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Original Article

Genetic parallelism underlying repeated bill divergence in the island scrub-jay (*Aphelocoma insularis*) increases at higher genetic levels of organization

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Abstract

Whether the same genes underlie parallel adaptive trait evolution remains an open question in biology. The degree of genetic parallelism is expected to increase at higher genetic hierarchical levels (i.e., single nucleotide polymorphisms [SNPs] to genes to pathways to phenotype) due to the hierarchical nature of the genetic basis of traits, which genomic approaches can help elucidate. Previous research shows a large degree of variation in the extent to which phenotypic parallelism shares the same genetic mechanisms in nature. Here, we analyzed the degree of genetic parallelism underlying repeated divergence in bill morphology of island scrub-jays (*Aphelocoma insularis*), across three naturally replicated pine–oak ecotones on Santa Cruz Island, California, US. We analyzed 66,503 SNPs generated using restriction site-associated DNA sequencing in 161 island scrub-jays to identify candidate SNPs associated with environmental variation and divergence in bill morphology. We then examined signatures of parallelism in genomic regions containing candidate SNPs and the associated genetic pathways. We found little evidence for parallelism at the SNP or gene level, but substantial parallelism at the pathway level. Our results support the view that the degree of genetic parallelism underlying repeated phenotypes depends on the genetic level of organization being analyzed.

Keywords: *Aphelocoma insularis*, parallel divergence, genome-wide association, genotype-by-environment association, microgeographic adaptation, population genomics

Introduction

A fundamental goal in evolutionary biology is to understand the processes and mechanisms that underlie adaptation. The repeated evolution of similar phenotypes in similar habitats is considered strong evidence for the role of natural selection in nature (Arendt & Reznick, 2008; Bolnick et al., 2018; Endler, 1986; Harvey & Pagel, 1991; Schluter et al., 2004). Studies exploring repeated phenotypic evolution have categorized this repeated process of adaptation as either "parallel" or "convergent," where parallelism typically refers to the degree to which natural selection lead to the independent evolution of the same phenotypes among different populations or species occupying similar environments, whereas convergence refers to those cases where distantly related species or lineages occupying similar environments evolve similar phenotypes (see Arendt & Reznick, 2008; Cerca, 2023; Stuart, 2019; Waters & McCulloch, 2021). While there is some debate over the use of these terms, here we use the term "parallelism" to refer to the pattern of repeated evolution of the same phenotype among populations. Yet, whether parallel changes at the phenotypic level should also reflect changes in the same or different sets of genes remains an open question with conflicting results (Conte et al., 2012; Barghi et al., 2020; Poore et al., 2023; Yeaman et al., 2018). Here, we are interested in the genetic basis of parallelism and whether the same genetic changes underlie parallel phenotypic changes (e.g., single nucleotide polymorphism [SNP], gene, and genetic pathway; Bolnick et al., 2018; Cerca, 2023). An improved understanding of the genetic basis of parallel traits can inform long-standing questions concerning the extent to which evolution is a predictable and repeatable process (Blount et al., 2018; Gould, 1989; Stern & Orgogozo, 2008) and what role genetic architecture plays in this process (Barrett & Hoekstra, 2011; Ferris et al., 2015: James et al., 2023). Such insights are also relevant for predicting adaptive evolution of populations

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responding to changing habitats (Cosentino & Gibbs, 2022; Preite et al., 2019; Reid et al., 2016; Winchell et al., 2023), or emergence of novel pathogens (Palmer & Kishony, 2013). Yet, little consensus has been reached on when and if we should expect there to be evidence for genetic parallelism, even within closely related taxa (Bohutínská et al., 2021; Bolnick et al., 2018; Rosenblum et al., 2014).

Some of the debate regarding the discrepancy between patterns of phenotypic and genetic parallelism may, in part, be driven by the genetic architecture of polygenic traits and the level of biological organization being examined. Studies that investigate selection and genetic parallelism often do so by comparing candidate markers (e.g., SNPs) and/or candidate genes across replicate populations (Fraser & Whiting, 2020). Multiple studies have successfully used this methodology to demonstrate genomic parallelism at the level of SNPs (e.g., Colosimo et al., 2005; Hoekstra et al., 2006; Loh et al., 2013), genes (e.g., Nosil et al., 2008, 2018; Manceau et al., 2010; Moran et al., 2023; Stern, 2013; Walsh et al., 2019), and pathways (e.g., Birkeland et al., 2020; Cooper et al., 2014; Jacobs et al., 2020). Studies that are successful at showing genetic parallelism have generally done so in traits controlled by relatively few genes of large effect, which are more likely to be detected in genome wide scans (Hoban et al., 2016; Tiffin & Ross-Ibarra, 2014; Wellenreuther & Hansson, 2016). However, demonstrating genomic parallelism for complex polygenic traits (e.g., Shi et al., 2016; Wood et al., 2014) can be particularly problematic when thousands of associated SNPs are spread across the genome, each with a small effect on phenotypic variance (Yengo et al., 2022). Indeed, studies examining polygenic traits tend to find that even if heritability for a trait is high, it may account for little phenotypic variance (e.g., human height; Visscher, 2008; Wood et al., 2014).This gap between heritability estimates and trait variation has lead to a debate over what causes this "missing heritability" (Manolio et al., 2009). One explanation for this missing heritability is that most polygenic traits are shaped by a large number of interconnected genes that are associated with a smaller number of core genes and pathways (i.e., the "omnigenic" model; Boyle et al., 2017). Such complexity increases the likelihood of genetic redundancy in a trait in which changes to multiple SNPs or genes can produce the same phenotype through a shared genetic pathway (Láruson et al., 2020). Within this framework, we would expect the evolution of complex adaptive parallel phenotypes by selection to exhibit reduced parallelism at the SNP or gene level, but increased parallelism at the pathway level (e.g., Chaturvedi et al., 2022; Rose et al., 2018; Ryan et al., 2023; Walden et al., 2020). While recent studies have begun to look at parallelism across genetic levels by incorporating comparisons of SNPs, genes, and pathways to fully characterize genetic parallelism in suites of polygenic traits (e.g., Bohutínská et al., 2021; Daub et al., 2013, 2017; Szukala et al., 2023), there are a dearth of studies that explicitly test the hypothesis that genetic parallelism scales with genetic levels of organization.

Here, we investigate genetic parallelism hierarchically from SNPs to genes to genetic pathways by analyzing replicate instances of adaptation of a complex, polygenic trait in the island scrub-jay (*Aphelocoma insularis*), a bird species that is endemic to Santa Cruz Island, California, US (Delaney & Cheek, 2022). Despite the jay's entire distribution being limited to a small island (250 km^2), they exhibit parallel phenotypic divergence between different pine and oak habitats on the island. Jays in pine-dominated habitat have longer, shallower bills compared to jays in oak dominated habitat (Langin et al., 2015). Specifically, this pattern is repeated across three bishop pine (*Pinus muricata*) and island scrub oak (*Quercus pacifica*; Junak, 1995) ecotones in the west, central, and east regions of the island (Figure 1A, Figure S1; Fischer et al., 2009; Langin et al., 2015; Walter & Taha, 1999). This "microgeographic" divergence in bill morphology in island scrub-jays is thought to be adaptive, as it mirrors the same "macrogeographic" pattern described in California (*A. californica*) and Woodhouse's (*A. woodhouseii*) scrub-jays, in which similar patterns of divergence show longer, shallower bills are more efficient at extracting pine seeds from pinecones while shorter, deeper bills are more efficient at hammering open acorns (Bardwell et al., 2001; Langin et al., 2015; Peterson, 1993; Richardson et al., 2014). The observed differences in bill morphology are heritable, which suggests divergent selection is acting on bill morphology between oak and pine habitat types (Cheek et al., 2022; Langin et al., 2015). Bill morphology in birds also appears to have a polygenic basis and multiple potential candidate SNPs occur near or within genes that have been identified in pathways associated with bill development including bone morphogenic protein (*BMP*; Abzhanov et al., 2004, 2006; Badyaev et al., 2008), transforming growth factor beta (*TGF-beta*; Mallarino et al., 2011), and Wingless-related integration (*Wnt*) signaling pathways (Cheek et al., 2022; Lundregan et al., 2018). Given this background, the island scrub-jay provides an excellent system to explore genetic parallelism across the SNP, gene, and pathway genetic levels.

We used restriction-site associated DNA sequencing (RADseq) of 161 island scrub-jays sampled across each of three pine–oak ecotones on Santa Cruz Island to address the question: To what extent does genetic parallelism underly parallel adaptive phenotypic divergence and does it increase with increasing genetic levels of organization (SNP, gene, and pathway)? First, we quantified population-wide divergence in bill length and depth across each of the pine–oak ecotones (following Langin et al., 2015). Second, we estimated potential population-wide genetic structure, as tests for selection can be biased by underlying population structure (Battey et al., 2020; Lotterhos & Whitlock, 2015). Third, we used genotype–environment association (GEA) and genome-wide association (GWA) approaches to test two alternate hypotheses regarding the genetic underpinnings of habitat-linked divergence in bill morphology. If the genetic basis underlying bill morphology in any single population is controlled by a limited number of genes of large effect (e.g., Abzhanov et al., 2004, 2006; Lamichhaney et al., 2015, 2016), we expect that genetic parallelism would be observed across all hierarchical genetic levels (SNPs, genes, and pathways). Alternatively, if bill length is shaped by multiple, redundant genes of small effect within a limited number of key pathways critical in shaping bill morphology (e.g., *BMP*, *TGF-beta*, and *Wnt*), we may expect to observe SNPs and genes that are unique to different ecotones, but genetic parallelism only at the higher genetic levels of organization (i.e., genetic pathways). Finally, we combined multivariate analysis of allele frequency changes among each of the pine–oak ecotone replicate pairs with identified candidate loci to ex-

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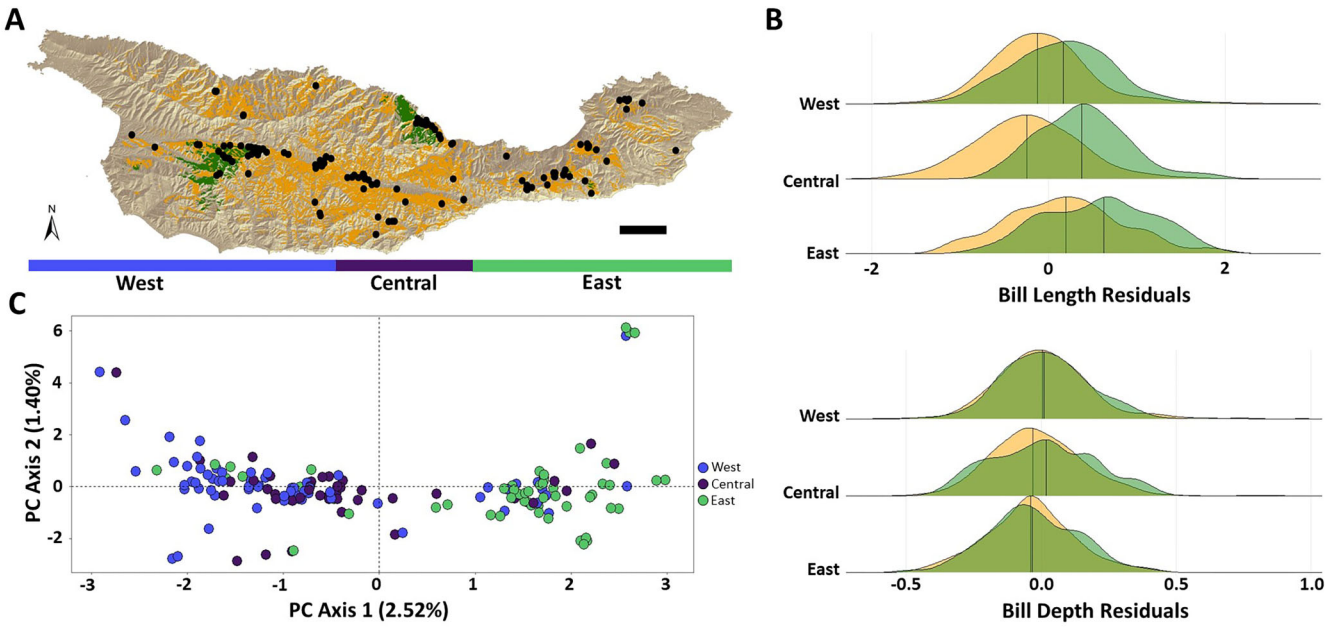

Figure 1. Island scrub-jays exhibit repeated habitat-linked phenotypic divergence at a microgeographic scale. (A) Study area of Santa Cruz Island, California, US, and habitat type (green, pine; orange, oak). Colored bars show the regional breakdown of Santa Cruz Island. Black bar represents 4 km. Genotyped island scrub-jays (black dots; $n = 161$) were sampled from each of the pine–oak ecotones (west oak = 30, central oak = 25, east oak = 23, west pines = 36, central pines = 17, and east pines = 30). Note that the sampling locations obscure the eastern pine stand, see Figure S1 for a higher resolution map. Divergence in bill morphology corrected for body size (B) was observed in bill length, but not bill depth, within western and central pine–oak ecotones using measurements from 1,484 island scrub-jays (western oak = 457, central oak = 435, eastern oak = 89, western pines = 356, central pines = 56, and eastern pines = 91). Density distributions of bill measurements by region are shaded by habitat (green, pine; orange, oak). Black lines represent the medians of bill length measurement corrected for body size by corresponding habitat and region. (C) Principal components analysis showing the genetic relationships of 161 island scrub-jays along the first two PC axes based on 63,355 SNPs. Colored circles correspond to individuals colored by which pine stand they are geographically closest to using the region breakdown of panel A.

plore signals of genetic parallelism at specific regions across the genome.

Materials and methods

Sample collection

Juvenile and adult male and female island scrub-jays were captured using either mist nets or box traps from each of the three pine–oak ecotones from 2009 to 2021 (Table S1). Morphological measurements were collected from each captured individual using digital calipers to record: bill length, measured from the anterior end of the nares to the tip of the bill; bill depth, measured at the anterior end of the nares; and tarsus length (to ± 0.01 mm). Wing chord and tail length were measured with a ruler (to ± 0.5 mm).

Whole blood samples were taken from the brachial vein and preserved in Queen's lysis buffer (Seutin et al., 1991). High-quality genomic DNA was extracted from blood samples (~50 μl) with DNeasy Blood and Tissue Extraction kits (Qiagen) using the manufacturer's recommended protocol.

Bill morphometrics

Langin et al. (2015) demonstrated repeated patterns of divergence in island scrub-jay bill morphology between individuals captured in pine versus oak habitats. These findings were consistent regardless of sex or age for a sample of 463 individuals measured by the same person. To determine if such a pattern of phenotypic divergence is replicable across many observers, we used generalized linear mixed models implemented in the lme4 R package (Bates et al., 2015) to determine whether habitat explains variation in bill length and depth. Body size in island scrub-jays is correlated with age and sex in addition to bill morphology (Langin et al., 2015), so we summarized body size using the first axis generated from a principal components analysis (PCA) on tarsus, wing, and tail lengths which explained 55.01% of the variation. We chose not to incorporate body weight in our measure of body size because body mass in birds can vary drastically depending on the season or the time of day (Clark, 1979). Only jays captured in August through December were included in morphometric analyses to minimize any seasonal effects on bill length variation due to wear (Langin et al., 2015). We also excluded juvenile individuals that were banded as nestlings as their bills change rapidly between fledging and reaching their formative plumage at approximately 6 months of age (Delaney & Cheek, 2022).

To assign individuals to habitat type, we used ARCGIS software and R scripts to calculate the % pine and % oak and % "other" (e.g., grasslands, or other habitat unsuitable for island scrub-jays) within a 300-m radius of each scrub-jay sampling location (the diameter of the largest island scrub-jay territory; Caldwell et al., 2013) using a reclassified 2005 vegetation map of Santa Cruz Island (Langin et al., 2015; The Nature Conservancy, 2007). Individuals were classified as pine if they were within 300 m of a pine polygon (Cheek et al., 2022; Langin et al., 2015). The jays are considered a single population structured by a pattern of isolation-by-distance likely driven by limited dispersal (Cheek et al., 2022; Langin et al., 2015). We therefore assigned individuals to the west, central, or east pine–oak ecotone bycalculating the distance from each capture location to the nearest pine

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polygon and assigning individuals based on the pine stand to which they were closest to geographically (Figure 1A, Figure S1).

We included habitat type as model predictors and included bander (the observer who measured the jay) as a random effect. We then used the residuals from a linear mixed model regression of bill length on our body size index and included individual ID as a random effect to account for repeated measures. We used these residuals as a body size-corrected measure of bill length and depth in our analyses. We determined the significance of the model using all individuals, and then reran models using individuals from each pine–oak ecotone to test the significance of bill morphology divergence by region.

Genotyping

Reduced representation techniques, such as RADseq (Baird et al., 2008) have made the generation of genome-wide genetic data more accessible in nonmodel systems by subampling the genome at both coding and noncoding regions to efficiently provide sufficient marker density to detect signatures of genetic parallelism (Andrews et al., 2016; Catchen et al., 2017; Whiting et al., 2022). We used the RADseq method described by Ali et al. (2016) to conduct targeted genotyping of SNPs across 179 individuals using the restriction enzyme *PstI*.

We built RADseq libraries following the bestRAD protocol described in Ali et al. (2016). Briefly, DNA was normalized to a final concentration of 50 ng in a 10 μl volume and digested with restriction enzyme PstI (New England Biolabs, NEB). The fragmented DNA was then ligated with PstI specific adapters with unique barcodes prepared with biotinylated ends, samples were pooled and cleaned using 1 × Agencourt® AMPure XP beads (Beckman Coulter). Pooled and clean libraries were sheared to an average length of 400 bp with a M220 Covaris Focused-ultrasonicator to ensure appropriate length for sequencing, and an Illumina NEBNext Ultra DNA Library Prep Kit (NEB) was used to repair blunt ends and ligate on Trueseq adaptors to the resulting DNA fragments. We size selected using a Sage Science Blue Pippin for 350–550 bp fragments to select DNA fragments with an average length of 450 bp. Libraries were enriched with Polymerase Chain Reaction (PCR) and cleaned again with Agencourt® AMPure XP beads. We assessed final library fragment size distributions and concentrations on an Agilent Tapestation 2200 and Qubit 2.0 fluorometer. The resulting libraries were sequenced on two S4 300 Cycle lanes of an Illumina NovaSeq 6000 for paired end 150 bp sequencing at the University of Oregon Genomics Core Facility (gc3f.uoregon.edu).

We used FASTQC v.0.11.8 (Andrews, 2010) to assess data quality. Individuals were demultiplexed and read quality (Phred score) was assessed using a sliding window approach implemented in the process_radtags function from STACKS v.2.53 (Catchen et al., 2011, 2013). We discarded reads with an uncalled base or with low quality scores (Phred score $\leq$10). We removed PCR duplicates using *clone_filter* from STACKS version 2.41 (Rochette et al., 2019). During preliminary quality control, we discovered a high number of SNPs after bp position 142, followed by a sharp decrease in SNPs, suggesting high error rates toward the ends of our reads. We therefore trimmed reads to 140 bp using TRIMGALORE! version 0.6.7 (Martin, 2011). We mapped clean, trimmed, reads to the California scrub-jay reference genome (GCA_028536675.1; DeRaad et al., 2023) using BWA-mem v. 0.7.17 (Li & Durbin, 2009), and used the default parameters in the *ref_map.pl* in STACKS to create a RAD loci catalog. We applied a coarse filter using populations: -p 1, -R 0.3, -min_mac 3. We discarded individuals with high missingness (data missing for >80% of SNPs) before filtering with the R package *radiator* (Gosselin et al., 2020) using the following settings: retain SNPs with global minor allele count (MAC) >3; retain loci with coverage between 6 and 100 per individual; retain loci with <20% missing data across individuals; retain one SNP per RADtag, keeping the SNP with highest MAC. We removed 2,394 SNPs that deviated significantly ($p < .0001$) from Hardy–Weinberg as these were likely genotyping errors (Gosselin et al., 2020). We used the *WHOA* package version 0.0.2.999 (Anderson, 2019) to assess heterozygote miscall rates for all sites. We did not remove putative siblings based on recommendations from Waples & Anderson (2017). We used R version 4.1.2 for all analyses (R Core Team, 2020).

As the California scrub-jay genome is a scaffold-level assembly and unannotated (scaffold N50 = 11.5 Mb), we mapped scaffolds to individual chromosomes of the zebra finch genome assembly (GenBank accession GCA_008822105.2) using the default parameters of SATSUMA SYNTENY version 2.1.0 (Grabherr et al., 2010). We then used custom R scripts modified from Van Doren et al. (2017) to reorder our island scrub-jay VCF file relative to the zebra finch genome and remove SNPs where chromosomal positions could not be determined. We imputed missing genotype values for all SNPs (22.1% missing data total) using BEAGLE v. 5.1, which uses a localized haplotype cluster model to impute missing genotypes (Browning & Browning, 2016), with 25 iterations and an N_e setting of 350 (Cheek et al., 2022). This imputed data set was used for analyses requiring complete data frames: PCA , GEA tests, and GWA).

Island scrub-jays are not obviously sexually dimorphic in size. However, female corvids including scrub-jays produce a rapid, broad-band vocalization referred to as a "rattle" call (Delaney & Cheek, 2022; Goodwin, 1976; Woolfenden & Fitzpatrick, 2020). We sexed individuals using individual or partner rattle call vocalizations observed in the field, or sex assignments from previous studies (Caldwell et al., 2013; Desrosiers et al., 2021; Langin et al., 2015). To assess the remaining individuals of unknown sex, PCRs were performed using the CHD1F/CHD1R primer set (Çakmak et al., 2017), available through Bento Bird Lab (Bento Bioworks Ltd., UK) following the manufacturers thermal cycling protocol.

Population structure

We calculated observed (H_O) and expected (H_E) heterozygosity of variant sites, inbreeding coefficient (F_{IS}), and nucleotide diversity (π) across all sites (variant and fixed) from our unimputed filtered dataset using the *populations* program within STACKS. We estimated effective population size (N_e) using the LD method of NEESTIMATOR version 2.1 (Do et al., 2014). Prior to estimating N_e, we identified and removed candidate adaptive markers using partial RDA (pRDA) and removed SNPs with more than 10% missing data to estimate N_e using a higher quality SNP dataset of presumptive neutral markers. Physically linked loci can can

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have a larger effect on N_e estimates than either population size or sample size (Waples et al., 2016). To account for this bias in our estimate of N_e, we calculated bias corrections for N_e and jackknifed confidence intervals based on 31 chromosomes for the zebra finch genome in NEESTIMATOR. We visualized population structure of the individual-based data using PCA, which attempts to visualize how genetic variation is distributed across a reduced number of orthogonal axes without underlying assumptions of genetic groups. We compared these results to ADMIXTURE version 1.3 (Alexander et al., 2009), which is an ancestry estimation method that tests for discrete population structure by sorting individuals into a number of hypothetical clusters or populations (K) composed of genetically similar individuals. We used the imputed, neutral data from BEAGLE for our PCA analyses. Because there are three pine–oak ecotones, we tested K = 1–6 in ADMIXTURE to determine if each pine and oak ecotone represent a distinct group and used a cross-validation plot to determine the best value of K. Finally, we tested for isolation-by-distance using genetic distance based on the proportion of shared alleles (Bowcock et al., 1994) using ADEGENET (Jombart, 2008; Jombart & Ahmed, 2011), and pairwise geographic distances between individuals calculated using the *geodist* package to measure "geodist" (Padgham & Sumner, 2020). We tested for isolation-by-distance using a Mantel test with 10,000 permutations and the "Pearson" method in the *vegan* r package (Oksanen et al., 2013).

Identification of loci associated with habitat

To search for patterns of parallel adaptive variation between pine and oak habitat, we grouped all sampled jays into the three pine–oak ecotones according to which pine stand they were geographically closest to (Figure 1A, Figure S1). We identified candidate adaptive markers associated with habitat using pRDA (hereafter referred to as RDA-GEA), a multivariate GEA analysis that is well suited to identify weak, polygenic signatures of selection related to environmental variables (Capblancq & Forester, 2021; Forester et al., 2016, 2018). We used the % pine and % oak within a 300-m radius of each scrub-jay sampling location as our environmental predictors. We accounted for population structure using longitude of each individual's sampling location as a third "conditioned" matrix in the RDA-GEA (Capblancq & Forester, 2021) because previous research showed that isolation by distance (i.e longitude) was a strong predictor of interindividual genetic differentiation (Cheek et al., 2022; Langin et al., 2015). We tested the significance of both the global model and model terms (% pine and % oak) for each pine oak ecotone using the anova.cca function in *vegan* with 10,000 permutations. Loci loading ± 2.5 standard deviations from the mean loading on the two RDA axes were identified as significant candidate loci (Forester et al., 2018). We determined the predictor with which each outlier locus was most strongly associated based on the absolute value of the predictor correlation at a given SNP.

Identification of loci underlying variation in bill morphology

We increased the sample size of individuals measured for bill morphology reported in previous studies (Langin et al., 2015). We found that jays that occur in pine have longer bills relative to jays living in oak habitat, but we do not observe significant divergence in bill depth. To test if genetic divergence mirrors parallel phenotypic divergence in bill length, we first performed a PCA on measurements of wing, tail, and mean tarsus length for the 161 genotyped individuals that passed our quality control filters. We extracted values from the first PC axis, which explained 75% of the variance, as an index of overall body size. We then performed a regression of bill length on this index of body size while including the person who banded the birds as a random effect and used the residuals as a body size-corrected measure of bill length in our analyses. One juvenile female captured in the east ecotone was a clear outlier (>3 standard deviations from the mean of the 161 jays genotyped) and was removed from further analyses. We then used a separate RDA (hereafter referred to as RDA-GWA) to model the effect of all 66,503 imputed loci on our body size-corrected measures of bill length while controlling for population structure by using longitudinal coordinates of sampling localities as a proxy for isolation-by-distance. We identified outlier SNPs associated with variation of bill length using the criterion of loading scores ± 2.5 SD from the mean loading.

Functional annotation of loci and quantifying parallelism

Visual inspection of linkage disequilibrium (LD) plots using LD calculated within a 500-kb genomic window in VCFTOOLS 0.1.16 with minimum and maximum alleles setting of 2 (Danecek et al., 2011) suggested high linkage between biallelic loci separated by ≤25 kb (Figure S3). We therefore input our zebra finch-mapped SNPs in BEDOPS version 2.4.41 (Neph et al., 2012) and output all genes within 25 kb of outlier SNP coordinates in the annotated zebra finch genome. We then used the R package biomart (Durinck et al., 2005, 2009) to extract gene ontology (GO) information for each gene found within our query sequences using the available zebra finch Ensembl database. We identified Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among genes associated with outlier loci using enrichKEGG function implemented in clusterprofiler 4.8.0 (Yu et al., 2012). We used a Fisher's exact test implemented in the SuperExactTest R package (Wang et al., 2015) to quantify parallelism at each genetic level (SNPs, genes, and pathways).

Identification of parallel evolution

We tested if repeated evolution of bill morphology within island scrub-jays occurs due to parallel allele frequency differences between jays sampled in pine and oak habitat using *AF-vapeR* (Whiting et al., 2022). This multivariate method identifies parallel regions of the genome using allele frequency change vectors from multiple population pairs, such that each pair represents a change in allele frequency from Population A to Population B (Whiting et al., 2022). We assumed that genomic regions that show a similar degree of change between replicate ecotone pairs are indicative of parallelism and potential evidence of selection on those genomic regions. Specifically, jays living in pine habitat are more closely related to individuals in adjacent oak habitat than to jays in other pine stands (Cheek et al., 2022; Langin et al., 2015). Therefore, we tested for allele frequency changes in specific regions of the genome between

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each of our three, replicate pine–oak ecotone populations pairs. Because LD noticeably decays after 100 kb (Figure S3), we scanned our genomic data using nonoverlapping windows of 10 SNPs (median physical window size of 119 kb) and determined which windows show significant eigenvalues by running 10,000 permutations against a randomized null distribution. Windows were identified as outliers if the observed eigenvalues were above 95% of our randomized null distribution to identify regions indicative of weak parallelism between our replicate population pairs. We considered an allele frequency change as fully parallel across our three replicated populations if each population had the same sign loading on the first eigenvector. This approach allowed us to determine if SNPs and/or genes identified as potential candidates by our RDA-GEA and RDA-GWA occur within genomic regions that may have undergone repeated selection between our pine–oak replicate pairs.

Results

Parallel divergence in phenotype

We confirmed phenotypic parallelism as bill length differed between island scrub-jays captured in pine and oak habitats on Santa Cruz Island (Figure 1B) using 1,047 individuals in addition to 437 of the individuals originally reported in Langin et al. (2015; Table S2). Jays captured in pine ($n = 503$) had longer bills compared to jays captured in oak ($n = 981$; p-value bill length $< .001$; 95% confidence interval [0.307 mm, 0.431 mm]) after correcting for body size. This pattern of divergence was repeated across the west and central pine–oak ecotones (n west $= 814$; p-value bill length west $< .001$; 95% confidence interval [0.352 mm, 0.620 mm]; n central $= 491$; p-value bill length central $< .001$; 95% confidence interval [0.310 mm, 0.587 mm]). Divergence of bill length was in the expected direction (i.e., longer bills in pine), but not statistically significant in the east (n east $= 180$; p-value bill length east $= .134$; 95% confidence interval [−0.086 mm, 0.684 mm]). Contrary to Langin et al., 2015, we found that bill depth did not vary significantly between individuals captured in pine versus oak habitat (p-value bill depth $= .656$; 95% confidence interval [−0.0218 mm, 0.0138 mm]). Thus, we focused only on bill length in subsequent analyses.

Genotyping

We sequenced 179 individuals sampled across all three pine–oak ecotones on Santa Cruz Island (Figure 1A, Figure S1). We obtained a total of 789,466,582 reads with an average of 1,972,737 reads per individual following clone_filter. Of our trimmed reads, 91.3% were mapped to the California scrub-jay reference genome. After filtering, the final data set included 69,268 SNPs genotyped in 161 individuals with overall missingness of 22.1% and average depth of coverage of 5.7 × (standard deviation = 2.0x, range = 2.1–13.5x). Heterozygote miscall rates were low (mean = 3.1%). We mapped 81.4% of the California scrub-jay scaffolds to the zebra finch genome. This resulted in 66,503 loci with chromosome-level positional information.

Population genetics

We calculated the following population genomic parameters in STACKS using the unimputed matrix of 66,503 SNPs and 161 island scrub-jays: $H_O = 0.204$, $H_E = 0.210$, $F_{IS} = 0.033$, and $\pi = 0.00062$. The N_e estimate was 325.5 based on 36,919 unimputed neutral SNPs with less than 10% missing data. The jackknifed 95% confidence interval was 260.0–423.5. The ADMIXTURE results using 66,503 unimputed SNPs found that K = 2 was the "best" supported value of K by minimizing cross-validation error in ADMIXTURE (Figure S2). Mantel tests using 63,355 neutral SNPs indicated weak spatial population structure (p-value $= .0651$; $r = 0.03419$) consistent with a pattern of isolation-by-distance primarily driven by longitude according to our PCA results (Figure 1C).

Parallel signatures of selection driven by habitat at higher genetic levels

The relative proportion of pine and oak habitat within a 300-m radius of each individual's capture location was associated with patterns of genetic variation we found in our RDA-GEA (Table S3). This pattern was repeated in each of the pine–oak ecotones based on RDA-GEAs of individuals captured in the west, central, and east regions of Santa Cruz Island (Table S3). Triplots of the two RDA axes for each model show individuals (colored circles) arranged in ordination space relative to their relationship with the predictor variables (black arrows, Figure 2A). The RDA-GEA identified 3,576 SNPs in the west ecotone, 3,813 SNPs in the central ecotone, and 3,125 in the east ecotone that are associated with pine and oak habitat (Table S4). However, only 15 of these SNPs (0.15%) were shared across all 9,933 unique SNPs identified between the three ecotones (Figure 2B). We identified a total of 8,136 genes that were within 25 kb of our candidate loci that were realigned to the annotated zebra finch reference genome, 330 (4.05%) of which were shared across all three ecotones (Figure 2C). Finally, of the 8,136 potential candidate genes, we identified 1,178 genes that were within 160 genetic pathways (Table S5). Of the 160 pathways, 136 (85%), were shared across all three ecotones. We observed significant (p-value $< .001$) overlap between the three ecotones only at the pathway level, but not SNP or gene (p-value $= 1$), level according to our Fisher's exact test (Figure 2D, Table S5).

Hierarchy of parallelism scales in bill length

Our RDA-GWA analysis of variation in body size-corrected measure of bill length identified 1,105 SNPs in the west ecotone, 1,180 SNPs in the central ecotone, and 1,253 SNPs in the east ecotone (Figure 3A). However, none of the 3,459 SNPs flagged by our RDA-GWA were shared across all three ecotones. A total of 3,701 genes were within 25 kb of our candidate SNP loci, 26 (0.70%) of which were shared across GWA analyses (Table S5; Figure 3B; Figure S4). Potential candidates found in the GO annotation reports included multiple genes (e.g., *SMPD3*, *NFIA*) associated with the bone morphogenetic protein pathway. Furthermore, 10 genes identified in the RDA-GWA analyses were also flagged by the GEA (Table S5). Our RDA-GWA flagged 80 genes that occur within 151 genetic pathways (Figure 3C), some of which are known to affect bill morphology including MAP

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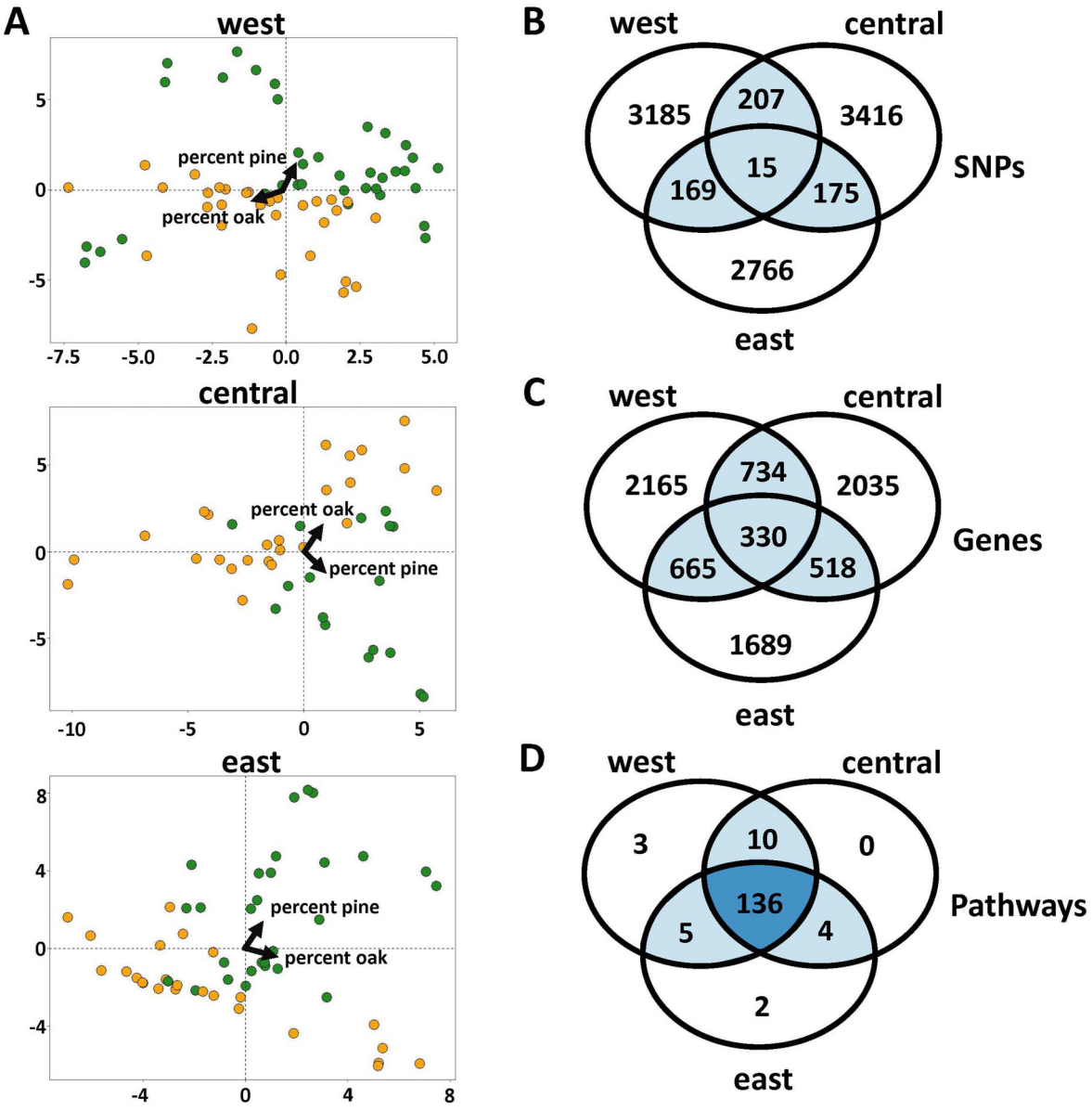

Figure 2. Evidence of repeated habitat-linked selection between 161 island scrub-jays sampled in pine and oak habitat. GEA using redundancy analysis showed habitat-linked genetic divergence in the west, central, and east ecotones. Colored points show where individual samples load for RDA axes 1 and 2 shaded by which habitat they were captured in (green, pine; orange, oak) based on a pRDA conditioned on the geographic location of each individual using 66,503 SNPs as the response and relative proportion of pine and oak habitat within a 300-m radius of sampling locality as the predictors (black vectors). The west, central, and east panels (A) represent results of three different RDAs of individuals grouped by which pine stand they are geographically closest to. Venn diagrams overlap in candidate SNPs (B), genes (C), and pathways (D) across the three pine–oak ecotone RDAs show genomic parallelism increases at higher genetic levels in island scrub-jays.

kinase activity (*MAPK*), *TGF-Beta*, calcium, and *Wnt* signaling pathways (Figure S5; Table S5; Yusuf et al., 2020). Of the 151 pathways identified, 85 (56%) were shared between all three ecotones (Figure 3C). A Fisher's exact test of overlapping SNPs, genes and pathways identified by our GWA reveal significant overlap (p-value $< .001$) at the pathway, but not SNP or gene level (p-value $= 1$).

Parallelism between replicates

The *AF-VapeR* analysis identified 2,914 outlier windows on the first three eigenvectors using 99% quantile cutoff from the randomized allele frequency matrices, and 186 outlier windows on eigenvectors 1 and 3 using a cutoff of 99.9%. No outlier windows were fully parallel (i.e., all three pine–oak ecotone population pairs experienced identical allele frequency changes within these windows) using these stricter cutoffs. We identified 166,616 outlier windows on the first three eigenvectors using the 95% cutoff, of which, 4,836 were fully parallel. None of the candidate SNPs we identified in our RDA-GEAs that were associated with habitat variation were found within these fully parallel outlier windows. However, of the 3,459 unique SNPs associated with variation in bill morphology across our three RDA-GWA analyses, 914 loci were found within 479 fully parallel outlier windows using the 95% quantile cutoff. Seven of the annotated genes identified by all three RDA-GWA analyses (*SLIT3*, *NIPSNAP2*, *HIVEP3*, *RBFOX1*, *MRPS17*, *AUTS2*,

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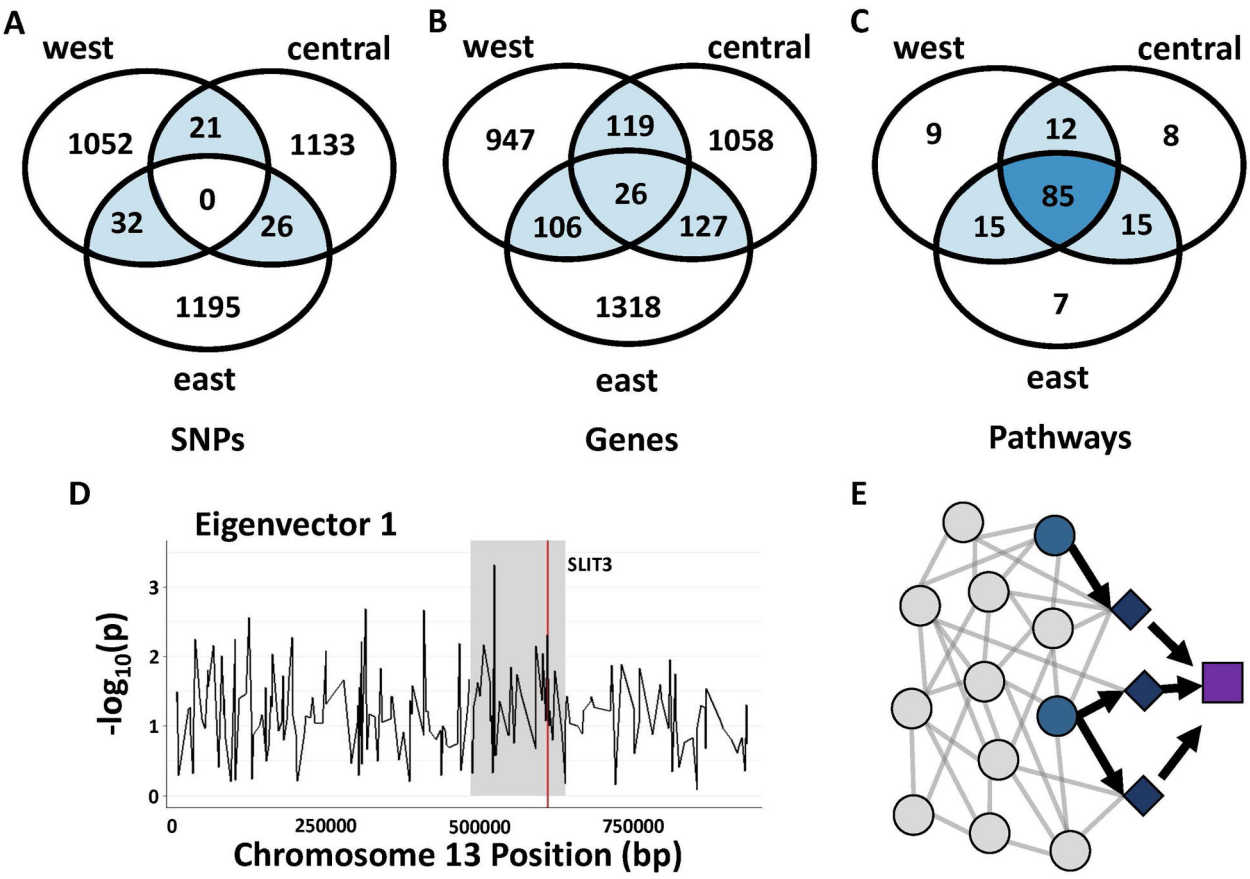

Figure 3. Genetic parallelism increases at higher genetic levels in a complex trait. Venn diagrams show that overlap in candidate SNPs detected by GWA analysis (A), genes (B), and pathways (C) associated with variation in bill morphology between island scrub-jays sampled in three pine–oak ecotones increases at higher genetic levels. Genetic parallelism among pine–oak population pairs within chromosome 13 (D) identified as a significant loading on eigenvector 1 within the window (highlighted gray box). Here, the significant window overlaps with the location of the *SLIT3* gene (red line). These results are consistent with the omnigenic model of polygenic genetic architecture shown in (E), in which many peripheral genes (gray circles), and SNPs within them, may have indirect effects (gray lines) on core genes (dark blue circles) that have direct effects (black arrows) within pathways (blue diamonds) that affect traits (purple square; Figure adapted from Fagny & Austerlitz, 2021).

and *LOC100230978*) also overlapped in six fully parallel windows (Figure 3D).

Discussion

The degree of concordance between phenotypic and genetic parallelism has been central to debates about the definitions of parallelism and convergence (Arendt & Reznick, 2008; Elmer & Meyer, 2011; Rosenblum et al., 2014). Yet, evidence for genetic parallelism is likely to be dependent on what level researchers focus their efforts in the genetic hierarchy and the genetic architecture of the trait. We tested for genetic parallelism across hierarchical genetic levels within a single continuously distributed population of jays, which exhibit isolation-by-distance across Santa Cruz Island. In this context, we expected the standing genetic variation to be relatively spatially homogenous because of weak isolation by distance and genetic parallelism to be observed across all hierarchical genetic levels (Bohutínská et al., 2021; Conte et al., 2012; Ord & Summers, 2015). Surprisingly, we found that genetic signatures of habitat-linked selection and genetic associations with bill morphology did not overlap significantly at the SNP level between three different pine–oak habitat replicates despite only being a few kilometers from each other. In contrast, we observe some overlap at the gene level, and significant overlap (i.e., parallelism) at the pathway level. Moreover, we observe some parallel changes in allele frequencies across the three ecotones that also overlap with a moderate fraction of candidate SNPs associated with bill morphology. Our findings suggest that microgeographic adaptation in island scrub-jays is shaped by multiple, redundant genes of largely small effect within a limited number of key genetic pathways.

Genetic parallelism within an omnigenic hierarchy

If we interpret the observed increase in parallelism at higher molecular levels in island scrub-jay bill morphology through an omnigenic or polygenic perspective, then we would expect a pattern where most SNPs and genes associated with phenotypic variation to have relatively small effects. However, some loci with larger effects should be limited to a few highly conserved genetic and developmental pathways (Figure 3E; Boyle et al., 2017). Other studies have also found such parallelism at higher genetic levels both within (e.g., Kingsley et al., 2009; Schiebelhut et al., 2023; Zhang et al., 2021) and between species (e.g., Manceau et al., 2010; Natarajan et al., 2015; Urban et al., 2021). Such results suggest that despite multiple potential genetic routes to the same

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phenotypic outcome (Yeaman et al., 2018; Láruson et al., 2020) these routes become more limited when moving from SNPs to genes, and genes to pathways.

In birds, several studies have implicated the same genetic pathways and genes involved in shaping bill morphology (e.g., *BMP*, calmodulin; Abzhanov et al., 2004, 2006; Badyaev et, al.,2008; Mallarino et al., 2011; Yusuf et al., 2020). We also found multiple candidate SNPs and genes associated with bill length that occurred within fully parallel windows identified using *AF-vapeR*. The gene *SLIT3* is a notable example of such parallelism, which has GO terms associated with anatomical structure development and is involved in several genetic pathways important for regulating *BMP* including *Wnt* and *TGF-beta* signaling (Gong & Si, 2023; Jiang et al., 2022). We identified *SLIT3* in all three RDA-GWA analyses and found that *SLIT3* occurred in a window that contained allele frequency changes that were fully parallel (repeated between all three pine stands) according to *AF-vapeR* (Figure 3D). However, we only observed fully parallel windows using the least stringent quantile cutoff of 95%, and only about 9% of these windows overlapped with candidate SNPs associated with bill morphology. Because a vast majority of the windows were unique to *AF-vapeR* and exhibited weakly significant evidence of full parallelism, it is possible that selection is a relatively weak force driving phenotypic divergence in this system relative to other evolutionary mechanisms, such as habitat selection due to phenotype-dependent dispersal (Edelaar and Bolnick, 2012; Clancey et al., 2024). The polygenic architecture underlying bill divergence may also reduce the impact of selection by minimizing the effect selection has on individual loci that explain little variance (Manolio et al., 2009). Collectively, these results reinforce the view that polygenic selection is likely a complex process (Barton & Olusanya, 2022; Szép et al., 2021), where selection on a given SNP may be weak because the path from genomic variation to phenotypic variation can take multiple routes (Láruson et al., 2020).

Parallelism and genetic architecture of bill morphology

Our morphological analyses show parallel divergence in bill length between scrub-jays living in pine versus oak habitat (Cheek et al., 2022; Clancey et al., 2024; Langin et al., 2015). While the divergence in bill length in island scrub-jays is relatively small in absolute terms (~1 mm), it mirrors adaptive differences in mainland *Aphelocoma* species (Bardwell et al., 2001; McCormack & Smith, 2008; Peterson, 1993). Indeed, similar relative differences in bill morphology have been shown to have consequential impacts on feeding performance, and ectoparasite control, which presumably affect fitness (see Bardwell et al., 2001; Clayton et al., 2005; Moyer et al., 2002). Habitat-linked differences in feather stable isotope composition and foraging behavior among island scrub-jays (Yeatts et al., 2025) provides additional evidence that the observed variation in bill morphology is linked to different diets under selection (Cheek et al., 2022; Langin et al., 2015, 2017). Furthermore, the 10 genes detected in both our RDA-GEA and RDA-GWAS included genes associated with pathways involved in bill morphology including *Wnt* signaling pathways (Lundregan et al., 2018; e.g., *SLIT3*, *ZNF385D*, *NPAS3*, *KCNQ5*, and *ASTN2*; Table S5). If differential wear and tear between habitats were driving differences in bill morphology, we would not expect to find shared genes linking habitat-linked selection associated to variation in bill morphology. Several candidate loci within or near genes that we identified in all three RDA-GWA analyses contained GO terms that were associated with skeletal and cartilage development (e.g., *SMPD3* and *NFIA*). These genes are also involved in *BMP* signaling, which is attributed to diversification of avian bill morphology (Abzhanov et al., 2004; Badyaev et, al.,2008; Mallarino et al., 2011; Yusuf et al., 2020). We also identified multiple growth factors and genes unique to one or two regions that are involved in pathways that likely impact the regulation of *BMP*, including *Notch*, *TGF-beta*, *MAPKs*, and *Smads* (Massagué, 2003; Fuentealba et al., 2007; Sapkota et al., 2007; Brugmann et al., 2010; Rahman et al., 2015; Table S5). This evidence provides additional support that bill morphology in island scrub-jays is a polygenic trait and likely the result of multiple, interacting pathways as seen in other bird species (Bosse et al., 2017; Lundregan et al., 2018; Gamboa et al., 2022).

Population genetics and spatial variation in selection

Both theoretical and empirical studies demonstrate that genetic parallelism should be high between closely related populations due to the increased likelihood they share the same ancestral pool of genetic variants (Bohutínská et al., 2021; Conte et al., 2012; Ord & Summers, 2015; Waters & McCulloch, 2021). We found evidence for a relatively small N_e (~325), as was previously found using fewer loci (Cheek et al., 2022) and low nucleotide diversity (π) relative to that observed in the mainland congener (DeRaad et al., 2022). We did not observe a significant impact of temporal variability on genetic diversity (see Supplementary material). While we observed some spatial structuring between island scrub-jays living in the east and west regions of Santa Cruz Island according to our ADMIXTURE results (Figure S2), our Mantel tests revealed a weak but positive relationship between geographic distance and genetic distance. Therefore, we believe the K = 2 result from ADMIXTURE is largely due to isolation-by-distance driven by limited dispersal (Figure 1C; Cheek et al., 2022; Langin et al., 2015). We therefore expected that parallelism should have been high at the SNP and gene level given the observed lack of population structure and recent shared ancestry (Delaney & Wayne, 2005; McCormack et al., 2011). Instead, we found limited parallelism at the SNP level between island scrub-jays residing in replicate pine–oak ecotones.

While the causative factors driving a lack of genetic parallelism at the SNP level underlying parallel traits remains a topic of discussion (Barghi et al., 2020; Bolnick et al., 2018; Elmer & Meyer, 2011), what is clear is that genomic parallelism is not common, even in highly controlled experiments (e.g., Bailey et al., 2017; Lenski, 2017; Poore et al., 2023). One potential factor beyond the scope of this study is the potential impacts of noncoding regions which could lead to patterns of phenotypic convergence that would not be within identifiable coding genes (Shakya et al., 2025). However, if selection is targeting the same genomic regions to generate the repeated divergence among pine oak population pairs in island scrub-jays, then we would expect repeated divergence in at least a few putative candidate SNPs within parallel genomic regions because of shared genetic variation (i.e.,

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population sorting; Lee & Coop, 2017, 2019; MacPherson & Nuismer, 2017; Waters & McCulloch, 2021). However, none of the SNPs flagged by our RDA-GEA analysis were found within any of the parallel windows identified in our *AF-vapeR* analysis of the three pine–oak population pairs. Because these parallel outlier windows were unique to AF-VapeR, it suggests that some of the parallel windows we observed may have originated through nonselective processes (Whiting et al., 2022). For example, the same windows could have reached fixation in each of the ecotones via genetic drift (Borowsky, 2018; Lee & Coop, 2019) or shared ancestral variation (Waters & McCulloch, 2021).

Another potential explanation for the observed lack of parallelism in our RDA-GEA analysis is that several dimensions of environmental variation likely cause quantitative differences in selection between pine and oak habitats on Santa Cruz Island. The eastern pine stand, for example, is smaller and more fragmented than the central or western pine stands, which could impact the overall strength of habitat-linked selection in that region. Coastal fog, temperature, and topography may also interact to form a climatic gradient along the east west axis of the island, with more arid conditions in the eastern region (Fischer et al., 2009; Morrison et al., 2011; Gamboa et al., 2022). Indeed, some of the genes that were shared among ecotones in the RDA-GEA analysis included GO annotations related to temperature (e.g., *HTR1B*, *GRM1*, *ZNF423*, and *NR1H3*), while several genes related to water homeostasis were unique to different regions (e.g *SCNN1G*, *EXT1*, and *EXT2*). Thus, despite the expectation that pine and oak habitats impose similar divergent selection, quantitative environmental differences likely exist among the qualitatively similar habitats (see Auld & Brand, 2017; Bailey et al., 2015; Kaeuffer et al., 2012; Schiebelhut et al., 2023). Future studies should move beyond bill morphology and investigate potential physiological traits in island scrub-jays that could be linked to climate related environmental features (e.g., thermal tolerance and feather structure; Gamboa et al., 2022). Given the island scrub-jay is vulnerable to catastrophic population declines due to climate change and disease (Bakker et al., 2020), it is critical that population management focuses on maintaining genetic variation across the genome to maintain adaptive genetic potential.

Conclusion

We found strong evidence for parallelism at the pathway level, but little evidence for parallelism among candidate SNPs associated with bill morphology and environmental variation. Our results support the idea that genetic parallelism in polygenic traits may be best explained by considering their polygenic and omnigenic basis, in which a very large number of SNPs affect a given trait through a smaller number of core pathways. Furthermore, we found only weak evidence of parallelism across the genome, which suggests selection alone may be a relatively weak force in this system and other evolutionary drivers of divergence, such as habitat dependent dispersal may play a more significant role. Overall, our findings imply that parallelism at the phenotypic level does not guarantee parallelism across all genetic levels even within a single continuously distributed population.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

All associated code and data are available on the Dryad Digital Repository (DOI: 10.5061/dryad.hx3ffbgpf).

Ethical statement

All work with living birds was approved by the Institutional Animal Care and Use Committees at Colorado State University (IACUC: #887) and the Smithsonian Institution.

Author contributions

C.K.W, P.A.H, R.G.C., T.S.S., and W.C.F designed the study. R.G.C collected samples with support from C.K.G., S.A.M., T.S.S., and W.C.F, generated the genomic data with guidance from P.A.H. and W.C.F., analyzed the data and wrote the paper with input from all authors.

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Conflict of interest

The authors declare they have no conflict of interests.

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