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The genetic basis of plumage coloration and elevation adaptation in a clade of recently diverged alpine and arctic songbirds

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Abstract

Trait genetic architecture plays an important role in the probability that variation in that trait leads to divergence and speciation. In some cases, speciation may be driven by the generation of novel phenotypes through the recombination of genes associated with traits that are important for local adaptation or sexual selection. Here, we investigate the genetic basis of three plumage color traits, and one ecological trait, breeding elevation, in a recent avian radiation, the North American rosy-finches (*Leucosticte* spp.). We identify unique genomic regions associated with each trait and highlight 11 candidate genes. Among these are well-characterized melanogenesis genes, including Mitf and Tyrp1, and previously reported hypoxia-related genes including Egln1. Additionally, we use mitochondrial data to date the divergence of rosy-finch clades which appear to have diverged within the past 250 ky. Given the low levels of genome-wide differentiation among rosy-finch taxa, and evidence for extensive introgression in North America, plumage coloration and adaptation to high elevations have likely played large roles in generating the observed patterns of lineage divergence. The relative independence of these candidate regions across the genome suggests that recombination might have led to multiple phenotypes, and subsequent rosy-finch speciation, over short periods of time.

Keywords: genome-wide association, melanogenesis, hypoxia-inducible factor, rosy-finches

Speciation is complex and is often described as a non-linear continuum wherein a diverse array of factors influences the likelihood of the evolution of reproductive isolation (Gavrilets, 2003; McKay & Zink, 2014; Nosil et al., 2017). Because speciation is complex, it can be challenging to understand the genomic and environmental contexts that are most likely to contribute to population differentiation and ultimately speciation. Indeed, organismal natural history (e.g., dispersal ability, strength of selection) and genomic architecture (e.g., the presence of differentiated sex chromosomes, the genetic basis of adaptive traits, or the linkage of different genomic regions) will all influence the likelihood of population divergence and speciation (Dufresnes et al., 2021; Flaxman et al., 2014; Matsubayashi et al., 2010). Despite the many avenues that lead to speciation, the combination of strong sexual selection and ecological opportunity can be a powerful driver of divergence (Gabrielli et al., 2020; Wagner et al., 2012). When both adaptive and sexually selected phenotypes are generated by relatively independent loci of large effect, the recombination of these genomic regions can produce novel phenotypes that can both succeed in open niche space and be favored by sexual selection (Marques et al., 2019). As a result, the recombination of traits influenced by multiple selective pressures (i.e., natural selection and sexual selection) may result in relatively short divergence times, as is seen in adaptive radiations such as cichlids (Wagner et al., 2012) and Darwin's finches (Boag & Grant, 1981; Podos et al., 2013). African lake cichlids show a strong correlation between diversification and both ecological opportunity and strength of sexual selection (Wagner et al., 2012). Additionally, the strength of extrinsic and intrinsic factors is likely highest across complex and heterogeneous landscapes that allow for the subdivision of small, isolated populations (Naciri & Linder, 2020). Here, we explore the genomic conditions underlying divergence by investigating the genetic basis of multiple phenotypes in a North American clade of alpine and arctic tundra specialist songbirds in the rosy-finch genus *Leucosticte* (Figure 1).

Although rosy-finches appear to be a recent radiation with low levels of genetic differentiation, they exhibit variation both in plumage coloration and breeding elevation (Figure 1A). Rosy-finch breeding elevation varies with the locally maximum elevation of mountains, and ranges from over 4,000 m in the southern end of their distribution to sea level along the Alaska coast, and the Aleutian and Pribilof islands.

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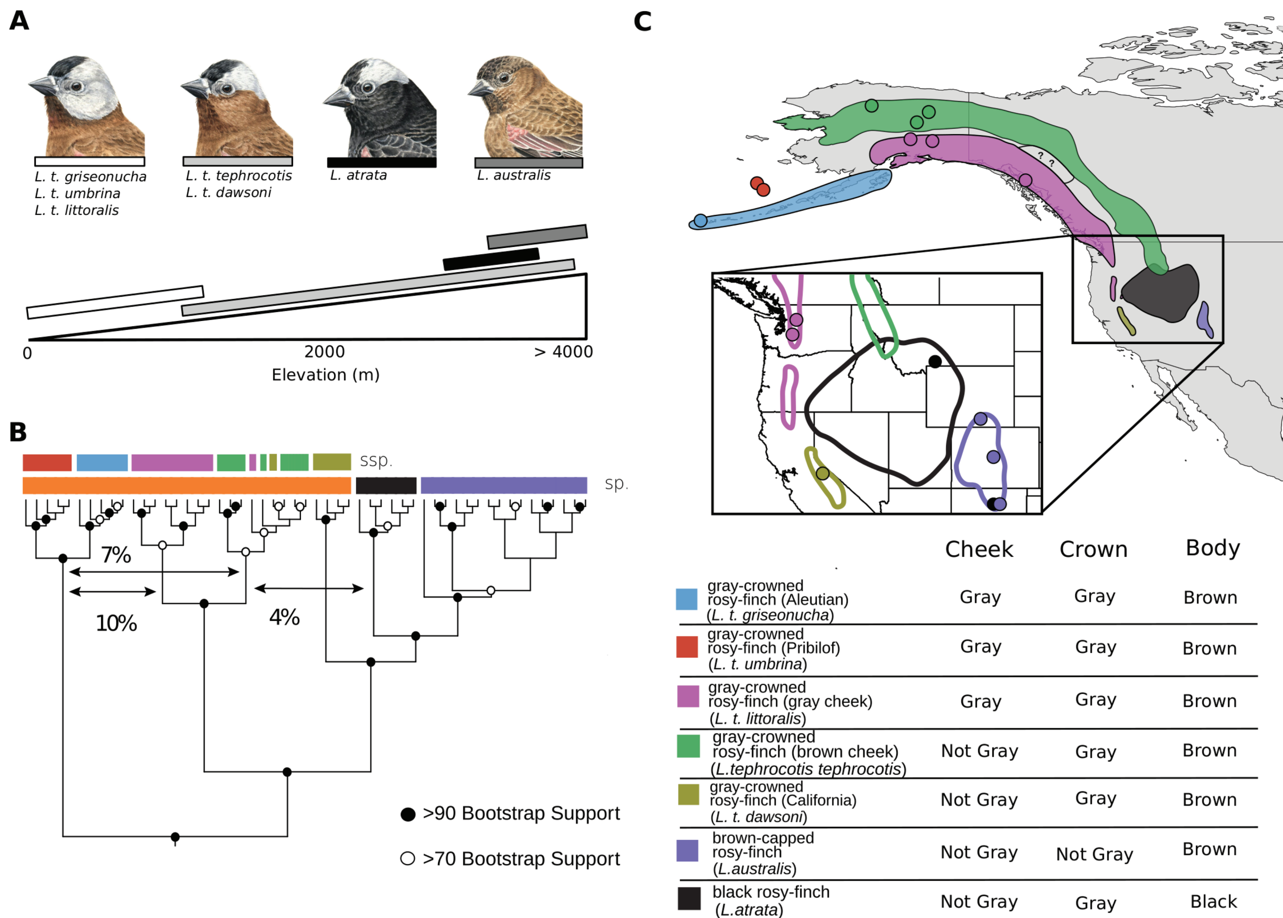

Figure 1. Rosy-finch plumage coloration and distribution. (A) The four rosy-finch plumage phenotypes and the subspecies they include. Gray bars indicate the elevational distribution of each phenotype. (B) Phylogenetic relationships reconstructed from Funk et al. (2021). Arrows indicate the genome percentage shared as a result of introgression. Clade bar colors correspond to the legend in panel C and represent species (bottom row) and subspecies (top row) classifications. The orange clade bar represents all subspecies of *L. tephrocotis*. (C) Distribution of North American rosy-finches with sampling localities shown as dots and phenotype coding for each taxon. Rosy-finch illustrations by Liz Clayton Fuller.

Rosy-finches also display patch-specific color variation including the presence or absence of gray on the crown or cheek, and two main body colors—brown and black. While specific trait combinations form the basis of species description (American Ornithologists' Union, 1998; Johnson, 1972) and align with well-supported phylogenetic clades (Funk et al., 2021), not all of the above traits are specific to a single taxon (Figure 1A, B). Overlapping morphologies, recent divergence times, and gene flow likely homogenize background genetic variation, and make rosy-finches well suited for tests of genome-wide associations.

We focus our analyses on testing for associations between genetic variation and both plumage color and adaptation to high elevations given that these traits appear to vary the most among rosy-finches and might have played an important role in rapid lineage divergence. Although loose correlations exist between plumage color and elevation in rosy-finches (e.g., birds lacking a crown patch are all high elevation), the correlation of multiple traits is likely to be a common signature in systems where intrinsic and extrinsic factors both contribute to speciation. As a result, the genetic basis and architecture of these traits are important targets for investigation. The evolutionary characteristics highlighted above, including recent divergence times and the presence of gene flow, increase the suitability of rosy-finches for genotype-phenotype associations.

Furthermore, coloration and elevational adaptation have been the subject of many previous studies of other taxa and have contributed to a rich literature describing the genes involved in both (Cheviron & Brumfield, 2012; San-jose & Roulin, 2017).

High-elevation environments impose different stressors than those at sea level, such as colder temperatures and decreased oxygen levels. As a result, populations may demonstrate a number of physiological adaptations to cope with the stress of high-elevation environments, including variation in metabolic characteristics related to energy consumption, or blood flow traits influencing oxygen delivery such as hemoglobin concentration, oxygen binding, and capillary density (Beall, 2007; Simonson et al., 2010; Yi et al., 2010). Comparative studies in humans (Beall, 2007) and birds (Graham & McCracken, 2019; Lim et al., 2019) demonstrate that while a number of different adaptive strategies exist, selective pressures may favor convergent changes at the molecular level on a few key pathways affecting oxygen homeostasis such as the hypoxia-inducible factor (Pamenter et al., 2020; Semenza, 2010). The hypoxia-inducible factor (HIF) pathway is one of the most commonly reported targets of selection in high-elevation-adapted populations (Pamenter et al., 2020). HIF is a transcription factor considered to be a master regulator of oxygen homeostasis

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(Semenza, 2010) that interacts with a number of genes frequently reported in studies of hypoxic adaptations including Epas1, Epo, and Egln1 (Dong et al., 2014; Graham & McCracken, 2019; Pamenter et al., 2020; Simonson et al., 2010).

The genetic basis of melanin-based traits has been well-characterized in humans (Serre et al., 2018) and in the study of avian plumage coloration (Gao et al., 2018; Inaba et al., 2019; Takeuchi et al., 1996). A number of initial studies focused on pigment changes across the entire body, such as yellow versus black body plumage (Theron et al., 2001). Many of these studies highlighted the role of the Mc1r gene in generating such drastic differences in plumage coloration (Baião et al., 2007; Cooke & Cooch, 1968; Mundy et al., 2004; Theron et al., 2001). Recent studies continue to highlight the influence of Mc1r in producing whole-body color changes (Campagna et al., 2022). However, as genomic data have allowed for more fine-scale admixture mapping and association studies, identifying the genetic basis of smaller plumage patches has become a common goal (Abolins-Abols, et al. 2018; Brelsford et al., 2017; Estalles et al., 2022; Stryjewski & Sorenson, 2017; Toews et al., 2016). Many of these studies identify independent, narrow genomic regions associated with individual plumage patch traits, suggesting a high degree of modularity to plumage coloration and patterning (Funk & Taylor, 2019).

We predict that, similar to other avian systems with patch-specific color differences, rosy-finch plumage patch colors are controlled by individual regions of the genome. We suspect that a similar genetic architecture may underlie adaptations to high elevations and that, together, unique combinations of genomic regions related to melanin-based traits and high-elevation adaptations have generated the observed phenotypic and ecological diversity in rosy-finches and have contributed to the divergence and speciation of multiple lineages within a short evolutionary time frame.

Methods

Sampling

We used genomic sequence data from 67 individuals previously sequenced as part of Funk et al. (2021). These included 11 Asian rosy-finches (*Leucosticte arctoa*) and 56 samples spanning the range of the three recognized North American rosy-finch species (Supplementary Table S1; American Ornithologists' Union, 1998; Clements et al., 2019), including five of the six gray-crowned rosy-finch subspecies (*Leucosticte tephrocotis tephrocotis*, n = 8; *Leucosticte tephrocotis littoralis*, n = 9; *Leucosticte tephrocotis griseonucha*, n = 5; *Leucosticte tephrocotis umbrina*, n = 5; and *Leucosticte tephrocotis dawsoni*, n = 5), the black rosy-finch (n = 6), and the brown-capped rosy-finch (n = 18). This sampling encompasses both the geographic and phenotypic distribution of North American rosy-finches and came from a combination of tissue and blood derived from museum collections and a 2018 field season. Asian rosy-finches were only included in the divergence dating portion of this study and were dropped for all association analyses.

Reference genome and sequence processing

To map reads, we used a Dovetail-generated, chromosome-level reference genome (Dovetail Genomics, CA, USA) of a brown-capped rosy-finch (*Leucosticte australis*; DMNS:Bird:52416, NCBI JANIJU000000000) new to this study. We annotated genomic features using the program Liftoff (Shumate & Salzberg, 2021) with the most recent zebra finch annotations that were available at the time of analysis (NCBI GCA_008822105.2). We numbered chromosomes according to the zebra finch using Satsuma (Grabherr et al., 2010) and visualized syntenic blocks using Satsuma's Chromosome Paint. In the discussion of our results below, we refer to different alleles as reference and alternate, and note here that our reference genome is from a high-elevation individual that lacks many melanin-based traits that are of interest in this study.

Details of the bioinformatic and filtering steps can be found on github (https://github.com/erikrfunk/whole_genome_bioinformatics/blob/master/rosyfinch_notes.md). Briefly, we performed a quality assessment for each sample using fastQC v0.11.8 (www.bioinformatics.babraham.ac.uk/projects/fastqc) before and after trimming reads. We used Trimmomatic v0.32 (Bolger et al., 2014) in paired-end mode to perform adapter removal and trim reads using a sliding window when the average phred score dropped below 20. We aligned short reads to the reference genome using the BWA-MEM algorithm (Li & Durbin, 2009), and sorted and indexed the alignment files using SAMtools v1.9 (Wysoker et al., 2009). We used the mpileup and call commands in bcftools to call variants for each individual (Li, 2011), filtering out sites with a quality score below 80. We also filtered out variants with a minor allele frequency of less than 0.05, and that had an average depth of less than one or greater than nine to remove variants possibly coming from paralogous loci. We required 75% of individuals (42 out of 56) to have data at a given site for it to be retained in our final dataset. Finally, we confirmed there were no siblings in the sampled birds using the Ajk statistic (--relatedness) from VCFtoolsv0.1.15 (Danecek et al., 2011).

Divergence dating

To provide context for the timing of rosy-finch diversification, we dated divergence events using BEAST v2.6.6 (Bouckaert et al., 2019). We extracted cytochrome *b* (Cyt *b*, 1143 bp) sequences from all genomes, including 11 Asian rosy-finches (*Leucosticte arctoa*) for a total of 67 individuals across four rosy-finch species, and aligned them using the MUSCLE plug-in in MEGA (Tamura et al., 2021). Preliminary investigation of Cyt *b* identified seven brown-capped rosy-finches with mitochondrial haplotypes more closely related to the Asian rosy-finch haplogroup than to the North American haplogroup despite the deeply nested nuclear genomic phylogenetic position of brown-capped rosy-finches (see Funk et al., 2021). Because this pattern can be driven by retained ancestral polymorphism during range expansion (Excoffier & Ray, 2008; Streicher et al., 2016), the inclusion of these individuals in the dating analysis would overestimate divergence dates. To correct for this, we present results from analyses using only the brown-capped rosy-finches with a North American haplotype.

To generate phylogenetic trees, we partitioned the alignment by codon position. Using PartitionFinder v2.1.1 (Lanfear et al., 2012), we set models of evolution as HKY+I, HKY, and TRN+G for each codon position respectively. We used a strict molecular clock with a rate of 0.0105 based on the 2.1% pairwise divergence rate estimated for the avian

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Cyt *b* gene (Weir & Schluter, 2008). We used a coalescent tree model and ran two independent analyses for 400 million iterations, assessing convergence of the posterior using Tracer v1.7.1 (Rambaut et al., 2018). We evaluated convergence by ensuring a stationary trace with all ESS values greater than 200 and used independent runs to ensure convergence on the same divergence dates. We stored 10,000 trees from the posterior and generated a final maximum clade credibility tree after burning in 10% using the program TreeAnnotator packaged with the Beast software distribution.

Genome-wide associations

We tested for genetic variants associated with three discrete plumage traits, including crown, cheek, and body color. We classified all individuals as either "gray" or "not gray" at the crown and cheek patches, and as having "brown" or "black" body plumage (Figure 1C). We also tested for genetic variants associated with high-elevation adaptation. We included only birds collected during the breeding season and scored birds using the elevation of the sampling site. To assess the effects of trait classification, we ran this association test treating elevation both as a continuous trait and as a discrete trait, using a break in sampled elevation at 1,500 m to categorize individuals as high- or low-elevation individuals. The results from these two analyses did not differ and we present here the associations recovered from the analysis that treated elevation as a continuous variable. Altogether, we present four association tests for plumage color and breeding elevation.

We tested for genetic associations with each of the above traits using a Genome-wide Efficient Mixed Model Analysis (GEMMA) (Zhou & Stephens, 2012). We calculated a centered relatedness matrix for all individuals using the -gk 1 argument, and ran the model using the Wald test allowing for a proportion of missingness per site of 0.1 (5 individuals with a missing genotype at most) for each SNP using the arguments -lmm 1 and -miss 0.1. To account for multiple comparisons we used an adjusted *p*-value significance threshold of 1×10^{-6}. For each plumage association test, we ran additional analyses using sample site elevation as a covariate. We did not include plumage as a covariate in our elevation association tests because individuals that lack plumage patches (i.e., brown-capped rosy-finches) make up a large proportion of the high-elevation birds included in our analyses. Preliminary runs suggested a poor model fit in our analysis of body plumage color association, likely due to population structure. To improve this model, we added three principal component (PC) axes as covariates and reran the model. PC scores were generated using the R package SNPRelate v1.19.3 (Zheng et al., 2012) for all North American taxa. We present here only the results of the association model with PC axes as covariates.

We classified the putative location of significantly associated SNPs as intergenic, upstream, downstream, 5' or 3' untranslated region, intronic, synonymous, or missense, based on their position relative to coding regions using SnpEff (Cingolani et al., 2012). We generated a list of genes associated with either plumage color or elevation by filtering annotated genes by those that contained or were near (within 10 kbp) associated single nucleotide polymorphisms (SNPs). We prioritized this list by the relevance of a gene's described function and highlight 11 candidate genes that are likely to have the largest influence on the rosy-finch phenotypes of interest (Table 1). Low diversity in regions surrounding an associated SNP could suggest strong selective pressures on adaptive variants. To further investigate patterns of differentiation at associated SNPs in each of the candidate genes we generated plots of heterozygosity and genotypes of all individuals at each associated SNP. We constrained our plots to include only SNPs related to a candidate gene as annotated by SnpEff, and calculated heterozygosity in R (v3.6.3) using the package vcfR (Knaus & Grünwald, 2017).

Results

Sampling and reference genome

The final brown-capped rosy-finch reference genome HiRise assembly was 1.052 Gb consisting of chromosome-length scaffolds. This included a longest scaffold of 151 Mb, with 90% of the genome assembled into 19 scaffolds of at least 14.9 Mb. A total of 19,990 genes (out of the 21,049 included in the zebra finch annotation) were annotated by Liftoff across all scaffolds. The bioinformatic and filtering steps of the short-read sequence data described in Funk et al. (2021) produced a total of 9,530,180 SNPs.

Divergence dates

Phylogenetic analysis of the Cyt *b* gene identified two primary clades: Asian rosy-finches and North American rosy-finches (Figure 2). Divergence dates estimated from a molecular clock suggest the age of the split between these two taxa occurred ~1.075 million years ago (843,000–1.33 million years 95% highest posterior density), and suggest the diversification of the North American clade occurred ~253,000 years ago (171,000–336,000 years 95% highest posterior density). Consistent with previous phylogenetic analyses that have examined mitochondrial data in rosy-finches (Drovetski et al., 2009), we did not recover any clades that correspond to taxa within North America.

Table 1. Candidate genes related to **a** plumage color and **b** high-elevation adaptation.

a. Candidate gene	Function
Ap3b1	Melanosome biogenesis and Tyr recruitment
Asip	Distribution of melanin pigment and production of eumelanin versus pheomelanin
Edn3	Melanocyte production
Mitf	Regulates transcription of many key melanogenesis genes
Mlana	Melanosome biogenesis
Tyrp1	Synthesis of melanin and maintenance of melanosome structure
b. Candidate gene	**Function**
Aggf1	Angiogenic factor promoting proliferation of endothelial cells
Aldh1a1	Retinol metabolism and metabolic responses to high-fat diet
Egln1	Primary regulator of HIF-1-alpha
Jmy	Stress responsive protein upregulated during hypoxia

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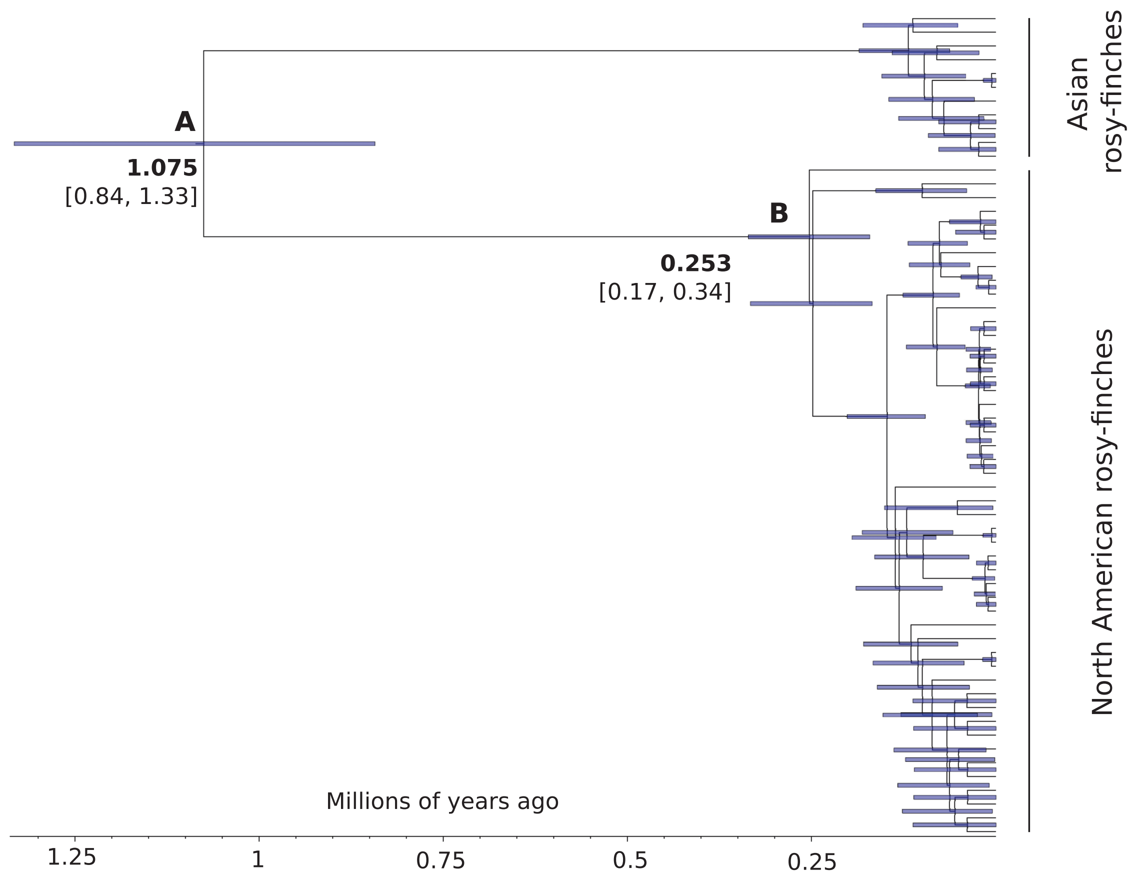

Figure 2. Divergence dates estimated from cytochrome b using a molecular clock rate of 2.1% pairwise divergence (numbers shown in millions of years). Labeled nodes correspond to (A) the divergence of North American rosy-finches and Asian rosy-finches, and (B) the diversification of North American rosy-finches. Bold numbers indicate the mean node age while numbers in brackets represent the 95% highest posterior density.

Genome-wide associations

Across all four association tests, we detected a total of 2,459 significant SNPs. Additionally, we identified 1,313 genes that either contain or are near significant SNPs (within 10 kbp). The distribution of these associations across the four tests, and class of each variant relative to coding regions can be found in Supplementary Table S2, and a complete list of genes can be found in Supplementary Table S3. We provide details on the results of each test below and highlight candidate genes related to each trait.

Cheek coloration

Contrasting birds that have gray cheeks with birds that do not have gray cheeks revealed two strong peaks of association (Figure 3A). The first peak, located on chromosome 12, contains nine intron variants related to melanocyte-inducing transcription factor (Mitf). This gene plays a central role in melanogenesis in humans as a master regulator of transcription, responsible for initiating transcription of a number of well-described melanin genes such as tyrosinase (Tyr), tyrosinase-related protein 1 (Tyrp1), and melan-A (Mlana) (Du et al., 2003; Kawakami & Fisher, 2017). The second association peak is located on chromosome 20 and contains six intron variants related to endothelin 3 (Edn3). Similar to Mitf, Edn3 is also involved in the melanogenesis pathway and is important for the production of melanocyte cells (Saldana-Caboverde & Kos, 2010).

Across both genes, all taxa lacking gray cheeks are fixed for the reference allele at nearly all associated sites while taxa that do possess gray cheeks are almost entirely heterozygous, or homozygous for the alternate allele (Figure 3C and Supplementary Figure S1). For example, brown-capped rosy-finches, black rosy-finches, and the *dawsoni* and *tephrocotis* gray-crowned rosy-finch subspecies are fixed for the reference allele at all but one significantly associated SNPs in Edn3, while the *littoralis*, *umbrina*, and *griseonucha* subspecies of gray-crowned rosy-finch are either heterozygous or homozygous for the alternative allele (we note again here that the reference sequence is from a brown-cheeked, high elevation individual). However, one SNP in an Mitf intron shows the reverse pattern, where individuals with gray cheeks are instead completely fixed for the reference allele. Heterozygosity varies widely across Mitf with few distinguishable patterns (Figure 3B and Supplementary Figure S2); however, taxa with gray cheeks demonstrate lower heterozygosity across an extended region of Edn3 that includes two associated SNPs (Supplementary Figure S2).

Crown coloration

We contrasted rosy-finches that possess a gray crown patch with those that do not and recovered one primary peak of association, with as many as six additional smaller, but still strongly associated, peaks (Figure 3D). In total, 501 SNPs were recovered as significantly associated with crown color, with over 100 of these relating to a single gene on the Z chromosome,

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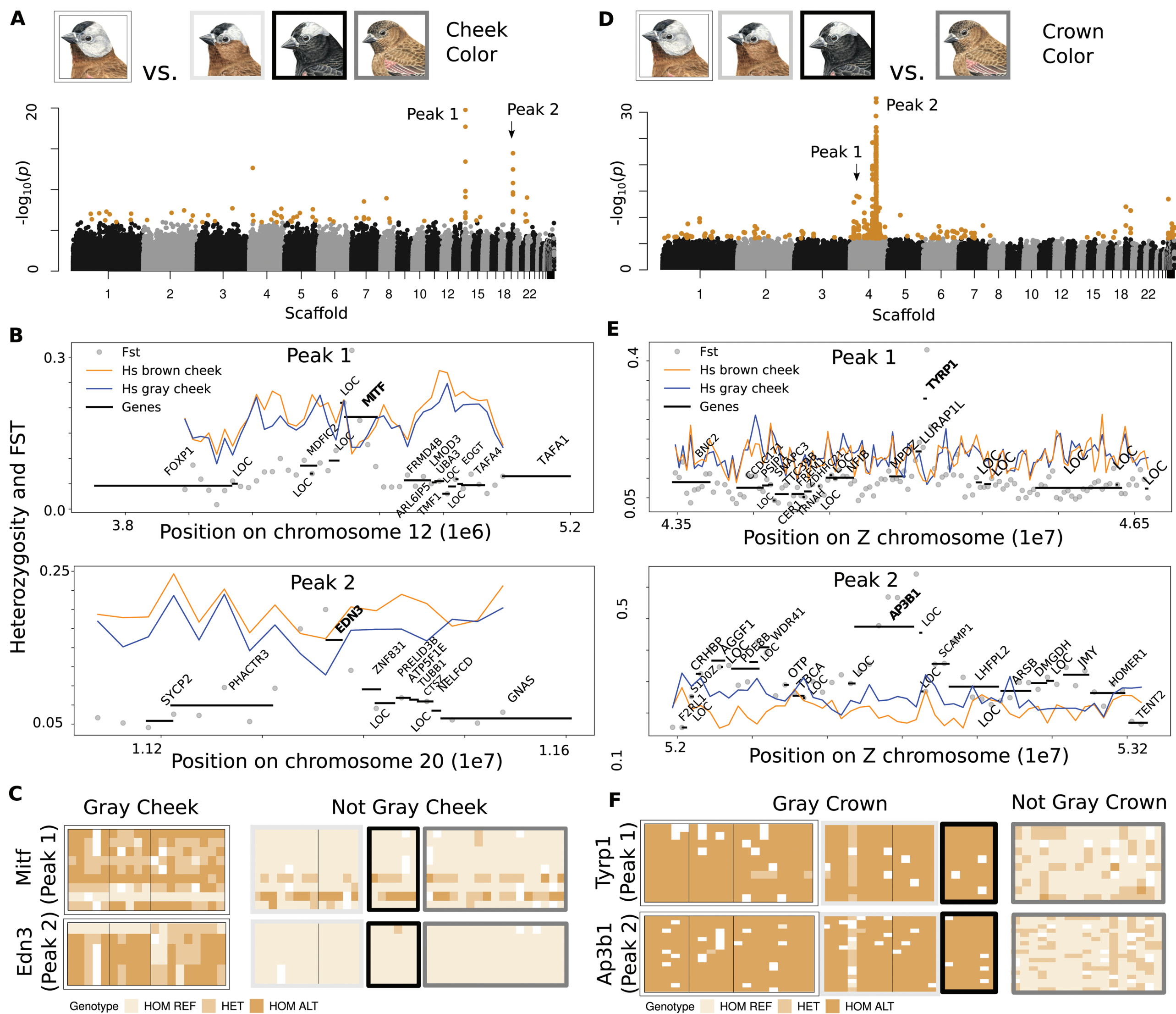

Figure 3. Plumage Associations. (A, D) Manhattan plots of genome-wide associations with cheek patch and crown patch respectively, with significant SNPs ($p < 1 \times 10^{-6}$) shown in orange. Scaffolds are ordered by size. (B, E) F_{ST} (gray dots) and heterozygosity in 25kb windows across the respective peaks of association for individuals with (blue) and without (orange) each plumage patch. Gene regions are shown as black lines where the height of a gene on the y-axis reflects the average F_{ST} within the gene region. (C, F) Genotypes of all individuals at significantly associated SNPs from top representative candidate genes. Individuals homozygous for the reference allele are shown in beige, homozygous for the alternate are shown in orange, heterozygotes are intermediate, and white represents missing. Only some Ap3b1 genotypes are shown here for space. See Figure S1 for all significantly associated genotypes.

adaptor-related protein complex 3 subunit beta-1 (Ap3b1). This gene recruits Tyr, a key enzyme in melanin biosynthesis and regulates protein trafficking of organelles such as melanosomes (Feng et al., 1999; Jing et al., 2014). Two additional melanin-related genes were also recovered as containing significantly associated SNPs, including Tyrp1 (Z chromosome), a direct target of Mitf in melanocytes and the enzyme responsible for synthesizing melanin (Sarangarajan & Boissy, 2001), and agouti signaling protein (Asip; chromosome 20), a widely studied gene that influences the production of eumelanin versus pheomelanin (Bultman et al., 1992). While the majority of the variants related to these genes are located upstream or in introns, we identified one missense mutation located in an exon of Tyrp1.

Across nearly all of the associated SNPs related to genes described above, including the missense mutation in Tyrp1, taxa with gray crowns are homozygous for the alternative allele, while taxa without gray crowns are either heterozygous or homozygous for the reference (Figure 3F and Supplementary Figure S1). Heterozygosity across Ap3b1 is broadly lower in taxa without gray crowns, including multiple regions where heterozygosity declines in taxa without gray crowns while heterozygosity stays the same, or greatly increases, in taxa that do have gray crowns, possibly suggesting sites under selection. The large number of associated SNPs related to this gene make it difficult to interpret the role of any single variant. Conversely, in both Tyrp1 and Asip, heterozygosity at multiple SNPs is lower in taxa with gray crowns. In both genes, this includes upstream variants, but also intronic variants, and the Tyrp1 missense variant (Figure 3E and Supplementary Figure S2).

Body plumage coloration

Our test for associations with body color resulted in over 999 associated SNPs (Supplementary Figure S3) and 837 associated genes containing or near (within 10 kbp) associated

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SNPs. While it is possible that multiple genes influence body color, this result is more likely the product of weak statistical power given few individuals in our dataset with black body plumage ($n = 6$). Additionally, the level of genetic divergence between black rosy-finches may be too great to distinguish associated SNPs from noise using this association approach.

While the noise that is present in our test for association with body color prevents any confident conclusions regarding specific candidate genes, 44 of the genes containing associated SNPs have been previously described as being involved in melanin pigmentation (Baxter et al., 2019). We include a brief discussion of some of these genes in Supplementary Text S1.

Elevation

Rosy-finches show a decline in maximum breeding elevation from southern to northern latitudes. While we recovered relatively few SNPs that were associated with breeding elevation (seven SNPs; Figure 4A, B), the associations we report relate to three genes with strong potential as candidate genes contributing to high-elevation adaptation. Similar to findings from multiple high-altitude adapted taxa (Bigham et al., 2009; Graham & McCracken, 2019; Simonson et al., 2010), we identified two associated variants in an intron of Egln1, a well-described component of the HIF pathway (Semenza, 2010). Additionally, our association test identified three intron variants related to Aldh1a1, a gene involved in alcohol metabolism and likely related to high-fat diets (Kiefer et al., 2012; Ziouzenkova et al., 2007). The role this gene plays in adaptation to high elevations is complex and will be discussed further below. Both Egln1 and Aldh1a1 show fixation of the alternate allele in lower elevation taxa, with the reference allele continuously increasing in frequency with elevation gain (Figure 4C and Supplementary Figure S1).

Associations with non-focal phenotypes

In each of the above association tests, we identified significantly associated SNPs located in or near genes whose functions likely influence non-focal phenotypes. For example, we identified candidate genes likely related to plumage coloration in our test for association with elevation, and vice-versa. Specifically, our analysis of cheek color associations detected a single intron variant in aldehyde dehydrogenase 1 family member A1 (Aldh1a1). This gene appears unlikely to influence melanin-based plumage traits, but instead may play an important role in adaptation to elevation.

Additionally, the second and third largest association peaks related to crown color were located at genes likely involved in adaptation to hypoxic environments such as high elevations. One of these genes, junction mediating and regulatory protein, p53 cofactor (Jmy), interacts with HIF-1-alpha, and is upregulated during hypoxia (Coutts et al., 2011). The other gene, angiogenic factor with G-patch and FHA domains 1 (Aggf1), plays an important role in angiogenesis (Liu et al., 2014) and has previously been identified as an altitude-adaptation gene under selection in Tibetan and Dahe pigs (Dong et al., 2014). Heterozygosity across Aggf1 between taxa varies the most of any gene identified across all four association tests (Supplementary Figure S4), with lower heterozygosity in individuals without a gray crown across nearly the entire gene region with the exception of three upstream variants. Similarly, Jmy shows multiple large stretches of lower heterozygosity of the same individuals.

Lastly, tests for association with elevation identified significantly associated SNPs upstream of Mlana. This gene acts as a target of Mitf in the melanogenesis pathway and does not have an obvious influence on adaptation to high-elevation or hypoxic environments.

Discussion

The genetic factors that promote phenotypic, niche, and species diversity are numerous and context dependent. That said, possessing multiple adaptive traits that are controlled by distinct regions of the genome may facilitate diversification. This has been proposed as a mechanism underlying adaptive radiations, wherein diversity is generated within short periods of time. We tested for genetic associations with three melanin-based plumage traits and adaptation to high elevations in a recent North American avian radiation of rosy-finches, and identified seven plumage-color-related, and four elevation-related candidate genes that all contain strongly associated SNPs. Our results describe the genetic basis for a suite of phenotypes likely under sexual and natural selection, and we report relatively few genomic regions associated with each trait.

Melanogenesis

Across all analyses, we found all but one of the candidate genes to be involved in either the melanogenesis or HIF pathway. Because all three plumage traits tested here are melanin-based, it is unsurprising that all seven candidate plumage genes interact with the melanin biosynthesis pathway; however, the genes identified here interact at a variety of stages in this process. Edn3 appears to play a more central role in the development and proliferation of melanocyte cells themselves (Saldana-Caboverde & Kos, 2010), and in some cases can result in spotted or patchy melanin deposition and patterning (Baynash et al., 1994). Other genes identified here are involved further down the synthesis pathway including the synthesis of melanin polymers (Tyrp1), melanosome biogenesis (Mlana) (Aydin et al., 2012; Zheng et al., 2020), or as transcription factors of key enzymes (e.g., Mitf) (Kawakami & Fisher, 2017).

Understanding genetic associations with melanin-based plumage traits is difficult in the absence of experimental manipulations. How does the alteration of a gene that is involved in melanogenesis only result in a partially altered phenotype? No rosy-finches have been recorded (to our knowledge) with gray cheeks, but not a gray crown. The difference in the roles of each of these sets of genes suggests that different parts of the rosy-finch phenotype might build upon each other in a stepwise manner (Figure 5A). For example, variation in Tyrp1 and Ap3b1 may completely prevent the generation of some melanin pigments given the importance of their roles in melanin synthesis (Jing et al., 2014; Sarangarajan & Boissy, 2001). Importantly, we detected variation in these genes in rosy-finches with the least complex melanin traits (i.e., brown-capped rosy-finches; Figure 1A), including a lack of gray cheeks, gray crowns, and black bodies. We also detect a large number of associated SNPs (over 100) in Ap3b1, possibly suggesting a relaxation of selective pressures in the rosy-finch species that lack gray cheeks, a gray crown, or a black body.

If the production of a gray crown is driven by genotypic variation in Ap3b1 and Tyrp1, the remaining phenotypes can be explained entirely by varying the extent to which

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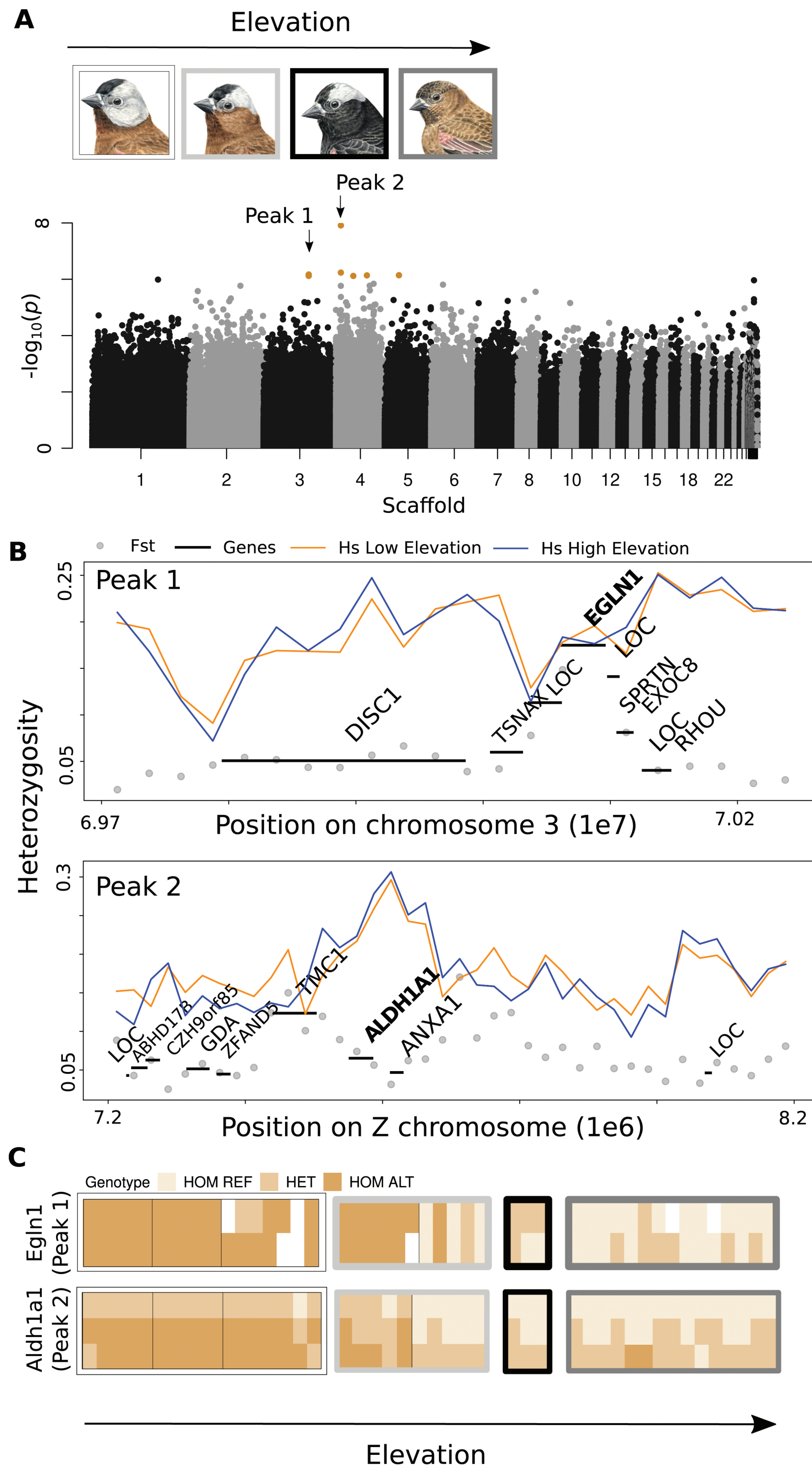

Figure 4. Associations with elevation. (A) Manhattan plot of genome-wide associations with elevation, with significant SNPs ($p < 1 \times 10^{-6}$) shown in orange. (B) F_{ST} (gray dots) and heterozygosity in 25kb windows across the respective peaks of association for individuals at high (blue) and low (orange) elevations. Gene regions are shown as black lines where the height of a gene on the y-axis reflects the average F_{ST} within the gene region. (C) Genotypes of breeding individuals at two significantly associated SNPs from Egln1 and three from Aldh1a1. Individuals homozygous for the reference allele are shown in beige, homozygous alternatives are shown in orange, heterozygotes are intermediate, and white represents missing data.

these pigments are deposited throughout a bird's plumage. Variation in genes that influence color patterning through the development or proliferation of melanocyte cells, such as Edn3, may further modulate which patches are present in a given phenotype, with Edn3 variants determining the presence or absence of a gray cheek patch. Although we lack the power in this dataset to provide a more in-depth interpretation of associations with black body color, we note the lack of associated SNPs relating to melanocortin 1 receptor (Mc1r), a finding common in other whole-body color changes in avian

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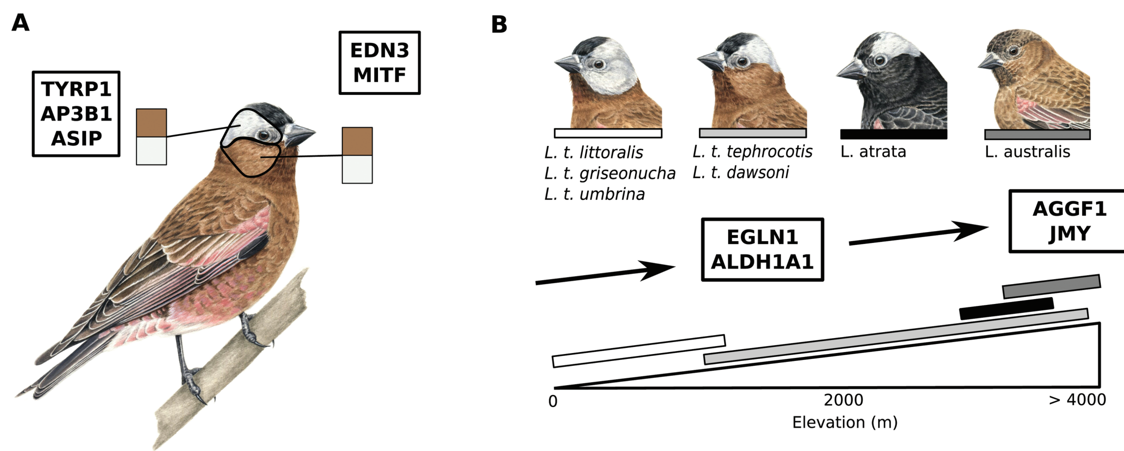

Figure 5. Genes underlying plumage coloration and elevational adaptation. (A) Five primary candidate plumage genes and their function in rosy-finch phenotype. Variation in Mitf and Edn3 result in cheek patch differences, while variation in Ap3b1, Tyrp1, and Asip result in crown patch differences. (B) Four candidate elevation genes showing shared variation at Egln1 and Aldh1a1 among high-elevation birds, and unique variation in Aggf1 and Jmy in brown-capped rosy-finches.

plumage (Theron et al., 2001). Broadly, the interpretations we provide here regarding the role of each candidate gene are limited by the phenotypes observed in our sampled individuals. Further investigation using a previously described contact zone (French, 1959; Mewaldt, 1950), or through captive crosses, would allow for validation of the independence and stepwise function of these sets of candidate genes and a test of the hypotheses outlined above (Baiz et al., 2021; Toews et al., 2016).

Hypoxia-inducible factor (HIF) pathway

All four of the candidate genes we identified as being associated with breeding elevation, and potentially related to high-elevation adaptation, play either direct roles in, or interact with, the HIF pathway. Of particular interest is our finding of SNPs associated with elevation in Egln1. This gene is considered to be one of the main oxygen sensors of the cell and is responsible for degrading HIF-1 under normal oxygen levels but not under hypoxic conditions (Metzen et al., 2005), which then triggers multiple downstream effects including the regulation of hemoglobin (Simonson et al., 2010). Variation in Egln1 has been implicated in adaptation to high altitude in Tibetan humans (Simonson et al., 2010; Xiang et al., 2013), Andean humans (Bigham et al., 2009), and in ducks (Graham & McCracken, 2019).

The other candidate gene detected by our association test with breeding elevation was Aldh1a1. The upregulation of HIF with Aldh1a1 activity (Ciccone et al., 2018) may suggest a direct regulatory role for this gene in hypoxia-related adaptation, however, the role of Aldh1a1 in fat metabolism might also suggest a metabolic role (Braun et al., 2016; Kiefer et al., 2012). Specifically, previous studies have demonstrated an increased capacity for fat metabolism over glycogen and carbohydrates resulting from the energetic strain of low-oxygen conditions (Cheviron et al., 2012; Young et al., 1982). The parallel changes in elevation and genotype frequencies at this gene suggest possibly adaptive benefits of this gene via either HIF or fat metabolism pathways, or both.

Previous phylogenomic analysis of rosy-finches highlighted only one major uncertainty in topology (Funk et al., 2021). This uncertainty involved the placement of a clade of individuals from the California population as either: (1) sister to black rosy-finches and brown-capped rosy-finches (Figure 1B), or (2) as sister to only black rosy-finches (see Funk et al., 2021; Supplementary Figure S2). We suggest that shared variation in high-elevation adaptation candidate genes could be responsible for generating this conflict in one of two ways. The first is that high-elevation variants evolved ancestrally, and that selection on standing variation resulted in the parallel evolution of these high-elevation taxa (Colosimo et al., 2005). Given the detection of introgression among many rosy-finch clades (Funk et al., 2021), the second possibility is that high-elevation variants evolved in the lineage leading to black rosy-finches and brown-capped rosy-finches, and that connectivity between California rosy-finches and black rosy-finches resulted in the introgression of beneficial high-elevation alleles. While these scenarios are likely difficult to distinguish, further sampling from California, and geographically proximate populations in Oregon, will provide additional insight into the likelihood of each of these possible evolutionary histories.

Significantly associated SNPs relating to the other two elevation adaptation candidate genes, Aggf1 and Jmy, were only recovered in one of the two highest elevation populations. As these two populations are allopatric (California vs. Colorado), we might expect that each should harbor unique variation (we kept these populations grouped to maintain sample sizes and reduce signals of population genetic structure). Our detection of private alleles in these populations, along with our finding of overall decreased heterozygosity across these genes, provides evidence that these may be recently derived adaptive variants that have swept to high frequencies. The identification of Aggf1 and Jmy in different populations and different genomic regions from the other elevation-related candidate genes also suggests an independent and additive effect of genes relating to different challenges of coping with hypoxic conditions (Figure 5B), such as increased fat metabolism (Aldh1a1) or angiogenesis (Aggf1) in addition to broad regulatory changes (Egln1). Although we do not identify any candidate genes related to elevation private to the other high elevation population (California), we have not included any tests here that would be capable of detecting this variation but suggest unique variants could exist in the California population as well.

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Correlated associations

In three of our four association tests, we report melanin and high-elevation adaptation candidate genes related to non-target phenotypes, including a melanin-related gene associated with elevation (Mlana), and elevation genes associated with crown patch (Jmy, Aggf1) and cheek patch (Aldh1a1). While this could initially point to the linkage of genes underlying color and high-elevation adaptation, the geographic distribution of co-varying phenotypes relative to the trait being tested likely provides the most parsimonious explanation. Although including correlated traits as covariates in association models can sometimes reduce spurious signals, traits that are too tightly correlated can remove all signals entirely. For example, rosy-finches that lack gray cheek patches are some of the highest elevation birds, while many gray-cheeked individuals occur at the lowest elevations. Additionally, as discussed above, one of the two populations of the highest elevation breeding rosy-finches are also the only birds that lack a crown patch. We suggest these genes are not truly linked and are instead the result of correlations between plumage traits and elevation. Co-varying phenotypes, and consequently, co-varying candidate genes, might be a common feature of speciation, particularly in systems where short times to speciation have been driven by multiple selection pressures acting on a suite of phenotypes. Our results highlight a broader challenge for association studies with non-model organisms and in understanding the linkage and genetic basis of traits that might be driving speciation.

Rosy-finch diversification

Divergence date estimates indicate that North American rosy-finches have only recently diverged from Asian rosy-finches (<1 million years), and that diversification of North American species likely occurred only within the last 250,000 years. Yet in that time, multiple divergent rosy-finch species and subspecies have evolved that possess different plumage phenotypes and breed across a range of elevations. We highlight a number of patterns from our results that may have contributed to the diversification of these birds.

One of the primary patterns we recognize is the location of associated SNPs relative to coding regions. Specifically, of the SNPs associated with candidate genes we identified here, only two are hypothesized to encode missense mutations while all remaining SNPs are located in introns or are located up or downstream of protein-coding regions. This pattern suggests a possible role for gene expression differences in the production of the observed phenotypic differences. This would be consistent with many other bird systems that demonstrate patch-specific plumage color through variation in cis-regulatory elements (Abolins-Abols et al., 2018; Campagna et al., 2017; Estalles et al., 2022; Funk & Taylor, 2019; Toews et al., 2016). Additionally, the number of intronic variants recovered here is also quite high, and though intron function can be variable, the associated SNPs from these regions may play important roles in regulating gene expression, splice variation, or meiotic recombination (Fedorova & Fedorov, 2003; Gallegos & Rose, 2017).

While many of the associated SNPs demonstrate a pattern in their position relative to coding regions, candidate genes also show similarity in their chromosomal position, with 6 of the 11 genes located on the avian sex chromosome, the Z chromosome. These results follow from previous patterns often referred to as the fast X effect (or in ZW systems such as birds, the fast Z) (Mank et al., 2007), wherein sex-linked genes accumulate variation faster due to the decreased population size of the Z chromosome and allow selectively beneficial mutations to move to high frequency faster due to hemizygosity in the heterogametic sex (Vicoso & Charlesworth, 2009). The presence of multiple melanin- and elevation-related genes on the Z chromosome may promote the divergence of rosy-finch lineages through the increased frequency of alleles locally adapted to the environment and through the maintenance of linkage disequilibrium of female preference with male sexual traits (such as plumage coloration) (Irwin, 2018; Qvarnström & Bailey, 2009).

Summary

Overall, we detected several candidate genes associated with plumage coloration and breeding elevation in rosy-finches. Although we identified a number of genes on the Z chromosome, many candidates are located in distinct regions of the chromosome separated by stretches of undifferentiated and unassociated SNPs (Backström et al., 2006; Semenov et al., 2018), or on separate chromosomes entirely. These results support a genetic architecture in which distinct regions of the genome are responsible for key traits that are likely important in the divergence of multiple rosy-finch lineages. More broadly, the shuffling of large-effect loci related to plumage color through meiotic recombination may be a particularly strong driver of diversification in birds or other organisms with strong visual acuity via pre-mating sexual selection (Estalles et al., 2022; Stryjewski & Sorenson, 2017; Turbek et al., 2021). In combination with loci locally adapted to the environment, the association of independent genomic regions with multiple phenotypic elements might be particularly conducive to generating novel phenotypes, increasing species diversity in short evolutionary time frames.

Supplementary material

Supplementary material is available online at *Evolution* (https://academic.oup.com/evolut/qpac064)

Data availability

The short read sequence data associated with this project are available for download from NCBI's SRA database under project number: PRJNA659436 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA659436). Links to each individual's SRA upload can be found in Table S1. The reference genome generated for this project is deposited on NCBI under the accession: JANIJU000000000 (https://www.ncbi.nlm.nih.gov/nuccore/JANIJU000000000.1/). Input files and supplementary materials can be found on Dryad (https://doi.org/10.5061/dryad.4xgxd25dt).

Author contributions

E.R.F. and S.A.T. conceived the idea for this project. E.R.F. led the analyses and writing with guidance from S.A.T. G.M.S., K.W., and J.J.W. provided important sampling, and S.A.T., G.M.S., and K.C.R. contributed to the reference genome.

Conflict of interest: Editorial decisions were made independently of S.A.T. who is an Associate Editor of Evolution.

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