / kbp).

We evaluated the genome-wide threshold for significance at a false discovery rate of 1% ($\alpha = 0.01$) in two ways. First, using a Bonferroni correction on the total number of SNPs tested (threshold = 9.24; $P = 5.69e^{-10}$) and second, as the 99th percentile across the distribution of differences following 1,000 permutations of bill hue onto observed genotypes (threshold = 9.03; $P = 9.29e^{-10}$). As results were qualitatively identical with respect to using either significance threshold approach (523 versus 576 significant SNPs), we adopted the permutation derived threshold as it preserves important features of the data while making fewer assumptions. Results were visualized by log-transforming the p-values, changing their signs, and generating Manhattan plots using the R package fastman.

We defined association peaks as clusters of at least five SNPs above our genome-wide significance threshold that were separated by <50 kb from the next significant SNP. We scanned for protein-coding genes within 100 kb upstream and downstream of each association peak in R using the annotation file for the zebra finch reference genome (GCF_003957565.2). Of the eleven identified association peaks (Table S5), we focused subsequent attention on the three autosomal peaks and the single Z chromosome peak that contained the most SNPs significantly associated with color variation genome-wide, included SNPs observed to be highly differentiated between subspecies, and encompassed genes previously associated with or plausibly linked to carotenoid-based color variation. The functional significance of all SNPs above genome-wide significance was evaluated using SnpEff v5.2a[76]. We used outgroup information from the black-throated finch and zebra finch to polarize alleles associated with bill color variation to their subspecies of origin.

Following Zhou et al.[87], the proportion of genetic variance explained (i.e., PVE) by the most strongly associated SNP variant in each association peak was estimated as:

$$PVE = \sigma_g^2 * (\beta^2 + SE^2)/\sigma_p^2$$

Where genotypic variance was represented by σ_g^2 and phenotypic variance by σ_p^2, respectively. Estimates of allelic effect size (β) and its standard error (SE) were inferred for each SNP by GEMMA.

CYP2J19 copy number evaluation

The study by Mundy et al.[13] of the zebra finch that first identified C*YP2J19* as a candidate oxidative ketolation enzyme reported two copies of this gene on chromosome 8. The authors proposed that the second copy had arisen due to tandem duplication with one specialized for color vision (denoted C*YP2J19A*) and the other for color ornamentation (C*YP2J19B*)[13]. However, only a single copy of C*YP2J19* is present in the long-read assembly of the zebra finch reference genome (labeled C*YP2J19A* in GCF_003957565.2). We examined evidence for two copies of C*YP2J19* in the long-tailed finch (*acuticauda* and *hecki*) and black-

throated finch (*atropygialis* and *cincta*) in three ways. First, we used our linked-read data to calculate molecular barcode sharing between 1 kb windows on chromosome 8 using Wrath[77] and found no evidence of structural variant differences between taxa. Specifically, we found no evidence of a tandem duplication difference between taxa associated with *CYP2J19*. Second, we evaluated whether samples from all four taxa exhibited an increase in relative depth of coverage on chromosome 8 in 1 kb sliding windows across *CY2PJ19A* (chr8:3167181-3178161). An increased depth of coverage would indicate that reads from two copies of this gene were mapping to the same genomic location: a ~2-fold increase in coverage would be expected if this was the case. We did not detect any increase in depth of coverage for reads mapping to *CYP2J19A* (chr8:3167000- 3179000) that would indicate that *CYP2J19* exists in more than a single copy in the genus *Poephila:* relative depth of coverage *acuticauda* = 0.95, *hecki* = 0.96, atropygialis = 0.93, *cincta* = 0.95). Third, the top BLAST v2.5.0+[78] hit for the long-range PCR assembly from Mundy et al.[13] of zebra finch *CYP2J19A* and *CYP2J19B* (KX024636) against the current zebra finch reference genome shares 97.5% sequence identity with a contiguous region of chromosome 8 containing *CYP2J19A* and the gene *CYP2J40* (score = 44662, evalue = 0.0). Like *CYP2J19*, *CYP2J40* is a cytochrome P450 that presumably resulted from tandem duplication, and we posit that it may have been the source of what is referred to as *CYP2J19B* in prior work[13].

Geographic and genomic clinal analysis

One-dimensional Bayesian analyses of cline position and shape were performed using a subset of 30 transect populations with at least 2N = 10 samples available using the R package BAHZ[79]. Geographic clines were modeled for the most strongly associated SNP in each of the four focal GWAS association peaks and for genome-wide hybrid index. We determined the best-fitting cline model based on changes in allele frequencies across our sampling transect using two parameters, cline center and width.

We evaluated our set of 649 ancestry informative markers in linkage equilibrium (see population genetic analyses above) for evidence of biased introgression between *acuticauda* and *hecki* using the ggcline function of gghybrid[74]. We used our genome-wide hybrid index and genotype data from 982 wild-caught samples to model the genomic cline center (c) and steepness (v) for each SNP marker and the hybrid index. The genomic background was represented as genomic clines for 100 randomly drawn ancestry-informative markers used to infer hybrid index. Due to LD-pruning, the *TTC39B* SNP most strongly associated with bill hue variation and used in geographic cline analysis (chrZ:67547840) was not included in the set of ancestry informative markers used for genomic cline analysis. Instead, a SNP tightly linked with chrZ:67547840 is among the set of ancestry informative markers: chrZ:67498406 (r^2 = 0.90, D' = 0.95).

Evaluating epistasis between color loci

We evaluated evidence of an epistatic relationship between *CYP2J19* and *TTC39* by examining the effect on phenotype of genotype at these two genes in tandem. We first quantified mean bill hue (H3) for the 508 individuals with phenotype data carrying zero, one, or two copies of the *acuticauda* allele of the SNPs most strongly associated with hue variation at these loci: chr8:3144828 (*CYP2J19*) and chrZ:67547840 (*TTC39B*). We tested for differences in mean H3 between each genotype combination using Tukey's HSD test following an ANOVA using functions from the 'lm' and 'stats' packages in R. We calculated additive (a) and dominance (d) effects for alleles at both SNPs using linear regression. To infer additive effects, we recoded genotypes as 0, 1, or 2 based on the number of copies of the *acuticauda* allele an individual carried. To infer dominance effects, we recoded genotypes as 0 or 1 if individuals were homozygous or heterozygous, respectively. Finally, we tested for evidence of epistasis by comparing goodness of fit for linear models of H3 against *CYP2J19* genotype, *TTC39B* genotype, and the interaction between *CYP2J19* and TTC39 genotypes using ΔAICc comparison. Model comparison was performed using the function 'dredge' from the 'MuMIn' package in R. For this analysis, we compared model fit using all samples with bill color data available (i.e., individuals from pops. 2 – 33, N = 508) and in the subset of individuals with

an otherwise *hecki* genetic background (i.e., individuals from pops. 20 – 33, N = 234). For graphical purposes (i.e., Figure 4F), we visually evaluated mean H3 of each genotype combination along a 55 nm gradient between yellow-billed *acuticauda* (mean H3 = 530 nm, N = 243, pops. 2-10) and red-billed *hecki* (mean H3 = 585 nm, N = 185, pops. 28-33).

Haplotype assembly and phasing

Molecular phasing of SNPs on chromosomes 2, 8, and Z was performed using haplotagging LR molecular barcode information with HapCUT2[80]. Phasing was performed using a BAM file containing LRs mapped to the zebra finch reference genome and a VCF file containing variant calls and diploid genotypes for the same individual. As female samples are hemizygous for the Z chromosome, and as a result are expected to already be phased apart from the pseudo-autosomal region, we did not include them in our HapCUT2 pipeline. Phasing performance, measured as N50 haplotype length, was evaluated as follows. We first compared the distribution of individual N50 haplotype lengths for each chromosome between the 1229 samples with LR data and low sequencing depth (e.g., 1.4×) against the 40 samples with standard short-read data and deep sequencing depth (e.g., 30.0×). We next directly compared N50 haplotype lengths for the 20 individuals in our dataset that were sequenced using both linked-read and short-read approaches to control for the effects of sample high-molecular weight DNA quality. LR information greatly increased phasing performance compared to short-read (SR) data: we recovered an 18-fold improvement in the length of phased blocks, with a median N50 of 19.6 kbp using LR data (versus 1.1 kbp for SR data; Figure S3; Table S6). Of the 18 samples used as technical replicates, phased block N50s were on average 29-fold longer when utilizing LR information (LR: 20.4 kbp, SR: 0.7 kbp) despite these samples having an average depth of coverage 13-fold lower (LR: 2× depth, SR: 26×; Figure S3; Table S6).

Selection

Scans for signatures of selection were performed on four regions of the genome containing the GWAS association peaks most strongly associated with variation in bill color. We defined each region as a genomic window $\pm$1 Mb of each association peak. The mean size of each association peak was 0.13 Mb (range from 0.03 to 0.33 Mb), and so the mean genomic window evaluated was 2.13 Mb (range from 2.03 to 2.33 Mb), respectively. This approach allowed us to investigate evidence of selective sweeps at each association peak against the genomic background where peaks are located. Evidence of positive selection was evaluated based on summary statistics of haplotype structure and Ancestral Recombination Graph (ARG) inference to test for and estimate the strength and timing of selection, as well as estimate the full allele frequency trajectory. We focus on ARG-based inference as these approaches are more informative and direct compared with haplotype homozygosity statistics and site frequency spectrum (SFS) approaches; which are themselves low-dimensional summaries of the ARG[25,38,88].

We phased all variants on chromosomes 2, 8, and Z in samples with linked-read information using HapCUT2[80]. We next used this LR-phased haplotype data to generate ARGs from our four top association peaks $\pm$ 1 Mb using Relate v1.1[25] and screened for lineages carrying mutations that have spread faster than competing lineages. As the Relate Selection Test assumes no population stratification[25], we evaluated support for selective sweeps in *acuticauda* (pops. 1 – 7) and *hecki* (pops. 20 – 34) separately and did not test for selection within the hybrid zone populations between them.

We evaluated long-range haplotype homozygosity within populations using the integrated haplotype homozygosity score (iHS) and compared haplotype homozygosity between populations using cross-population extended haplotype homozygosity (xpEHH). Both statistics were calculated with phased genotype data using the R package rehh[81,89]. We focused on three groups of sampled populations for calculating summary statistics based on haplotype structure: (*i*) allopatric yellow-billed *acuticauda* (pops. 1 – 7), (*ii*) color-admixed *hecki* (pops. 20 – 27), and (*iii*) allopatric red-billed *hecki* (pops. 28 – 34) (Figure

S6). We did not include hybrid zone populations (pops. 8 – 19) in these calculations because of their demographic composition of recent generation hybrids.

We leveraged the LR supported phasing in our dataset using RELATE v1.1[25] to examine evidence of positive selection at the four genomic regions encompassing bill color association peaks. We first converted phased VCFs to the haps/sample file format used by Relate with the RelateFileFormats.sh script and prepared the input files using PrepareInputFiles.sh (both part of the Relate package). We ran Relate on 1928 haplotypes from males and females for autosomal chromosomes 2 and 8 and on 1206 haplotypes from males for the Z chromosome. We used the zebra finch reference sequence to polarize variants as ancestral or derived. We ran Relate with options -m 5.85e-9 -N 5e5 for the autosomal chromosomes and -m 6.5e-9 -N 2e5 for the Z chromosome to account for the difference in germline mutation rate and effective population size between these chromosome classes[90,91]. We supplied genetic maps for each chromosome generated from genotype calls from 70 allopatric *acuticauda* males using ReLERNN v1.0.0[82], a deep learning approach that uses recurrent neural networks. ReLERNN was run using the simulate, train, predict, and bscorrect modules with default settings apart from the mutation rates specified above and a generation time of two years. Inferred recombination rates were averaged in 1 Mb blocks in 50 kb sliding windows. We used the Relate script EstimatePopulationSize.sh with options -m 5.85e-9 (autosomal) or -m 6.5e-9 (chrZ) and -years_per_gen 2.

We extracted the genealogies of each of our focal groups (i.e., *acuticauda* pops. 1-7 and *hecki* pops. 20-34), re-estimated population size history for them, and used the output of the previous step as input for the Relate Selection Tests script DetectSelection.sh. This approach tests for evidence of positive selection on a particular variant based on the speed at which lineages carrying it spread relative to other 'competing' lineages[25]. We accounted for the demographic history of each group using the same tuning for mutation rate and generation time differences between chromosome classes as above. From the .sele files generated, we extracted p-values from the column "when_mutation_has_freq2" which tests for evidence of selection over the lifetime of a particular variant.

We further investigated the evolutionary history of each variant found to show evidence of positive selection within our four association peak regions using CLUES[40]. We used the Relate script SampleBranchLengths.sh on the genealogies of each of our groups to sample branch lengths from the posterior and account for uncertainty. We ran 100 samples (-num_samples 100) and accounted for the demographic history of each group using the .coal files output from EstimatePopulationSize.sh. We then ran CLUES (inference.py script) with the option -coal to account for demographic history. We fine-mapped variants using the likelihood ratio statistic generated by CLUES as suggested by Stern et al.[40] and focused on the variants within each association peak that show moderate to strong selection ($s > 0.003$). We used the plot_traj.py script to plot the results. As evidence from population genetic, geographic cline, and genomic cline analyses together suggested that allelic variation associated with bill color variation has predominately introgressed from subspecies *acuticauda* into subspecies *hecki*, we focused attention on estimating the strength and timing of selection, as well as the full allele frequency trajectory, within subspecies *hecki* (i.e., pops. 20-34).

We converted estimates of time from generations to years based on an estimated generation time for the long-tailed finch of two years. We inferred this approximate generation time based on equation 2 of Bird et al.[92]. This equation estimates generation time (*G*) in birds based on age of first reproduction (*F*), maximum longevity (*L*), and a scaling factor (z) tuned to the age of first reproduction. Following Bird et al.[92], we selected a scaling factor equal to 0.142, which was inferred from 301 species with an age of first reproduction (*F*) between 0.5 and 1.5 years and available data for F and L. In the long-tailed finch, the age of first reproduction in the wild is one year and the maximum longevity in captivity is eight years[92].

We note that, compared to the autosomes (Figures S4, S5, and S6), evidence of selective sweeps was more difficult to interpret on the Z chromosome. While results of geographic and genomic cline analyses were consistent with selection at SNPs chrZ:67498406 and chrZ:67547840 (Figure 4), neither locus showed evidence of a selective sweep in *hecki* based on ARG analysis (Figure S4). We interpret this to be the result in part of the degree of haplotype divergence between subspecies on the Z chromosome and the length of the introgressing region. Indeed, a highly differentiated region approximately 0.4 Mbp in length (from 67.5 to 67.9 Mb, mean allopatric $F_{ST} = 0.75$), encompassing *TTC39B*, shows evidence of introgression from *acuticauda* into *hecki*. Ancestral recombination graph approaches for detecting natural selection may be poorly suited in cases of adaptive introgression between highly divergent taxa[39], as such cases violate assumptions regarding the recent coalescence for haplotypes carrying selected alleles, and this problem might be especially pronounced for genomic regions with reduced rates of recombination such as avian sex chromosomes[66]. Indeed, support for selection on *TTC39B* within *hecki* came not from a variant that arose within *acuticauda* but rather for a variant found on an *acuticauda* haplotype background that appears to have originated within the hybrid zone after secondary contact (see Figure 6). Within populations of color-admixed *hecki*, this variant (i.e., chrZ:67480260) is strongly linked with the *acuticauda* allele of the Z-linked SNP most significantly associated with bill color variation (chrZ:67547840, $r^2 = 0.80$, $D' = 0.92$) and with the SNP exhibiting greatest genomic cline evidence of introgression from *acuticauda* into *hecki* (chrZ:67498406, $r^2 = 0.87$, $D' = 0.96$).

ADDITIONAL RESOURCES

SUPPLEMENTAL VIDEOS AND SPREADSHEETS

Table S1: Haplotagging multiplexing information for linked-read (LR) samples, related to STAR Methods.

Table S2: Metadata sequencing results and haplotagging performance for LR samples, related to STAR Methods.

Table S3: Metadata and sequencing results for short-read (SR) samples, related to STAR Methods.

Table S4: Hybrid index estimation results for wild-caught long-tailed finch samples, related to Figure S2 and STAR Methods.

Table S5: Summary of eleven bill color association peaks identified by genome-wide association studies, related to Figure 3 and STAR Methods.

Table S6: Phasing performance (N50) on chr2, chr8, and chrZ for LR and SR samples, related to Figure S3 and STAR Methods.

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