DISSERTATION

GENOMICS OF HABITAT-LINKED MICROGEOGRAPHIC ADAPTATION IN AN ISLAND ENDEMIC BIRD

Submitted by

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ABSTRACT

THE GENOMICS OF HABITAT-LINKED MICROGEOGRAPHIC ADAPTATION IN AN ISLAND ENDEMIC BIRD

A fundamental goal of evolutionary biology is to understand the mechanisms that maintain adaptive diversity. This dissertation focuses on the interplay of two key evolutionary mechanisms - natural selection and gene flow. While natural selection is often portrayed as a driving force of adaptive evolution, gene flow is assumed to disrupt selection by introducing maladapted alleles into locally adapted populations. Yet this paradigm is beginning to shift as a growing appreciation for the role gene flow may play in concert with natural selection to facilitate adaptative divergence. I explore this interaction of selection and gene flow in island scrub-jays (*Aphelocoma insularis*), a highly mobile bird experiencing local adaptation at a microgeographic scale. First, I demonstrated that observed differences in bill morphology between pine-oak ecotones are likely genetically based despite overall limited population genetic structure. Second, I found that the genetic underpinnings of divergent bill morphologies are highly parallel at higher genetic levels, which is indicative of selection acting on shared, but highly polygenic, molecular pathways. Finally, I tested alternate dispersal mechanisms potentially impacting patterns of limited gene flow and found evidence for sex-biased natal habitat preference shaping limited dispersal. Collectively, these results show how gene flow can enhance adaptive divergence at microgeographic scales.

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DEDICATION

For my father, L. Michael Cheek

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

Background

The contributions of divergent selection and gene flow in promoting adaptive divergence in natural populations have been the subject of long-standing debate in evolutionary biology (Mayr ,1963; Slatkin, 1987). While theory predicts that intermediate levels of gene flow may facilitate adaptive divergence by maintaining population sizes and genetic variation (Garant Garant, Forde, & Hendry 2007), most models assume gene flow disrupts adaptive divergence by swamping out locally adapted alleles (reviewed by Räsänen & Hendry, 2008; Tigano & Friesen, 2016). Consequently, many studies investigating local adaptation have done so using populations that occur well outside the dispersal capabilities of the focal species to negate impacts of gene flow (Lenormand, 2002; Richardson et al., 2014). Yet, a growing body of evidence supports the hypothesis that divergent selection may maintain adaptive phenotypic divergence in the presence of ongoing gene flow (Nosil, 2008; Pinho & Hey, 2010; Tigano & Friesen, 2016), even at small spatial scales (Andrew et al., 2012; Urban et al., 2017). These cases of 'microgeographic' adaptation occur within the dispersal capability of the study organism and allow researchers to explore the minimum spatial scale of adaptive evolution (Richardson et al., 2014).

Demonstrating microgeographic adaptation; however, is a difficult challenge in most systems as it requires multiple lines of evidence (Cheek et al., 2022). First, studies must characterize gene flow to determine if patterns of trait variation occur within the dispersal capacity of the study organism (Sexton et al., 2014). Second, evidence is needed that supports the hypothesis that a given environmental gradient imposes divergent selection pressure on the trait of interest (Anderson et al., 2015; Blanquart et al., 2013). Finally, it must be determined whether phenotypic variation is genetically based to infer whether adaptive evolution is underlying a given trait of interest (Hendry et al., 2001; Reid et al., 2016; Richardson & Urban, 2013; Turner et al., 2010). The advent of next generation sequencing technologies provides a highly adaptable toolset to explore each of these lines of evidence needed to effectively demonstrate microgeographic adaptation.

Unlike the classic model of isolated 'islands' of well-demarcated populations undergoing adaptive divergence, genetic diversity is often spread among wild populations that are continuously distributed across heterogenous landscapes (Batty et al., 2020; Bradburd & Ralph, 2019). This diversity commonly results in Isolation by Distance (Meirmans, 2012), which describes the case in which genetic differentiation increases with increasing geographic distance due to limited dispersal (Wright, 1943, 1946). In some cases, dispersal may be further restricted by physical barriers or environmental features that prevent gene flow between geographically adjacent populations. These barriers can facilitate genetic divergence when drift is stronger within populations than gene flow between populations (Rousset, 1997; Slatkin, 1993). This awareness of key factors that influence the spatial distribution of genetic variation has given rise to the fields of landscape genetics (Manel et al., 2003, 2010; Storfer et al., 2007) and landscape genomics (Schwartz et al., 2009; 2010) which seek to characterize genetic variation in relation to geography and the environment (Manel & Holdregger, 2013; Storfer et al., 2018). Empirical and theoretical examples show that patterns of genetic variation can reflect the dispersal patterns of species (Bolnick et al., 2009; Garant et al., 2002; Postma & Van Noordwijk, 2005; Postma et al., 2009), even to the extent of describing nonrandom dispersal mechanisms facilitating divergence (Edelaar et al., 2008; Nicolaus & Edelaar, 2018; Wang & Bradburd, 2014). Yet there is an ongoing call for more empirical evidence that explicitly tests for non-random dispersal and gene flow (Edelaar & Bolnick, 2012). Combining key objectives of landscape genomics while incorporating biological knowledge of the species is a possible approach to describe nonrandom dispersal and gene flow in wild populations (e.g., Bode et al., 2019).

Genome scan approaches to identify markers with higher genetic differentiation among populations (commonly measured by Fst, Savolainen et al., 2013) and association tests linking genotypes and the environment (genotype-environment associations, GEA, Rellstab et al., 2015) have been widely used to detect local adaptation in non-model species where little may be known about the specific phenotypic traits undergoing selection (Lotterhos & Whitlock, 2015). Yet many of these tests are inherently biased towards large effect loci undergoing strong selection that are less easily swamped by gene flow (Hoban et al., 2016). Indeed, much of the theoretical research has focused on local adaptation of a few loci of strong effect (Akerman & Bürger, 2014; Haldane,1930; Kawecki & Ebert, 2004; Lenormand, 2002; Ralph & Coop, 2010, 2015). While there are some examples of single genes or regions resulting in definitive adaptive changes in natural populations (Lamichaney et al., 2016, Prince et al., 2017), such cases are moderately rare (Lundregan et al., 2018). The challenge is to examine only true divergent loci (e.g., exhibiting higher *Fst* values) corresponding to environmental variation that is hypothesized to be related to divergent selection while also accounting for potential biases in sampling design (Flanagan et al., 2017), genomic library preparation, bioinformatic analyses and quality filtering (O'Leary et al., 2018). Multivariate GEA methods that test for associations across thousands of genetic variants simultaneously have been proposed as an attractive alternative as they test for associations among potentially covarying sets of genetic variants with multiple environmental features (Forester et al., 2016, 2018). Nature is highly complex; therefore these multivariate

GEA methods provide an intuitive approach to represent the genomic complexity found in nature (Capblancq & Forester, 2021).

Species vary greatly in the genetic architecture underlying complex traits, and relative strength of selection on those traits. Distinct lineages that independently adapted to similar conditions by similar, “parallel”, traits provide a valuable opportunity to explore power and limitations of natural selection on the adaptive evolution (Bolnick et al., 2018). Theoretical and empirical studies demonstrate that the same genes or genetic variants within genes (termed “genetic parallelism”) may be involved in parallel adaptation (Conte et al., 2012; Martin & Orogozo, 2013; Stern, 2013; Rosenblum et al., 2014). Yet the genetic variation that underlies the parallel phenotypic variation we observe, such as morphological or life history traits, are often the result of many genes contributing to the overall phenotype (Falconer & Mackay, 1996; Yeaman 2015). Therefore, the overall genetic signature of selection within such complex, highly polygenic traits may be weak because each gene has a limited overall impact and each variant within those genes explains a smaller amount of the observed phenotypic variation (Roesti et al., 2014; Rosenblum et al., 2014; Yeaman, 2015; Yeaman et al., 2018). Newly developed molecular methods that can characterize these polygenic traits, such as genome wide association (GWAS), have become increasingly accessible even in wild species (Ahrens et al., 2018; Barrett & Hoekstra, 2011; Santure & Garant, 2018; Wellenreuther & Hansson, 2016). Research that explores the genetic basis of parallel phenotypic traits will improve our understanding of how selection acts on complex traits and the predictability of adaptive evolutionary change.

Study system

The island scrub-jay (*Aphelocoma insularis*) is a medium sized passerine restricted to Santa Cruz Island which lies approximately 32 km off the coast of southern California. Island scrub oak (*Quercus pacifica*) comprises much of the island's contemporary woody vegetation (Junak, 1995), with three geographically distinct stands of bishop pine (*P. muricata*; Fischer et al., 2009) forming a mosaic of habitat diversity within a limited geographical area (250 km^2, Langin et al., 2015). The island scrub-jay system provides an ideal opportunity to tease apart the effects of selection and gene flow at microgeographic scales because 1) it occurs in both habitat types across the island, 2) there are no physical barriers to impede dispersal between habitats, and 3) it has been evolving in isolation for approximately 1 million years (McCormack et al., 2010) with no gene flow from outside the system to confound studies of adaptive divergence as seen in other island systems (Postma et al., 2009). Jays in all three pine stands have longer, shallower bills relative to jays in adjacent oak habitat and exhibit patterns of genetic discontinuity between habitat types and localized dispersal (Langin et al., 2015). This pattern is similar to adaptive differences observed in mainland scrub-jay species (Bardwell et al., 2011; Peterson, 1993). These findings suggest that adaptation to different foraging substrates (acorns or pinecones) has resulted in a pattern of microgeographic divergence.

Dissertation objectives

I propose the island scrub-jay provides an ideal system to study the interaction of gene flow and selection in facilitating divergence at a microgeographic scale. By examining how habitat composition and the landscape shapes the distribution of additive genetic variation within a single population exhibiting microgeographic divergence of a putative trait undergoing divergence, I will be able to parse out the effects of selective vs. non-selective evolutionary processes (Wang et al., 2013; Wang and Bradburd 2014). This system also provides a valuable opportunity to apply an evolutionary and ecological framework to the management and conservation of one of North America's most range restricted species. Below, I describe my proposed research and specific aims of each chapter.

The collective aim of my dissertation is to estimate the relative contributions of selection and gene flow in driving microgeographic adaptive variation. In my first study (Chapter 2), I demonstrated that repeated patterns of habitat-linked phenotypic divergence in island scrub-jays described by Langin et al., (2015) has a polygenetic basis using genome-wide association analyses. Furthermore, I tested whether variation in bill morphology is driven by selection and restricted gene flow between pine and oak habitats. I found that habitat type is strongly associated with multiple loci across the island scrub-jay genome using genotype-by-environment association analyses. Despite overall limited population genetic differentiation within the species, I identified signatures of isolation-by-distance and restricted gene flow between habitat types. These results provide strong evidence that phenotypic divergence observed in island-scrub jays is the result of differential selection between habitats and warrants further investigation into the relationship between bill morphology and habitat type.

In my second study (Chapter 3), I examined genomic data to determine the extent to which and at what molecular level genetic parallelism underlies repeated patterns of phenotypic divergence in island scrub-jays. I found that phenotypic divergence was associated with genomic parallelism at the pathway, but not SNP or gene level. Bill morphology is a highly polygenic trait with many genes likely contributing to the same molecular pathways that impact bill shape. Therefore, selection acting on standing genetic variation within the same molecular pathways linked to bill morphology can result in convergent phenotypes among pine-oak ecotones.

In my final study (Chapter 4), I combined a ten-year genetic pedigree with spatial mark-resight and nesting data to construct a spatially grounded pedigree to infer natal dispersal patterns in island scrub-jays. I found that male island scrub-jays exhibit extremely limited natal dispersal indicative of preference for environmental conditions similar to their natal site. Females are the dispersing sex, and I found several cases of females presumably dispersing >3km to reach their breeding territory. Yet females that fledged in pine appear to preferentially settle in pine habitat regardless of their bill morphology. These findings suggest that natal habitat preference of females and natal site fidelity of males limits gene flow between habitat types on Santa Cruz Island.

Elucidating the capacity for populations to diverge in the presence of gene flow is critical in aiding our understanding of divergence in nature (Richardson et al., 2014). These dissertation results take advantage of both indirect molecular techniques and direct field observation methods to provide empirical evidence of the evolutionary mechanisms that facilitate adaptation at a microgeographic scale. Specifically, I found that selection, coupled with limited gene flow between habitats, likely facilitates microgeographic adaptation in a mobile bird species.

Conservation implications

"We console ourselves with the comfortable fallacy that a single museum piece will do, ignoring the clear dictum of history that a species must be saved in many places if it is to be saved at all."[1]

— *Aldo Leopold, A Sand County Almanac*

The island scrub-jay is North America's only remaining single-island-endemic bird species (Morrison et al., 2014). While the population appears to be increasing with the recovery

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[1] Leopold, A. (1989). A Sand County almanac, and sketches here and there (Vol. 263). Oxford University Press, USA.

of native vegetation following removal of feral sheep and pigs (Sillett et al., 2012), their limited range and small population size makes the island scrub-jay vulnerable to catastrophic population decline due to disease, natural disasters, and climate related habitat loss (Bakker et al., 2020; Boyce et al., 2011; Morrison et al., 2011). Conservation practices must therefore take proactive steps to mitigate the impacts of climate driven habitat loss and increased extinction risk.

As stated by Aldo Leopold in his *Sand County Almanac*, a clear pattern in the conservation of wildlife is that isolated species are vulnerable. One proposed strategy to conserve the island scrub-jay is a translocation effort to re-establish a second population on nearby Santa Rosa Island where the jays historically occurred until the late 1800's (Morrison et al., 2014, 2011). This strategy would serve a two-fold purpose. First, as global temperatures rise, novel pathogens such as West Nile Virus have continued to spread causing massive losses of wildlife populations (Bakker et al., 2020; LaDeau et al., 2007). Corvids, including *Aphelocoma*, are highly susceptible to West Nile Virus, and island scrub-jays are likely extremely vulnerable due to their limited population size and immunological naivety (Boyce et al., 2011). A second population on Santa Rosa as well as vaccination strategies drastically lower species-wide extinction risk (Bakker et al., 2020). Second, like Santa Cruz Island, ranching and overgrazing by non-native ungulates on Santa Rosa Island caused severe reduction of native woody vegetation cover (Minnich, 1980; Rick et al., 2014; Van Vuren & Coblentz, 1987). Yet the recovery of oak chapparal and other woody vegetation is considerably less on Santa Rosa Island following the removal of introduced ungulates. Because island scrub-jays serve as important long-distance seed dispersers, the re-establishment of jays on Santa Rosa will likely accelerate the restoration of woody vegetation by as much as 200% over the next 100 years (Pesendorfer et al., 2017, 2018).

My findings show that the island scrub-jay does not face an immediate genetic risk of extinction despite their low population size. However, the results I present here show that island scrub-jays have much lower genetic diversity than their mainland counterparts (DeRaad et al., 2022), and exhibit habitat-linked microgeographic adaptation between pine and oak habitats on Santa Cruz Island (Cheek et al., 2022; Langin et al., 2015). The specific genetic underpinnings underlying this adaptation are also unique to each region of the island due to limited dispersal and natal habitat preference driving patterns of isolation-by-distance. Therefore, conservation managers should not manage island scrub-jays as a single panmictic group. Instead, island scrub-jays are best characterized as a spatially structured population exhibiting highly localized adaptation. Therefore, individuals from across the islands and from both pine and oak habitats should be prioritized in any translocation efforts to retain the maximal level of genetic diversity and evolutionary potential of a second population on Santa Rosa Island (Hohenlohe et al., 2020).

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CHAPTER 2: HABIAT-LINKED GENETIC VARIATION SUPPORTS MICROGEOGRAPHIC ADAPTIVE DIVERGENCE IN AN ISLAND-ENDEMIC BIRD SPECIES

Summary

We investigated the potential mechanisms driving habitat-linked genetic divergence within a bird species endemic to a single 250 km^2 island. The island scrub-jay (*Aphelocoma insularis*) exhibits microgeographic divergence in bill morphology across pine-oak ecotones on Santa Cruz Island, California (USA) similar to adaptive differences described in mainland congeners over much larger geographic scales. To test whether individuals exhibit genetic differentiation related to habitat type and divergence in bill length, we genotyped over 3,000 single nucleotide polymorphisms (SNPs) in 123 adult island scrub-jay males from across Santa Cruz Island using restriction site-associated DNA sequencing (RADseq). Neutral landscape genomic analyses revealed that genome-wide genetic differentiation was primarily related to geographic distance and differences in habitat composition. We also found 168 putatively adaptive loci associated with habitat type using multivariate redundancy analysis (RDA) while controlling for spatial effects. Finally, two genome-wide association analyses revealed a polygenic basis to variation in bill length with multiple loci detected in or near genes known to affect bill morphology in other birds. Our findings support the hypothesis that divergent selection at microgeographic scales can cause adaptive divergence in the presence of ongoing gene flow.

Introduction

Understanding the processes that generate and maintain adaptive phenotypic variation is a fundamental goal of evolutionary biology. Much research has focused on the interaction between gene flow and selection in shaping patterns of local adaptation across selective landscapes (Haldane, 1930; Räsänen & Hendry, 2008; Tigano & Friesen, 2016). The homogenizing effects of gene flow on local adaptation (Akerman & Bürger, 2014; Slatkin, 1973, 1987) have led to the dominant paradigm that isolation is generally a prerequisite for adaptive divergence among populations (Garant, Forde, & Hendry, 2007; Kaweki & Ebert, 2004; Lenormand, 2002; Nosil 2008; Yeaman & Whitlock, 2011). Yet there is mounting evidence that 'microgeographic' adaptation in the absence of geographic barriers may be more common than predicted by traditional models (reviewed by Richardson, Urban, Bolnick, & Skelly, 2014).

Microgeographic adaptation is a specific case of local adaptation that occurs within the 'dispersal neighborhood' of a species, defined as two standard deviations of the dispersal distribution of a population (Richardson et al., 2014; Wright, 1943, 1946). Early examples of microgeographic adaptation involved strong divergent selection over very small spatial scales in plants (Antonovics, 2006; Turner, Bourne, Von Wettberg, Hu, & Nuzhdin, 2010). Recent evidence indicates that adaptive divergence driven by strong divergent selection is also possible in highly mobile animals with significant levels of dispersal and gene flow (e.g., Hohenlohe et al., 2010; Mikles et al., 2020; Nacci, Proestou, Champlin, Marinson, & Waits, 2016; Torres-Dowdall et al. 2012; Urban et al., 2017). While many studies clearly demonstrate phenotypic and genetic variation consistent with hypotheses of microgeographic adaptive evolution (e.g., Charmantier, Doutrelant, Dubuc-Messier, Fargevieille, & Szulkin, 2016; Maciejewski, Jiang, Stuart, & Bolnick, 2020; Pequeno, Franklin, & Norton, 2021), determining the environmental factors and evolutionary and genetic mechanisms driving these patterns remains a difficult challenge (Barrett & Hoekstra 2011; Hoban et al., 2016). Studies that are successful at showing both genomic evidence of divergent selection and a genetic basis to diverging phenotypes at fine-spatial scales are generally restricted to traits controlled by few genes of large effect (e.g., Laurent et al., 2016; Linnen et al., 2013; Nosil et al., 2018; Pfeifer et al., 2018). However, many traits under selection are polygenic, making multiple loci of small effect difficult to detect in common selection tests such as F_{ST} outlier methods (Hoban et al., 2016; Lundregan et al., 2018; Tiffin & Ross-Ibarra, 2014; Wellenreuther & Hansson, 2016; Yeaman, 2015).

Spatial population genetic structure is another complicating factor in studies of local adaptation (Battey, Ralph, & Kern, 2020). Theoretical and empirical research has demonstrated that gene flow can erode genetic signatures of local adaptation by replacing locally adapted alleles with maladaptive immigrant alleles (Hendry, Taylor, & McPhail, 2002; Kaweki & Ebert, 2004; Lenormand, 2002; Postma & van Noordwijk, 2005; Räsänen & Hendry, 2008). Recent work, however, has illustrated how non-random dispersal with respect to genotype has the potential to mitigate maladaptive gene flow (Edelaar, Seipielski, & Clobert, 2008; Edelaar & Bolnick, 2012; Lowe & McPeek, 2014; Nicolaus & Edelaar, 2018). Non-random dispersal may involve multiple mechanisms, including individuals avoiding habitats to which they are not adapted (Wang & Bradburd, 2014; Edelaar et al., 2019), reduced fitness in non-optimal habitat (Karpestam, Wennersten, & Forsman, 2012; Richardson & Urban, 2013), assortative mating (Servedio, 2016), or a combination of mechanisms (e.g., 'magic trait', where a trait undergoing divergent selection also contributes to assortative mating; Servedio, Van Doorn, Kopp, Frame, & Nosil, 2011). We can therefore predict that non-random dispersal and subsequent directional gene flow would facilitate adaptation due to increased genome-wide differentiation (Edelaar &

Bolnick, 2012). Such segregation of adaptive genetic variation has even been demonstrated across fine spatial scales in mobile taxa (e.g., Bolnick et al., 2009; Camacho, Canal, & Potti, 2013; Camacho, Sanabria-Fernández, Baños-Villalba, & Edelaar, 2020), and could be a potential mechanism facilitating microgeographic adaptation in some systems.

While an increasing number of studies provide evidence for microgeographic adaptation (Richardson et al., 2014), conclusively demonstrating fine-scale adaptation requires a highly integrative analytical framework that provides multiple lines of evidence. First, adaptive trait variation must be documented across environmental gradients and within the dispersal neighborhood of the species in question. This background knowledge of the study species also provides *a priori* hypotheses about which environmental variables are most important for local adaptation. Second, there must be evidence for divergent selection across the environmental gradient that could drive the observed trait divergence (Ahrens et al., 2018; Blanquart, Kaltz, Nuismer, & Gandon, 2013; de Villemereuil, Frichot, Bazin, François, & Gaggiotti, 2014; Richardson & Urban, 2013). Lastly, some of the observed phenotypic variation must be genetically based (Hendry, Day, & Taylor, 2001; Reid et al., 2016; Turner et al., 2010). Recent studies have demonstrated that complex, multi-locus patterns of selection can be detected in heterogeneous landscapes in the presence of gene flow by using genome scan approaches (Forester, Jones, Joost, Landguth, & Lasky, 2016; Forester, Lasky, Wagner, & Urban, 2018; Lotterhos & Whitlock, 2015; Yeaman, 2015). We use such a framework in this study.

The island scrub-jay (*Aphelocoma insularis*), a medium sized bird restricted to Santa Cruz Island, California, USA, may represent a case of microgeographic adaptation. This species has been evolving in isolation for approximately 1 million years (Delaney & Wayne, 2005; McCormack, Heled, Delaney, Peterson, & Knowles, 2011) with no evidence of gene flow from outside the island to confound genetic patterns of differentiation, as seen in other island systems (Postma & van Noordwijk, 2005; Postma, den Tex, van Noordwijk, & Mateman, 2009). The 250 km^2 island is variable in topography, ecology, and climate. Coniferous forest was predominant on the island during the Pleistocene (Anderson, Starratt, Jass, & Pinter, 2009). Current vegetation cover on the island is a mosaic of coastal sage scrub, oak woodlands, and chaparral habitat dominated by island scrub oak (*Quercus pacifica*; Junak, 1995). Only three relict stands of bishop pine (*Pinus muricata*) remain in the western, central, and eastern regions of the island (Fischer, Still, & Williams, 2009; Walter & Taha, 1999; Figure 2.1).

Despite its very restricted range, the island scrub-jay exhibits phenotypic divergence in bill size and shape between different habitat types. Jays that occur in oak-dominated habitat have shorter, deeper bills compared to jays located in pine habitat (Langin et al., 2015). This striking pattern mirrors well-described adaptations seen in mainland populations of California (*A. californica*) and Woodhouse's scrub-jays (*A. woodhouseii*; Peterson, 1993), which are thought to be adaptive, as the short, stout bills of jays living in oak woodlands are more efficient for hammering open acorns, while long, shallow bills of jays living in pine-juniper forests are more efficient at extracting seed from pine cones (Bardwell, Benkman, & Gould, 2001). While this divergence is observed in mainland jays separated by hundreds of kilometers, the same pattern occurs across a few kilometers in island scrub-jays (Langin et al., 2015).

Here we investigate if this pattern of fine-scale phenotypic divergence in island scrub-jay bill morphology represents a case of microgeographic adaptation, which would require demonstrating divergent selection across the pine-oak ecotone, and whether the variation in bill morphology has a genetic basis, as seen in other avian systems (Abzhanov et al., 2006; Bosse et al., 2017; Lundregan et al., 2018). Prior studies using pedigree-based analyses suggested bill length and depth to be heritable (Langin et al., 2015). This previous work used a limited number of microsatellites to elucidate population structure on the island, but was not designed to test for divergent selection or identify loci underlying bill morphology (Langin et al. 2015). Genomic approaches that allow genotyping thousands of markers, such as single nucleotide polymorphisms (SNPs), provide greater statistical power to detect fine-scale genetic divergence (Allendorf 2017; Hohenlohe, Funk, & Rajora, 2021; Morin et al., 2004). Genomic data also allow detection of divergent selection between habitats, to test the role of selection in driving phenotypic divergence (Hoban et al., 2016), in addition to testing whether trait variation is genetically based using genome-wide association analyses (GWA; Santure & Garant, 2018).

We genotyped 123 adult male island scrub-jays using restriction-site associated DNA sequencing (RADseq) to address three questions: 1) Is gene flow restricted between habitat types? 2) Does divergent selection act across habitat types? and 3) Does divergence in bill morphology have a genetic basis? We first applied a neutral landscape genomics approach to test alternative hypotheses regarding the effects of variation in habitat type (pine versus oak) and other landscape features on genome-wide divergence. We predicted observed genetic divergence would be consistent with limited dispersal or non-random gene flow between pine and oak habitats, while also considering the effects of geographic distance and habitat quality. Second, we used a multivariate genotype-environment association (GEA) analysis to test if divergent selection across the pine-oak ecotone drives microgeographic divergence in bill morphology. Finally, we used two different genome-wide association methods to test the hypothesis that variation in bill morphology is genetically based, and that loci correlated with bill morphology are linked to candidate genes that are known to cause variation in bill morphology in other bird species.

Methods

Island scrub-jay sampling

Island scrub-jay blood samples were collected from 152 adult males during September through December, 2009 - 2011 (see Langin et al., 2015, for detailed field methods; Figure 2.1). We focused on males to eliminate variation in bill length driven by sex. Each captured jay was measured by the same person (K. Langin) using digital calipers to record bill length (to ±0.01 mm) 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. Wing chord and tail lengths were also measured with a ruler (to ±0.5 mm). 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.

RAD-sequencing and genotyping

We used RADseq to genotype thousands of anonymous loci throughout the island scrub-jay genome. We extracted genomic DNA from blood using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). The samples were individually barcoded and pooled following the protocol from Etter, Bassham, Hohenlohe, Johnson, and Cresko (2011). We generated RADseq libraries using the restriction enzyme *SbfI*, which targets an 8-bp cut site (5′ CCTGCAGG 3′). We ligated a unique 6-bp DNA barcode to each individual’s cut DNA before sample multiplexing was performed in equimolar proportions by groups of 14 individuals per pool. We sheared 80–100 µl of each pooled sample to an average fragment size of 500 bp using a Covaris ultrasonicator (Covaris Inc., Woburn, MA). Desired fragment sizes between 300-600 bp were separated by manual gel excision from the sheared DNA. We then sequenced 100 bp single-end reads on two Illumina HiSeq 2500 lanes at the Genomics Core Facility at the University of Oregon (gc3f.uoregon.edu).

Sequenced reads were quality-filtered and demultiplexed, and individual barcodes were removed using the "process_radtags" program in STACKS version 2.3b (Catchen Hohenlohe, Bassham, Amores, & Cresko, 2013; Rochette & Catchen 2017; Rochette, Rivera-Colón, & Catchen 2019). We aligned sequences to the Florida scrub-jay (*Aphelocoma coerulescens*) draft reference genome (Feng et al., 2020) using BWA-MEM (v. 0.7.17; Li & Durbin, 2009). We used the default settings of ref_map.pl in STACKS to identify SNPs and exported the raw SNP matrix using the "populations" module of STACKS (Catchen et al., 2013). We then visualized and evaluated this matrix for missingness and genotype miscall rates using the R Package, GENOSCAPERTOOLS (Anderson ,2020; https://github.com/eriqande/genoscapeRtools) and WHOA (Anderson, 2020; (https://github.com/eriqande/whoa). After this evaluation, individuals with greater than 24% missing data were removed by re-running populations with a whitelist.

Additional filtering was performed using the RADIATOR package (v.1.1.5; Gosselin, Lamothe, Devloo-Delva, & Grewe, 2020) in R (R Core Team, 2013). We filtered by global minor allele count (MAC=3) to reduce sequencing errors, assembly artifacts, and rare alleles (Linck & Battey, 2019). We then retained loci with coverage between 6x and 100x across individuals, removing loci with coverage too low for accurate genotype calling and loci with high coverage that likely reflect repetitive regions (e.g., paralogues). We retained markers that were genotyped in at least 70% of individuals and between positions 1-88 (removing the last 7 positions of our reads, which showed signatures of low-quality genotyping). We then selected the SNP with the highest minor allele count per contig to minimize effects of short distance linkage disequilibrium. SNPs with significant deviation from Hardy-Weinberg proportions (P-value < 0.0001) across all samples were removed.

As the Florida scrub-jay draft genome is a scaffold level assembly and unannotated (scaffold N50= 7.7 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 v2.1.0 (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 (7.01% missing) using BEAGLE (v. 5.1; Browning & Browning, 2016) with 25 iterations and an N_e setting of 370 (see Results). This imputed dataset was used for analyses requiring complete data frames: Principal Components Analysis (PCA; section 2.2), GEA tests (section 2.3), and genome-wide associations (GWA; section 2.4).

Is gene flow restricted between habitat types?

After filtering, we identified and removed loci showing signatures of selection using PCADAPT (Luu, Bazin, & Blum, 2016) with a false discovery rate of 10% to avoid confounding neutral demographic patterns with patterns generated by loci under selection when conducting downstream neutral population genetics analyses. We calculated population genomic statistics using 3,408 neutral markers across all sampled individuals (Figure 2.1) analyzed as a single population. Observed and expected heterozygosity, nucleotide diversity, and the inbreeding coefficient were calculated using the *populations* program in STACKS. Effective population size (N_e) was estimated using the linkage disequilibrium method of Waples, Larson, and Waples (2016), calculated in the R package STRATAG (Archer, Adams, & Schneiders, 2016). We calculated two individual-based genetic distances: genetic distance based on relatedness (Smouse

& Peakall, 1999) using used the R package POPGENREPORT (Adamack & Gruber, 2014), and the proportion of shared alleles (Bowcock et al., 1994) using ADEGENET (Jombart, 2008; Jombart & Ahmed, 2011).

We tested for population genetic structure across the island and between pine and oak habitats using a combination of methods. First, we tested for isolation-by-distance using both individual measures of genetic distance outlined above, and pairwise geographic distances calculated using the GEODIST package to measure "geodist" (Padgham & Sumner, 2020). We tested for isolation-by-distance using a Mantel test with 1,000 permutations and the "pearson" method in the VEGAN R package (Oksanen et al., 2013). Second, we used PCA implemented in VEGAN to visualize how genetic variation is distributed across a reduced number of orthogonal axes without an underlying assumption of genetic groups or spatial structure.

We tested for discrete population structure using snmf in the LEA R package (Frichot & François, 2015) and STRUCTURE (Pritchard, Stephens, & Donnelly, 2000). Because there are 3 pine stands, we conducted runs for K = 1–6 for both analyses to test the hypothesis that each pine and adjacent oak stand represent distinct populations. We ran snmf with 10 replicates per K value and retained the run with the smallest cross-entropy to choose the optimal number of clusters (Frichot, Mathieu, Trouillon, Bouchard, & François, 2014). The best supported value of K was the estimate for which the cross-entropy curve exhibited a plateau or a clear minimum value. We ran STRUCTURE with a burn-in length of 50,000 and a run length of 50,000 using the admixture model. We assumed K = 1 if its log likelihood value was highest, as the second-order rate of change in log likelihood cannot be calculated for K=1 (Evanno, Regnaut, Goudet, 2005).

Finally, because Santa Cruz Island is characterized by extremely variable and rugged terrain, we predicted that landscape features could influence dispersal and gene flow (Sillett Chandler, Royle, Kery, & Morrison, 2012; Langin et al., 2017a). To understand how different landscape features shape the genetic variation of island scrub-jays, we applied a linear mixed effects modelling approach to test the effects of landscape features on neutral population structure using maximum likelihood of population effects (MLPE; Clarke, Rothery, & Raybould, 2002; Row Knick, Oyler-McCance, Lougheed, & Fedy, 2017; van Strien, Keller, & Holderegger, 2012). MLPE uses individual-based genetic distances as the response and environmental resistances as fixed effects, with an additional random effect matrix of pairwise individual comparisons to control for their non-independence (Balkenhol, Cushman, Storfer, & Waits, 2016; Trumbo et al., 2019). Although MLPE has received criticism regarding its power to accurately capture how landscape features impact gene flow (Peterman & Pope, 2020), our goal here was to test the validity of *a priori* hypotheses regarding scrub-jay dispersal across habitat types (Langin et al., 2015), rather than finding the best supported resistance model. And MLPE is an effective method to infer associations between landscape features and gene flow (Kozakiewicz et al., 2019; Shirk, Landguth, & Cushman, 2017; Trumbo et al., 2019). We used the proportion of shared alleles and relatedness as our genetic distance response variables. The environmental resistances used as the explanatory variables were those that we hypothesized *a priori* are related to genetic distance in this system: topographically-corrected geographic distance (positive effect), low vegetation density (positive effect), and elevation (positive effect; Figure S2.1). Landscape data were converted to resistance surfaces using the Reclassify and Raster Calculator tools in ARCGIS v.10.6 (ESRI). We calculated vegetation density from chlorophyll reflectance in visual and near-infrared spectra (i.e., enhanced vegetation index) using a moderate resolution (250 m) imaging spectroradiometer data collected in 2015 (modis.gsfc.nasa.gov). Environmental resistances among individuals were calculated using CIRCUITSCAPE (McRae, 2006) for each landscape resistance surface. To test for resistance between pine and oak habitats, we calculated the percent pine and percent oak within a 300 meters 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 (The Nature Conservancy 2007; Langin et al. 2015). We then calculated the absolute differences in pine and oak composition by subtracting the percent pine and percent oak between all individuals to help identify transitions between these habitat types (ecotone; positive effect). Because collinearity can cause instability in parameter estimation in regression models (Dormann et al., 2012; Row et al., 2017), we tested each landscape variable for multicollinearity, both prior to and after calculating environmental resistances in CIRCUITSCAPE, to ensure Pearson's r correlations < 0.7 and variance inflation factor (VIF) scores < 5 in our final models.

We standardized landscape resistances to units of standard deviation centered on the mean (Row et al., 2017; van Strien et al., 2012). We ranked models using the Bayesian information criterion (BIC) and report the top models within five BIC units (Richards, 2015). We used standardized beta coefficients to assess the direction of effect (positive or negative) of each landscape variable on genetic distance to infer how genetic distance is affected by landscape features and which variables have the greatest impact on neutral genetic variation.

Does divergent selection act across habitat types?

To identify loci associated with habitat type (pine versus oak) while accounting for geography, we used a partial redundancy analysis (RDA; Forester et al., 2016) as our GEA test (hereafter referred to as GEA-RDA), implemented in VEGAN (Oksanen et al., 2013). This constrained ordination approach models multivariate response data (in this case, genetic data) as a function of linear combinations of predictor variables by combining multivariate linear regression and principal components analysis (Legendre & Legendre 2012). RDA has been shown to be an effective method to detect weak, multilocus signatures of selection due to its low false positive and high true positive rates (Forester et al., 2018), making it an ideal test for genotype-environment associations in this system. We modeled our complete 3,345 SNP dataset (see Results) as a function of percent pine and percent oak within a 300 m radius of each sampling location as habitat predictors of genetic divergence. We used the longitude of each individual's sampling location as a proxy for isolation-by-distance (see Results). Including longitude as a third 'conditioned' matrix in the RDA allowed us to control for potential effects of isolation-by-distance. Longitude was weakly correlated with percent oak ($|r| = 0.06$) and percent pine ($|r| = 0.35$); therefore, we were able to include longitude in our model without confounding patterns between highly correlated variables. This allowed us to reduce potential noise driven by continuous population structure in our analysis of genetic divergence associated with habitat composition. We tested the significance of both the global model and model terms (percent pine and percent oak) using the anova.cca function in VEGAN with 10,000 permutations. Further, we identified outlier loci based on the 'locus score', which are the coordinates (loading) of each locus in the ordination space. We defined outliers as loci with loadings ±2.5 standard deviations from the mean on the two RDA axes (Forester et al., 2018). We then determined the predictor with which each outlier locus was most strongly associated based on the absolute value of the predictor's correlation coefficient for each SNP.

Does divergence in bill morphology have a genetic basis?

Langin et al. (2015) found that the magnitude of difference in bill length between pine and oak habitats in island scrub-jays on Santa Cruz Island is similar to observed differences between pine/juniper and oak habitats in mainland populations of scrub-jays (Peterson, 1993). The bill length difference among mainland scrub-jays has also been shown to be adaptive for foraging on pine vs. oak (Bardwell et al., 2001). Therefore, we chose to focus on bill length as our putatively adaptive trait of interest for identifying SNPs associated with bill morphology. Because island scrub-jays show marked differences in bill morphology related to sex, age, and season (Langin et al., 2015), it is noteworthy that one of the strengths of this dataset is that all of our samples came from adult males that were measured by the same person (K. Langin) within the same four month period, thereby reducing other sources of variation in the phenotypic data to increase the likelihood we would be able to detect loci associated with a complex morphological trait (Visscher et al., 2017). Bill length is positively associated with body size (Langin et al., 2015). Therefore, we performed a principal components analysis (PCA) on measurements of wing and tarsus length and extracted values from the first PC axis which explained 68.9% of the variance as an index of overall body size. We then used the residuals from a regression of bill length on this index of body size as a body size-corrected measure of bill length in our analyses.

We used two different GWA tests to identify loci underlying variation in bill length using our imputed dataset from BEAGLE. We tested against the null hypothesis that bill morphology is entirely due to plasticity and therefore the marker effect size on bill length is zero. First, we used a Genome-Wide Mixed Model Association algorithm (GEMMA; Zhou & Stephens, 2012) which fits a univariate linear mixed model using marker genotypes as a fixed effect in association tests with a single phenotype while correcting for population structure using a kinship matrix (random effect). Hereafter we refer to this approach as GWA-GEMMA. By incorporating relatedness, we reduce the likelihood of spurious associations caused by neutral population structure (Sul, Martin, & Eskin, 2018). We applied a Benjamini-Hochberg correction for multiple testing to control for false discovery rate (FDR) and identified outlier SNPs based on a significance threshold of 0.05.

Univariate genome scan methods like GEMMA have been shown to be biased towards large effect loci undergoing strong selection (Hoban et al., 2016; Wellenreuther & Hansson, 2016). Indeed, a Bayesian sparse linear mixed model showed that much of the significant genetic variation associated with bill length may be attributed to one SNP (see Supplemental Material), which we would not necessarily expect, as bill morphology has been shown to be a complex polygenic trait in other systems (e.g., Bosse et al., 2017; Lundregan et al., 2018; Perrier et al., 2020). Our linear mixed model approach may therefore be biased towards these large effect SNPs while failing to detect the signal of smaller effect loci especially given our modest sample size. To address this limitation of GEMMA, we also used a partial RDA, which has been shown to be an effective method for identifying small-effect loci (Forester et al. 2018), to model the effect of all 3, 345 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 (hereafter referred to as GWA-RDA). Outlier loci were identified using the criterion of loading scores ±2.5 standard deviations from the mean to identify SNPs associated with variation of bill length.

Visual inspection of linkage disequilibrium (LD) plots using LD calculated within a 100 kb window in VCFtools 0.1.17 with a min and max alleles setting of 2 (Danecek et al., 2011) suggested high linkage between biallelic loci separated by 25 kb or less (Figure S2.2). We therefore input our zebra finch-mapped SNPs in BEDOPS v. 2.4.39 (Neph et al., 2012), and output all genes within 25 kb of variant site coordinates in the annotated zebra finch genome. We then used the R packages MYGENE (Mark, Thompson, Afrasiabi, & Wu, 2020) and BIOMART (Durick et al., 2005; Durinck et al., 2009) to extract accession identifications and ontology information for each gene found within our query sequences using the available zebra finch Ensembl database. We compared all the loci flagged by our two GWA methods and searched annotation reports for GO terms related to bill morphology. We inferred those genes with these terms would best support our hypothesis of genetically based divergence of bill morphology.

Results

Genotyping and filtering SNP matrices

We genotyped island scrub-jays from oak habitat (n=111), and western, central, and eastern pine stands (n=41; Figure 2.1) and obtained a total of 203,128,694 reads with an average of 1,336,373 reads per individual. Two individuals from the central oaks had low numbers of reads and were dropped from further analyses. Of the remaining reads, 91.8% were mapped to the Florida scrub-jay reference genome with an average coverage of 16.8x per locus per individual following BWA-mem alignment and genotyping in gstacks.

Initial processing of individuals with less than 24% missing data in STACKS resulted in a matrix of 26,160 SNPs and 124 individuals. Two individuals were found to be closely related in the detect_duplicate_genomes step of RADIATOR, so the individual with the least missing data in the pair was retained and the new matrix of 123 individuals and 25,815 SNPs was further filtered in RADIATOR (Table S2.1). The number of SNPs and individuals kept after each filtering step can be found in Table S2.1. This resulted in a final matrix of 3,409 SNPs.

PCADAPT detected one putatively adaptive outlier locus, which was removed for a matrix of 3,408 SNPs used for downstream neutral population genomic analysis (section 3.2).

Because synteny is highly conserved in birds (Backström et al., 2008; Zhang et al., 2014), we were able to map 96.3% of our Florida scrub-jay scaffolds to the zebra finch genome. This resulted in 3,345 loci with chromosomal level positional information for GEA (section 3.3) and GWA (section 3.4) analyses. PCADAPT detected three putatively adaptive outlier loci within this imputed, mapped dataset, which were removed for a matrix of 3,342 SNPs for our PCA analyses (below).

Population structure at neutral loci

We calculated the following population genomic parameters in STACKS using the neutral SNP matrix of 3,408 SNPs and 123 island scrub-jays: observed heterozygosity (H_o) = 0.256, expected heterozygosity (H_e) = 0.272, inbreeding coefficient (F_{IS}) = 0.066, and nucleotide diversity (p) across all sites (variant and fixed) = 0.00285. The effective population size (N_e) estimate was 346.8 with a 95% confidence interval of 327–368 based on parametric bootstrapping. Mantel tests indicated significant (P-value = 0.001) spatial population structure for both measures of genetic distance ($r_{PropShared}$=0.235; $r_{relatedness}$= 0.347) consistent with a pattern of isolation-by-distance primarily predicted by longitude according to our PCA results (Figure S2.3; Figure 2.2). We did not find evidence of discrete populations, and K=1 was the best supported value of K by minimizing cross validation error in snmf (Alexander, Novembre, & Lange, 2009; Figure S2.4) and maximum log-likelihood in STRUCTURE (Pritchard et al., 2000; Table S2.2).

MLPE analysis showed topographic distance and absolute difference in percent oak were consistently retained in top models and positively correlated with genome-wide genetic distance of island scrub-jays for both measures of genetic distance. Low vegetation density showed a negative correlation in models of the proportion of shared alleles, but was not retained in models of genetic relatedness. Absolute difference in percent pine was not retained in most models and showed a weak negative correlation to genetic relatedness (Table 2.1). None of our raw raster surfaces were highly correlated ($|r| < 0.70$) prior to running CIRCUITSCAPE. After calculating resistance surfaces, collinearity among environmental variables increased, due to CIRCUITSCAPE resistances being higher for individuals separated by greater geographic distances (McRae, 2006). Elevation was the only variable strongly correlated with other variables, with a variance inflation factor greater than 10, so it was removed from further analyses. Variables retained were topographic distance, low vegetation density, absolute difference in percent pine, and absolute difference in percent oak (Figure S2.1).

Evidence of divergent selection associated with habitat

The relative proportion of pine and oak within a 300-meter radius of each individual was significantly associated with genetic variation in island scrub-jays based on RDA (*P*-value percent pine = 0.001, *P*-value percent oak = 0.001). A triplot of our two GEA-RDA axes shows SNPs (dark grey points), and individuals (colored circles) arranged in ordination space relative to their relationship with the predictor variables (black arrows, Figure 2.3A). Of the 168 outlier SNPs that were ±2.5 standard deviations from the mean loading on RDA axis 1 (n=93) and RDA axis 2 (n= 72; Figure 2.3B), we found 82 SNPs that were most strongly correlated with percent oak, and 86 SNPs that were most strongly correlated with percent pine (Figure S2.5).

Identification of loci underlying variation in bill morphology

Using GWA-GEMMA, we detected 7 SNPs that were significantly associated with bill length after correcting for multiple tests (FDR; Figure 2.3C), while the GWA-RDA detected 91 additional SNPs (Figure 2.3D; Figure S2.6). SNPs did not overlap between our two GWA analyses, though three of the GWA-GEMMA SNPs were close to the GWA-RDA outlier threshold, indicating some congruence of adaptive signatures across these different methods. Additionally, 5 SNPs detected by GWA-RDA were also detected by our GEA-RDA, 3 of which corresponded to candidate genes associated with bill morphology (*COL14A1*, *PPP3CB*, *IGF1R*; Figure 2.3; Table S2.3).

We identified 254 genes within 25 kb of our variant sites flagged by the two GWA analyses and GEA. Potential candidate genes found in our GO term search of annotation reports (Table S2.4) included several genes previously identified in pathways associated with bill morphology (Abzhanov, Protas, Grant, Grant, & Tabin, 2004; Abzhanov et al., 2006; Badyaev, Young, Oh, & Addison, 2008; Bosse et al. 2017; Lundregan et al., 2018; Mallarino et al., 2011) including bone morphogenetic protein (*SMURF1*; *BMP15*), ossification (*PBX1*), calmodulin (*IQCB1*; *PPP3CB*; *HSPA2*), Smad proteins (*TRIM33*), MAP Kinase activity ("MAPK": *STK39*; *AKAP13*; *IGF1R*), transforming growth factor beta (*FN1*), Notch (*MFNG*; *TAFA4*; *TP63*), and Wingless-related integration ("Wnt": *ADGRA2*; *TRABD2B*) signaling pathways (Figure 2.3B, 2.3C, 2.3D).

Discussion

Divergence at a microgeographic scale

Evolutionary theory and empirical studies have long emphasized the importance of geographic isolation and reduced gene flow as a prerequisite for local adaptation to occur among populations. Nonetheless, local adaptation in the face of gene flow appears common even in taxa with a high dispersal potential (Richardson et al., 2014). For example, other avian systems have been shown to exhibit evolutionary responses in multiple morphological (Bertrand et al. 2016; Cornuault et al. 2014), and physiological traits (Gamboa et al. 2021; Mikles et al. 2020) within the potential dispersal radius of the study species. Understanding such fine-scale adaptation requires multiple lines of evidence including trait variation across environmental gradients and within the dispersal neighborhood of the species in question, evidence for divergent selection across the environmental gradient, and observed phenotypic variation that must be at least partially genetically based.

Our study demonstrates that island scrub-jays exhibit habitat-linked microgeographic divergence. Neither snmf nor STRUCTURE analyses revealed discrete population structure in island scrub-jays (Figure S2.4; Table S2.2), suggesting that the spatial scale of Santa Cruz Island is within the dispersal capabilities of island scrub-jays (Figure 2.2; Table 2.1). Our results indicate that despite this lack of structure, island scrub-jays exhibited a genome-wide pattern of isolation-by-distance (Figure 2.2; Table 2.1). We also observed relatively high genetic diversity comparable to estimates of mainland bird species (e.g., Mikles et al., 2020), and an N_e estimate larger than found in other vertebrate species on Santa Cruz Island (Funk et al., 2016; Trumbo, Funk, Pauly, & Robertson, 2021). Thus, localized isolation and genetic drift likely play a limited role in this system. Instead, we hypothesize that multiple, non-mutually exclusive processes including non-random gene flow and habitat choice (Edelaar & Bolnick, 2012), and/or divergent selection (Langin et al. 2015) are contributing to a pattern of microgeographic divergence in island scrub-jays.

Localized dispersal is common across a wide range of taxa (Sexton, Hangartner, & Hoffman, 2014) including *Aphelocoma* jays (Aguillion et al., 2017; Carmen, 1988; McCormack, Peterson, Bonaccorso, & Smith, 2008). Breeding island scrub-jays maintain and defend year-round territories (Caldwell et al., 2013; Collins & Corey, 1994; Curry & Delaney, 2020), and exhibit limited natal dispersal (Langin et al., 2015; Langin, Sillett, Funk, Morrison, & Ghalambor, 2017a), but most individuals live as non-territorial "floaters" for multiple years before a breeding territory becomes available (Curry & Delaney, 2020). Little is known about the movement patterns of jays during this transient period or about how dispersal decisions are affected by phenotypes or environmental context (Bolnick & Otto, 2013; Clobert, Le Galliard, Cote, Meylan, & Massot, 2009). Similar to the Florida Scrub-jay, island scrub-jays exhibit sex-biased dispersal but generally disperse up to 3 km from their natal territory (Langin et al., 2015). This distance is sufficient for the jays to move between adjacent oak and pine stands on Santa Cruz Island (Figure 2.1). Therefore, non-random dispersal via habitat selection might play a role in genetic differentiation even at this limited spatial scale (Edelaar et al., 2008; Edelaar & Bolnick, 2012; Nicolaus & Edelaar, 2018).

We found strong support for models containing multiple significant effects of landscape features on genetic distance in island scrub-jays. Topographic distance was the strongest predictor of genetic distance, but we also observed that landscape features related to pine-oak ecotones affect genetic distance. Absolute difference in oak habitat at the scale of jay home range size was positively correlated with both our measures of genetic distance (Table 2.1). In other systems, studies have found support for both natal habitat preference (Camacho, Canal, & Potti 2016; Piper, Palmer, Banfield, & Meyer, 2013) and phenotype habitat-matching (Benkman 2016; Camacho et al., 2013; Garant, Kruuk, Wilkin, McCleery, & Sheldon, 2005). Both mechanisms have profound evolutionary consequences in the recognition of suitable habitat and settlement decisions (Berner & Thilbert-Plante, 2015; Davis & Stamps, 2004; Edelaar, Siepielski, & Clobert, 2008) and are plausible hypotheses for reduced gene flow between habitat types in the island scrub-jay. Our working hypothesis for the lack of an effect of absolute differences in pine habitat could be attributed to the fact that most jays included in this study were found primarily in oak habitat. Oak chaparral is more widespread on Santa Cruz Island compared to pine woodland (Junak 1995), and most island scrub-jays occur in oak habitat because of pine habitat being rare relative to oak habitat (Sillett et al. 2012). Consequently, we had a relatively small sample size of jays with substantial pine woodland within their home range (Figure 2.1). This may explain the low variation in pairwise differences in pine habitat presence among jay home ranges and could constrain our ability to detect a positive association between genetic distance and pine habitat (Landguth et al. 2012; Lotterhos & Whitlock, 2015; Forester et al., 2016). Our models also indicate that low vegetation density was not positively correlated with genetic distance (Table 2.1). Therefore, we do not have evidence to suggest these fully flight-capable birds are incapable of dispersing across open areas despite their avoidance of marginal or barren habitats (Curry & Delaney, 2020).

We hypothesize that assortative mating could restrict gene flow across oak – pine ecotones and facilitate genome-wide divergence between these habitat types (Servedio, 2016). Island scrub jays appear to mate assortatively by bill morphology (Langin et al., 2015). While this pattern could be due to spatial autocorrelation as a function of isolation-by-distance (Urban, 2011), female calls associated with pair-bond formation and territory defense differ among oak and pine habitats (Langin et al., 2017b), suggesting a relationship between bill morphology and acoustic signals (e.g., Podos, 2001). Future work investigating mate-choice in island scrub-jays will be needed to determine the mechanisms underlying apparent environmental barriers to gene flow in this system (Bradburd & Ralph, 2019; Manel et al., 2010).

We found support for divergent selection related to adaptation to pine versus oak habitats in island scrub-jays. Individuals sampled in pine and oak habitat could clearly be separated based on their genotypes after controlling for isolation-by-distance (Figure 2.3A). These genetic signatures of selection are unlikely to be confounded by demographic processes due to the lack of discrete population structure in the island scrub-jay (Figure S2.4; Cushman & Landguth, 2010; de Villemereuil et al., 2014; Lotterhos & Whitlock, 2015). Further, our association tests support the hypothesis that observed differences in bill morphology between island scrub-jays living in pine and oak habitats have a genetic basis and are caused by many loci of small effect. We identified 96 unique SNPs out of our 3,345 SNP dataset that were strongly associated with variation in bill length using GWA-GEMMA and GWA-RDA, demonstrating at least a partial genetic basis to variation in bill morphology (Table S2.3). Notably, we found several candidate genes previously shown to be associated with variation in bill morphology, including those in the calmodulin pathway *(IQCB1*; *PPP3CB*; *HSPA2*; Abzhanov et al., 2006; Lundregan et al., 2018), and those involved in regulating BMP activity (*SMURF1*, *FN1*, *IGF1R*, *AKAP13*, *MFNG*, *STK39*, *TRIM33*; Abzhanov et al., 2004; Badyaev et al., 2008; Mallarino et al., 2011). One of the loci that was flagged by both our GEA-RDA and GWA was associated with the collagen trimmer, *COL18A1* (Figure 2.3B, 2.3D), which is thought to be involved in palate development and is a significant marker for bill length (Bosse et al. 2017; Table S2.4). If adaptive developmental plasticity or simple differential wear between habitats were the only factors driving differences in bill morphology, we would not expect to see any evidence for loci associated with bill variation (Endler, 1986; Ghalambor, Carroll, & Reznick, 2007; Kawecki & Ebert, 2004). In addition, we would not expect to find shared SNPs between our GEA-RDA and

GWA analyses, thereby linking selection associated with habitat to variation in bill morphology (Table S2.3).

Variation in bill morphology related to habitat gradients have been observed in several bird species and have been attributed to differences in local foraging substrates (Alonso, Fernández-Eslava, Edelaar, & Arizaga, 2020; Badyaev et al., 2008; Benkman, 1993, 2003). Bill depth and length have both been shown to respond rapidly to selection due to food resource availability (Grant & Grant, 1993; Grant & Grant, 2002). The observed differences in bill length between island scrub-jays living in pine and oak habitats mirrors patterns seen in mainland *Aphelocoma* jays, where bill shape varies adaptively with local resources, but on a spatial scale of a few kilometers instead of hundreds of kilometers (Bardwell et al., 2001; Langin et al., 2015; McCormack and Smith, 2008; Peterson, 1993). Although we have not quantified foraging behavior or other ways the bill is used in island scrub-jays within pine and oak habitats, prior research of mainland jays shows that bill morphology significantly affects foraging efficiency on different substrates (Bardwell et al., 2001), which may influence fitness (Pyke, 1984). Additional research quantifying the feeding performance on different diets relative to bill morphology will be needed to understand the potential fitness consequences of phenotype-habitat mismatch.

While not the focus of this study, our GEA analysis also flagged multiple SNPs correlated with habitat type that were associated with genes without a direct relationship to bill morphology (Table S2.3, S2.4). Previous research has found genetic differentiation across fine spatial scales in several avian species related to physiological and reproductive adaptive divergence between habitats (e. g., Charmantier et al., 2016; García-Navas, Ferrer, Sanz, & Ortego, 2014; Perrier et al., 2020; Mikles et al., 2020; Senar, Borras, Cabrera, Cabrera, & Björklund, 2006). We hypothesize that the signature of divergent selection at these loci could be attributed to environmental differences between habitats driven by the climate gradient induced by the California Current: Santa Cruz Island is characterized by more arid conditions in the east and a cooler climate in the west (Fischer et al., 2009; Morrison et al., 2011; Gamboa et al. 2021). Given that birds exhibit strong physiological responses to extreme heat (Mckechnie & Wolf, 2009), the island's pronounced east – west climate gradient could lead to divergence in physiological or behavioral traits in the island scrub-jay. Hence, our observed pattern of selection across habitats may involve adaptive differences in traits other than bill length.

Conservation Implications for a Range Restricted Species

Adaptability and resilience of wild populations, particularly those with restricted ranges, are threatened by rapid climate change, emphasizing the need to understand the evolutionary processes underlying genetic and phenotypic microgeographic variation (Hohenlohe et al., 2020; Funk, Forester, Converse, Darst, & Morey, 2019). The island scrub-jay is a species of conservation concern because it currently occurs on a single, small island (Morrison et al. 2011), is a key seed disperser (Pesendorfer, Sillett, Koenig, & Morrison 2016), and is vulnerable to catastrophic population declines related to climate change and disease (Bakker et al., 2020). Given that the adaptive divergence in island scrub-jays living in pine and oak habitats is genetically based, conservation planning should consider the implications of such microgeographic variation in management decisions (Langin et al., 2015). For example, instead of only using abundance as a metric of population viability, managers could incorporate information on adaptive variation driven by habitat heterogeneity to effectively conserve the adaptive capacity of individuals across their range; such capacity will likely only increase in importance as climates rapidly change (Hohenlohe et al., 2020; Funk, McKay, Hohenlohe, & Allendorf, 2012; Funk et al., 2019).

Conclusion

The island scrub-jay exhibits a remarkable repeated pattern of habitat-linked differences in bill morphology between adjacent pine and oak stands hundreds of meters apart. Prior evidence for a genetic basis to this pattern was based solely on pedigree-based heritability estimates that were potentially confounded by spatial autocorrelation in bill morphology (Langin et al., 2015; Langin, Sillett, Funk, Morrison, & Ghalambor, 2017a). In this study, we characterized fine-scale genetic variation using genome-wide SNP data to test for genomic signatures underlying this pattern of microgeographic divergence. Neutral landscape genomic analyses showed a significant pattern of isolation-by-distance and increased genetic distance associated with habitat variation consistent with divergence within the dispersal capabilities of island scrub-jays. In addition, we found signatures of divergent selection at the genomic level by identifying multiple SNPs from across the genome that were strongly associated with habitat composition and bill length across the pine-oak ecotone, and evidence that bill morphology has a genetic basis. Collectively, our results support the hypothesis that habitat-linked divergence in bill morphology in island scrub-jays represents microgeographic adaptation within a single population. The island scrub-jay system provides a robust framework to further test for microgeographic adaptation, and in so doing, also inform the conservation of one of North America's most range-restricted bird species.

Figures and tables

Table 2.1: Maximum likelihood of population effects (MLPE) neutral landscape genomic results. Response variables were individual-based genetic distances (proportion of shared alleles or relatedness). Fixed effects were topographic distance, low vegetation density, absolute difference in percent oak, and absolute difference in percent pine. Standardized beta coefficients (β) were used to assess the relative support for our *a priori* landscape resistance to gene flow hypotheses. The top supported models reported below are within 5 BIC units of the most supported model (different models are separated by a dotted line). Landscape resistance variables that had positive beta coefficients were considered to have a significant positive effect on genetic distance. Variables with negative beta interpreted here as nonsignificant.

Genetic Distance	**Landscape Feature**	**β**	**R^2**	**Delta BIC**
Proportion Shared	Topographic Distance	0.467	0.273	0.0
	Low Vegetation Density	-0.179		
	Absolute Difference Oak	0.047		
	Topographic Distance	0.478	0.271	2.57
	Low Vegetation Density	-0.193		
Relatedness	Topographic Distance	0.428	0.271	0.0
	Absolute Difference Oak	0.050		
	Topographic Distance	0.435	0.272	1.57
	Absolute Difference Oak	0.066		
	Absolute Difference	-0.041		

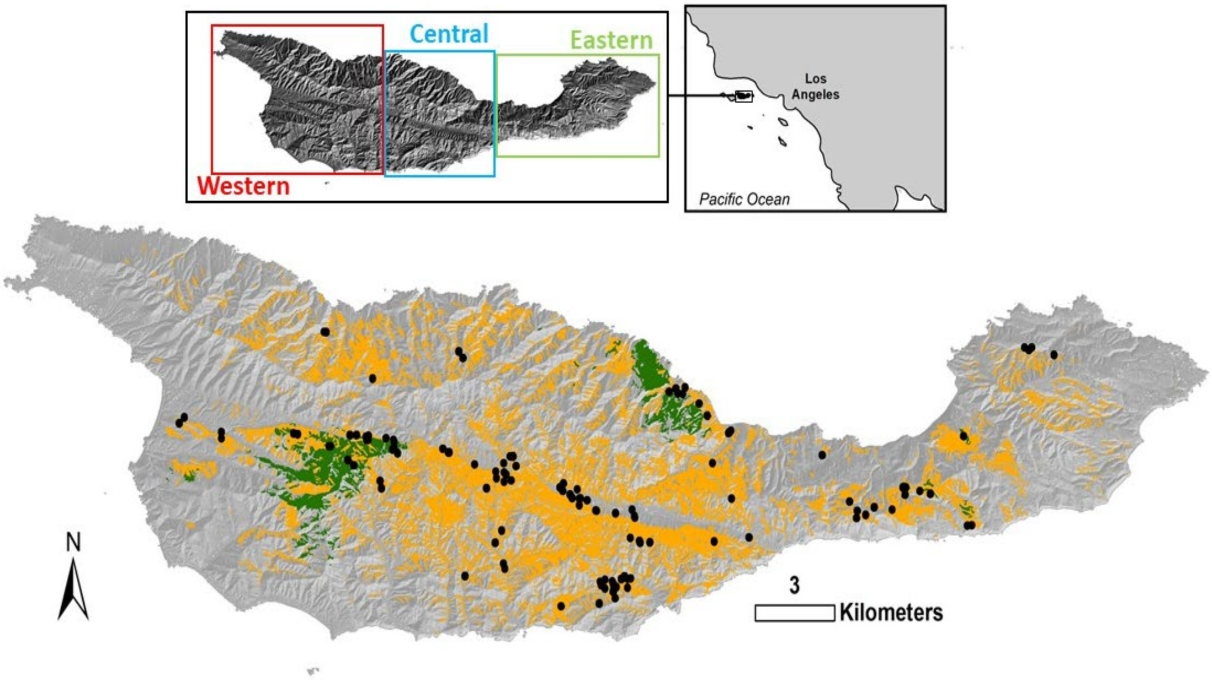

Figure 2.1. Study area of Santa Cruz Island, California, USA with sampling sites (black dots; n = 152) and habitat type (green, pine; orange, oak). Island scrub-jays were sampled from each of the pine-oak ecotones (western oak = 39, central oak = 60, eastern oak = 12, western pines = 25, central pines = 7, eastern pines = 9). Insets show regional breakdown of Santa Cruz Island and location of the island (in black) relative to mainland California.

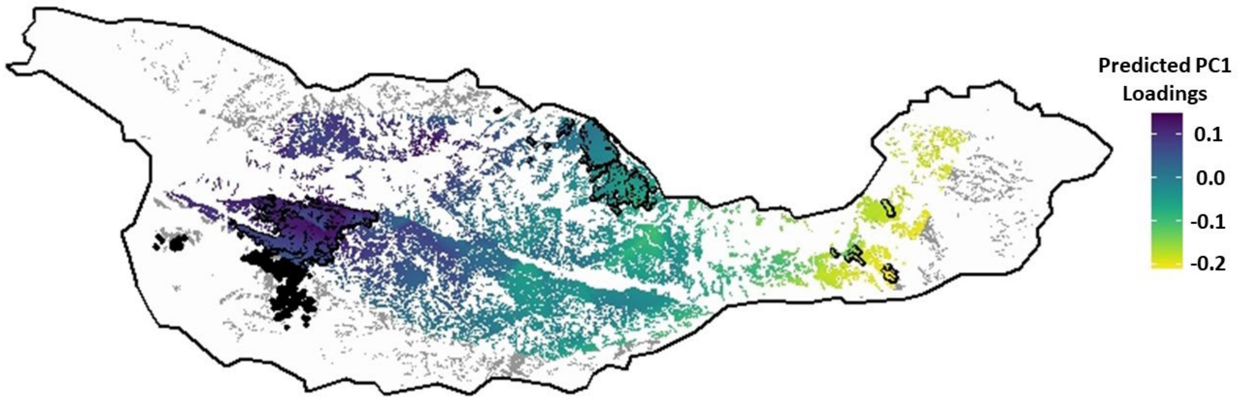

Figure 2.2. The island scrub-jay population exhibited continuous spatial genetic structure across Santa Cruz Island. Colored polygons represent woody vegetation where jays are found, with pine habitat outlined in black and the remaining shaded regions representing oak habitat. The color gradient is a continuous representation of the predicted neutral genetic surface for the first Principal Component (PC) axis for sampled tree stands (grey = unsampled tree stands). Jays are expected to have a similar genetic composition if they are in areas with similar colors.

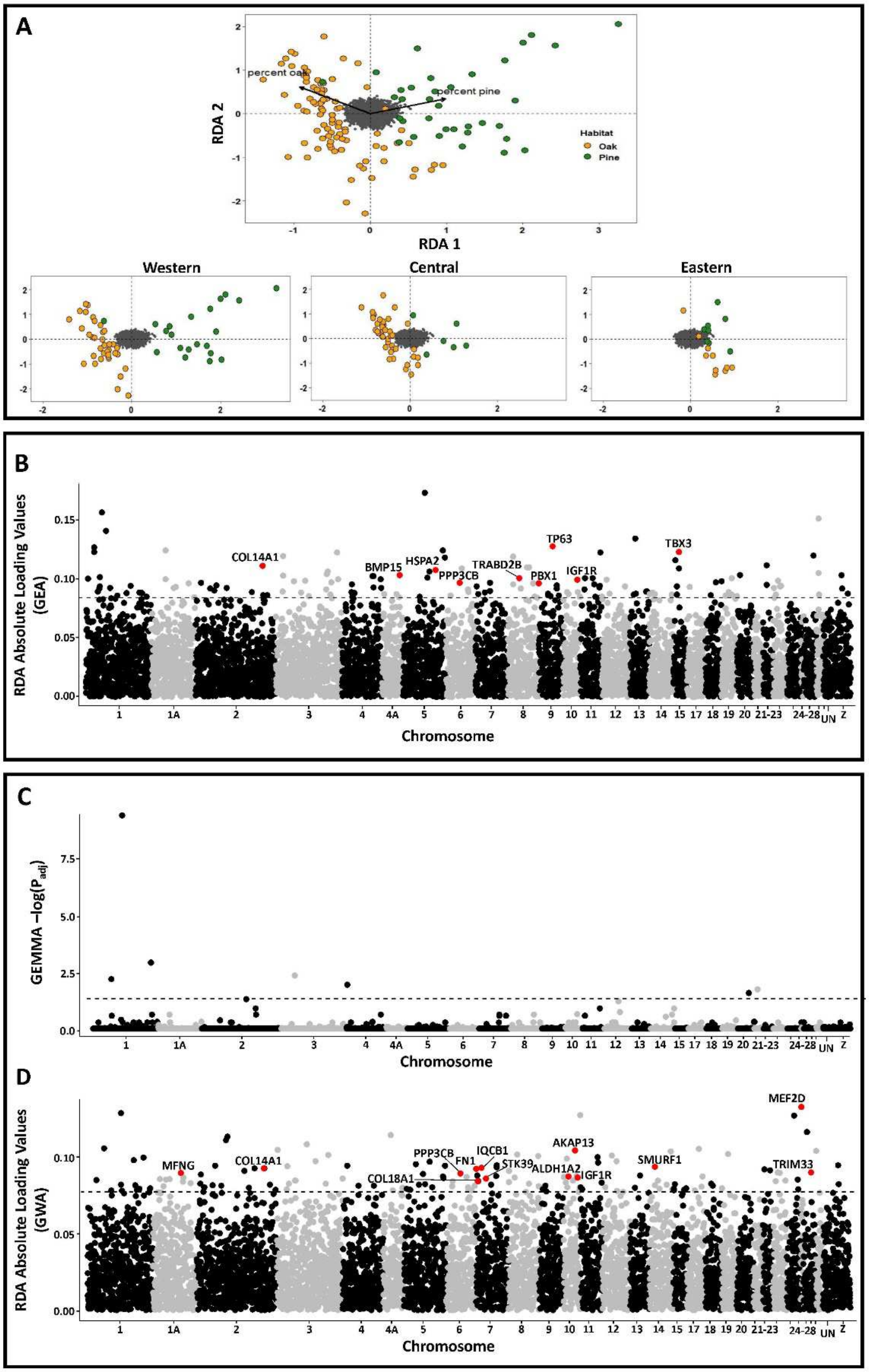
A
RDA 2
RDA 1
percent oak
percent pine
Habitat
Oak
Pine
Western
Central
Eastern
B
RDA Absolute Loading Values (GEA)
COL14A1
BMP15
HSPA2
PPP3CB
TRABD2B
TP63
PBX1
IGF1R
TBX3
Chromosome
1 1A 2 3 4 4A 5 6 7 8 9 10 11 12 13 14 15 17 18 19 20 21-23 24-28 UN Z
C
GEMMA −log(P_{adj})
Chromosome
D
RDA Absolute Loading Values (GWA)
MFNG
COL14A1
PPP3CB
FN1
IQCB1
STK39
COL18A1
AKAP13
ALDH1A2
IGF1R
SMURF1
MEF2D
TRIM33
Chromosome

Figure 2.3: Results of genotype-environment association (GEA) and genome-wide association (GWA) of 123 island scrub-jays. (a) Ordination plot of redundancy analysis (RDA) showing the habitat-linked (pine vs. oak) genetic divergence. Colored points show where individual samples load for RDA axes 1 and 2 based on a partial RDA conditioned on geographic location of each individual using 3,345 SNPs (shown as dark grey points in the center of the plot) as the response and relative proportion of pine and oak habitat within a 300-meter radius of sampling locality as the predictors (black vectors). The western, central, and eastern panels represent individuals grouped by which pine stand they are geographically closest to. Manhattan plot (b) shows the absolute value of the SNP loadings on RDA axis 1 of the RDA-GEA. Panels (c) and (d) show genome-wide association (GWA) results using univariate GEMMA and multivariate RDA, respectively, that identify SNPs associated with body size-corrected measures of bill length. The horizontal dashed line in panel (c) corresponds to the threshold for statistical significance (*P*-value = 0.05). Manhattan plot (d) shows the absolute value of the SNP loadings on RDA axis 1 of the RDA-GWA. The horizontal dashed lines in panels (b) & (d) show 2.5 standard deviations from the mean absolute loading value for the RDA-GEA and RDA-GWA. Red highlighted points in panels (b) and (d) represent outlier SNPs associated with candidate genes (labels) related to bill morphology based on previous studies. Black and grey colors distinguish different chromosomes numbered according to the zebra finch nomenclature.

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CHAPTER 3: GENETIC PARALLELISM UNDERLYING REPEATED BILL DIVERGENCE INCREASES AT HIGHER MOLECULAR LEVELS OF ORGANIZATION

Summary

Whether the same genes should underlie parallel adaptive trait evolution remains an open question in biology. Genomic approaches provide insights on how frequently the same genetic basis underlies parallel adaptive evolution. Yet there is large variation in the extent to which phenotypic parallelism shares the same genetic mechanisms in nature. Because genetic parallelism encompasses a hierarchy of mechanisms that connect genetic variation to phenotypic variation (i.e. single nucleotide polymorphisms [SNPs] to genes to pathways to phenotype), the degree of parallelism is expected to increase at higher hierarchical levels. 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, USA. We analyzed 66,503 SNPs generated using BestRAD in 161 island scrub-jays for which bills were measured 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 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 convergent evolution depends on the genetic level of organization being analyzed.

Introduction

A fundamental goal in evolutionary biology is to understand the processes and mechanisms that underly adaptation. The repeated evolution of similar traits in similar habitats is considered strong evidence of adaptation in nature (Endler, 1986; Harvey & Pagel, 1991; Schluter et al., 2004; Arendt & Reznick 2008; Schluter and Conte 2009; Bolnick et al., 2018). Studies exploring repeated phenotypic evolution have categorized the process as either "parallel" or "convergent" (Arendt & Reznick, 2008; Stuart 2019; Waters & McCulloch, 2021; Cerca 2023). There is an ongoing and circuitous debate on the usage of these terms (Arendt & Reznick, 2008; Elmer & Meyer 2011; Rosenblum, Parent & Brandt, 2014). For the purposes of this study, we use the term "parallelism", to refer to the degree to which natural selection acts on similar ancestral phenotypes to generate a continuum of derived parallel phenotypes that vary from being highly parallel to nonparallel across molecular levels (e.g., single nucleotide polymorphism [SNP], gene, pathway, trait, function; Bolnick et al., 2018; Cerca 2023). An improved understanding of genetic parallelism underlying parallel traits can inform long standing questions concerning the extent to which evolution is a predictable and repeatable process (Gould, 1989; Stern and Orgogozo 2008; Blount et al., 2018). Such insights are relevant for predicting adaptive evolution of populations responding to changing habitats (Reid et al., 2016; Preite et al., 2019; Cosentino & Gibbs, 2022; Winchell et al. 2023), or emergence of novel pathogens (Farhat et al., 2013; Palmer & Kishony 2013). Yet there is little consensus on what factors drive variation in the degree of genetic parallelism, even within closely related taxa (Rosenblum, Parent & Brandt, 2014; Bolnick et al., 2018; Bohutínska et al., 2021).

Some of the debate regarding the discrepancy between degree of phenotypic and genetic parallelism may, in part, be driven by methodology. 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, 2019). Multiple studies have successfully used this methodology to demonstrate genomic parallelism at the level of SNPs (e.g. Colosimo et al., 2005; Hoekstra, 2006; Loh et al., 2012), genes (e.g. Nosil, Egan & Funk, 2009; Manceau et al., 2010; ; Stern, 2013; Gallant et al., 2014; Nosil et al., 2018; Thirkildsen et al., 2019; Walsh et al., 2019), and pathways (e.g. Cooper et al., 2014; Jacobs et al., 2020; Birkeland et al., 2020). However, focusing on just the SNP or gene level of genetic parallelism results in several challenges. First, outlier tests used to detect potential candidate loci are biased towards SNPs of strong effect (Hoban et al., 2016). Given that most adaptive traits are complex and have a polygenic genetic architecture, this bias towards detecting single SNPs or genes of large effect may obfuscate our ability to detect the extent of parallelism, as it likely fails to identify many loci of small effect that influence adaptive divergence in nature (Rockman et al., 2012; Yeaman, 2015; Hoban et al., 2016; Wellenreuther & Hansson, 2016). Second, because complex traits (e.g human height; Wood et al. 2014; Shi et al. 2016) may have hundreds or even thousands of associated SNPs spread across the genome, each with a small effect on the phenotypic variance (Yengo et al., 2022), genome-wide association (GWA) studies examining polygenic traits tend to exhibit low heritability despite large sample sizes, leading to debate over the causes of this “missing heritability” (Manolio et al., 2009). Boyle et al. (2017) proposed an “omnigenic” model to explain this missing heritability by arguing 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 within regulatory networks. 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 molecular pathway (Láruson et al., 2021). Within this framework, we would expect that the evolution of adaptive parallel phenotypes by selection should exhibit little parallelism at the SNP or gene level, but increased parallelism at the pathway level (e.g. Rose et al., 2018; Walden et al., 2020; Chaturvedi et al., 2022; Ryan et al., 2023). While recent studies have begun to look at parallelism across molecular levels by incorporating comparisons of SNPs, genes, and pathways to fully characterize genetic parallelism in suites of polygenic traits (e.g. Daub et al., 2013, 2017; Bohutinska et al., 2021; Szukala et al., 2023), the hypothesis that genetic parallelism scales with molecular levels of organization in complex traits has not yet been systematically tested.

Here, we aimed to test this hypothesis by investigating genetic parallelism hierarchically from SNPs to genes to pathways. We analyzed replicate instances of adaptation of a complex, polygenic trait to different habitat types in the island scrub-jay (*Aphelocoma insularis*). The jays are single island residents, endemic to Santa Cruz Island, California, USA (Delaney & Cheek, 2022). Despite the jay's limited geographic range (250km^2), they exhibit parallel phenotypic divergence between pine and oak habitats on the island. Jays in pine-dominated habitat have longer, shallower bills compared to jays located in oak dominated habitat (Langin et al., 2015). Specifically this pattern is repeated across each of the three bishop pine (*Pinus muricata*) and island scrub oak (*Quercus pacifica*; Junak, 1995) ecotones in the west, central and east regions of the island (Walter & Taha, 1999; Fischer et al., 2009; Langin et al., 2015). This microgeographic divergence in bill morphology in island scrub-jays is thought to be adaptive, as it mirrors the same 'macrogeographic' patterns described in California (*A. californica*) and woodhouse (*A. woodhouseii*) scrub-jays, in which bill length variation is associated with foraging efficiency on pinecones and acorns (Peterson, 1993; Bardwell et al., 2001; Richardson et al., 2014; Langin et al., 2015). The observed differences in bill length are heritable and have a polygenic basis, which suggests divergent selection is acting on bill morphology between oak and pine habitat types

(Langin et al., 2015; Cheek et al., 2022). Given this background, the island scrub-jay provides an excellent system to explore genetic parallelism across the SNP, gene, and pathway molecular levels.

In this study, 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: Does the extent of genetic parallelism underlying parallel adaptive phenotypic divergence increase with increasing molecular level of organization (SNP, gene, pathway)? First, we quantified population-wide divergence in bill length and depth across each of the pine-oak ecotones (Langin et al., 2015). Second, we estimated potential population wide genetic structure because tests for selection can be biased by underlying population structure (Cushman & Landguth 2010; Lotterhos & Whitlock 2015; Battey et al., 2020). Third, we used genotype-environment association (GEA) and GWA approaches to test alternative hypotheses regarding the genetic underpinnings of local adaptation to habitat and divergence in bill morphology. We predicted that if the genetic basis underlying bill length in any single population is controlled by a few genes of large effect, then GWAS results will find the same SNPs that are highly parallel between replicate pine-oak ecotones and that genetic parallelism will be observed across molecular levels (SNPs, genes, pathways). Alternatively, if the genetic basis of bill length includes many genes of small effect, then GWAS results will find many SNPs and genes from across the genome that are unique to replicate ecotones, but genetic parallelism may be observed at higher molecular levels of organization because of the limited number of pathways available to affect phenotypes such as bill morphology. Finally, we combined multivariate analysis of allele frequency changes among each of the pine-oak ecotone replicate pairs with identified candidate loci to investigate signals of genetic parallelism at specific regions across the genome.

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. 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). 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 individuals that were banded as nestlings as their bills are not fully developed.

Whole blood samples were extracted 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. 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.

Bill morphometrics

Langin et al. (2015) demonstrated repeated patterns of divergence in island scrub-jay bill morphology between individuals captured in pine vs. 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.

To assign individuals to habitat type, we used ARCGIS software and R scripts to calculate the percent pine and percent oak within a 300 meter 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 meters of a pine polygon (Langin et al., 2015; Cheek et al., 2022). We calculated the distance from each capture location to the nearest pine polygon and assigned individuals to the west, central, or east pine-oak ecotone depending on the pine stand to which they were closest to geographically (Figure 3.1A).

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 re-ran models using subsets of individuals from each pine-oak ecotone to test the significance of bill morphology divergence by region.

Genotyping

We prepared libraries for 179 individuals following BestRAD library preparation protocol with some modifications (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 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 150bp 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., 2013; Catchen et al., 2011). We discarded reads with an uncalled base or with low quality scores (Phred score ≤ 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 and 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: principal components analysis (PCA; Section 2.4), GEA tests (Section 2.5), and genome-wide associations (GWA; Section 2.6).

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 (Goodwin 1976; Woolfenden and Fitzpatrick 2020; Delaney and Cheek 2022). We sexed individuals using individual or partner rattle call vocalizations observed in the field, or sex assignments from previous studies (Caldwell et al., 2013; Langin et al., 2015; Desrosiers et al., 2021). 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., United Kingdom) following the manufacturers thermal cycling protocol.

Population structure

We calculated H_O, H_E and nucleotide diversity (π) 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 Ne, we identified candidate adaptive markers using partial RDA (pRDA; see section 2.3) and removed SNPs with more than 10% missing data to estimate Ne using a higher quality SNP dataset of presumptive neutral markers (genetic data with candidate adaptive SNPs removed). To reduce bias in Ne estimates due to retaining linked loci, which can have a larger effect on Ne estimates than either population size or sample size (Waples et al., 2016), we corrected Ne and jackknifed confidence intervals based on 31 chromosome pairs in the zebra finch genome that mapped to California scrub-jay reference. We visualized population structure of the individual-based data using a model-free approach, PCA, and an ancestry estimation method, ADMIXTURE version 1.3 (Alexander et al., 2009). We used the imputed data from BEAGLE for our PCA analyses. Because there are 3 pine-oak ecotones, we tested K = 1-6 with the un-imputed dataset in ADMIXTURE and used a cross-validation plot to determine the best 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 calculated using the *geodist* package to measure "geodist" (Padgham & Sumner, 2020). We tested for isolation-by-distance using a Mantel test with 10000 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 by sorting jays according to which pine stand they were geographically closest to (Figure 3.1A). We identified candidate adaptive markers associated with habitat using partial RDA (pRDA; hereafter referred to as RDA-GEA), a multivariate genotype–environment association analysis that is well suited to identify weak, polygenic signatures of selection related to environmental variables (Forester et al., 2016, 2018; Capblancq & Forester, 2021). We used the percent pine and percent oak within a 300 meter radius of each scrub-jay sampling location (the diameter of the largest island scrub-jay territory; Caldwell et al., 2013) from a reclassified 2005 vegetation map of Santa Cruz Island (The Nature Conservancy, 2007; Langin et al., 2015; Cheek et al., 2022) 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 our previous research showed that longitude was a strong predictor of interindividual genetic differentiation (Langin et al., 2015; Cheek et al., 2022). We tested the significance of both the global model and model terms (percent pine and percent oak) 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

Bill morphology divergence in island scrub-jays has been shown to mirror well-described patterns observed between mainland scrub-jay populations occurring in pine/juniper vs. oak habitats (Peterson et al.,1993; Bardwell et al. 2001; Langin et al., 2015). We observe the same pattern – 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 (section 3.1). Therefore, we chose to focus on bill length to identify potential genetic markers associated with bill morphology as described in our previous study (Cheek et al., 2022). To determine if genetic divergence mirrors parallel phenotypic divergence in bill length, we performed a PCA on measurements of wing, tail, and mean tarsus length for the 161 genotyped individuals that passed our quality control filters (see section 3.3). 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 bander 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 and was removed from further analyses. We used a separate RDA (hereafter referred to as RDA-GWAS) 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. Outlier loci were identified using the criterion of loading scores ±2.5 SD from the mean to identify SNPs associated with variation of bill length.

Functional annotation of loci and quantifying parallelism

Visual inspection of linkage disequilibrium (LD) plots using LD calculated within a 500-kb 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 S3.2). 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 variant site 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, Zhao & Zhang, 2015) to quantify parallelism at each genetic level (SNPs, genes, pathways).

Identification of parallel molecular evolution

We tested if repeated evolution 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 pairs are indicative of parallelism and 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 (Langin et al., 2015). Therefore, we tested for allele frequency changes between each of our three, replicate pine-oak ecotone populations pairs. We scanned our genomic data using non-overlapping windows of 200 SNPs (median physical window size of 2.84 Mbp) 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-GWAS occur within genomic regions that may have undergone repeated selection between our pine-oak replicate pairs.

Results

Parallel divergence in phenotype

We confirmed that bill length differed between island scrub-jays captured in pine and oak habitats on Santa Cruz Island (Figure 3.1B) using a larger number of individuals than originally reported in Langin et al. (2015). Jays captured in pine (n=503) had longer bills compared to jays captures in oak (n=981; *p*-value bill length <0.001; 95% confidence interval [0.307mm, 0.431mm]) 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 <0.001; 95% confidence interval [0.352mm, 0.620mm]; n central= 491; *p*-value bill length central <0.001; 95% confidence interval [0.310mm, 0.587mm]). Divergence of bill length was in the expected direction (i.e. longer bills in pine), but not significant in the east (n east= 180; *p*-value bill length east=0.134; 95% confidence interval [-0.086mm, 0.684mm]). 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 =0.656; 95% confidence interval [-0.0218mm, 0.0138mm]).

Genotyping

We sequenced 179 individuals sampled across all three pine-oak ecotones on Santa Cruz Island (Figure 3.1A). 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: observed heterozygosity of variant sites (H_O) = 0.204, expected heterozygosity (H_E) = 0.210, inbreeding coefficient (F_{IS}) = 0.033, and nucleotide diversity (π) across all sites (variant and fixed) = 0.00062. The effective population size (Ne) 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 S3.1). However, mantel tests using 63,355 neutral SNPs indicated weak spatial population structure (*p*-

value=0.0651; r =0.03419) consistent with a pattern of isolation-by-distance primarily driven by longitude according to our PCA results (Figure 3.1C).

Parallel signatures of selection driven by habitat at higher genetic levels

The relative proportion of pine and oak within a 300-m radius of each individual's capture location was associated with genetic variation in island scrub-jays according to our RDA-GEA (Table 3.1). 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 3.1). 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 3.2A). 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 S3.1). Only 15 of these SNPs (0.15%) were shared across all 9,933 unique SNPs identified between the three ecotones (Figure 3.2B). 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 3.2C). Finally, we identified 1,178 genes that were within 160 molecular pathways (Table S3.2). Of the 160 pathways, 136 (85%), were shared across all three ecotones. We observed significant (*p*-value <0.001) overlap between the three ecotones only at the pathway level, but not SNP or gene (*p*-value =1), level according to our Fischer's exact test (Figure 3.2D, Table S3.2).

Hierarchy of parallelism scales in bill length

Our RDA-GWAS analysis identified 1,105 SNPs in the west ecotone, 1,180 SNPs in the central ecotone, and 1,253 SNPs in the east ecotone that were associated with variation in bill length (Figure 3.3A). None of the 3,459 SNPs flagged by our RDA-GWAS were shared across all three ecotones. A total of 3,701 genes were within 25 kb of our candidate loci, 26 (0.70%) of which were shared across GWAS analyses (Table S3.2; Figure 3.3B; Figure S3.3). Potential candidate genes found in the Gene Ontology annotation reports included multiple genes (e.g. *SMPD3*, *NFIA*) that are associated with the bone morphogenetic protein pathway. Furthermore, 10 genes identified in the RDA-GWAS analyses were also flagged by the GEA (Table S3.2). Our RDA-GWAS flagged 80 genes that occur within 151 molecular pathways (Figure 3.3C), some of which are known to affect bill morphology including MAP kinase activity (*MAPK*), *TGF-Beta*, calcium and *Wnt* signaling pathways (Figure S3.4; Table S3.2; Yusuf et al. 2022). Of the 151 pathways identified, 85 (56%) were shared between all three ecotones (Figure 3.3C). A Fischer's exact test of overlapping SNPs, genes and pathways identified by our GWAS reveal significant overlap (*p*-value <0.001) at the pathway, but not SNP or gene level (*p*-value =1).

Weak parallelism between replicates

The *AF-VapeR* analysis identified 248 outlier windows across the genome using the 95% quantile cutoff from the randomized allele frequency matrices. We failed to detect parallel windows using a stricter cutoff (e.g. 99%). Across these 200bp outlier windows, none of the candidate SNPs we identified in our RDA-GEAs that were associated with habitat variation were found. However, of the 3,459 unique SNPs associated with variation in bill morphology across our three RDA-GWAS analyses, 425 loci were found within the fully parallel outlier windows. All three pine-oak ecotone population pairs therefore experienced parallel allele frequency changes for these outlier windows associated with potential candidates. Three of the annotated genes identified by all three RDA-GWAS analyses (*SLIT3*, *NIPSNAP2*, *HIVEP3*) also overlapped in fully parallel windows (Figure 3.3D).

Discussion

The presence or absence of concordance between phenotypic and genetic parallelism has played a key role in the debates about the definitions of parallelism and convergence (Arendt & Reznick, 2008; Elmer & Meyer 2011; Rosenblum, Parent & Brandt, 2014). Yet, evidence for genetic parallelism is likely to be dependent on where researchers focus their efforts in the molecular hierarchy connecting genetic to phenotypic variation. We tested for genetic parallelism across hierarchical molecular levels within a single continuously distributed population where the standing genetic variation is expected to be broadly overlapping and genetic parallelism across all levels should be more likely (Conte et al., 2012; Ord & Summers, 2015; Bohutínská et al., 2021). Yet we found that genetic signatures of habitat-linked selection and associations with habitat and bill morphology do not nonoverlap significantly at the SNP level between individuals sampled in three different pine-oak habitat replicates. Instead, we observe some overlap at the gene level, and significant overlap (i.e. parallelism) at the pathway level. Moreover, we observe some subtle parallel changes in allele frequencies among individuals sampled in the three ecotones that also overlap with a moderate fraction of candidate SNPs associated with bill morphology. Taken together, our findings suggest that microgeographic adaptation in island scrub-jays is consistent with an omnigenic framework, in which bill morphology and traits potentially linked to habitat variation are associated with a core set of pathways, but less so with a set of genes or SNPs. These findings imply that parallelism at the phenotypic level does not guarantee parallelism across all genetic levels even within a single continuously distributed population. Below we discuss these results in more detail.

Genetic parallelism within an omnigenic hierarchy

If we consider genetic parallelism within the hierarchical omnigenic framework, then we expect that most SNPs and genes associated with variation in bill morphology will have relatively small effects and there should be many potential targets for polygenic selection, but there should also be some loci with larger effects limited to a few highly conserved molecular and developmental pathways (Figure 3.3E; Boyle et al. 2017). Our results are consistent with this perspective. Multiple case studies show such parallelism at higher molecular levels 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, 2016; Villoutreiz et al., 2020; Urban et al., 2021), suggesting that there are multiple potential genetic routes to the same biochemical outcome (Yeaman et al., 2018; Láruson et al., 2021).

In birds, several studies have implicated the same 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). If selection is shaping island scrub-jay bill morphology through onmigenic mechanisms, then we would expect that candidate SNPs associated with bill morphology that are unique to different ecotones could occur within broader genomic targets of selection (i.e. genes). Indeed, we found a number of candidate SNPs and genes associated with bill length that occurred within fully parallel windows identified using *AF-vapeR*. One notable example is the gene *SLIT3*, which has GO terms associated with anatomical structure development and is involved in several molecular pathways important for regulating *BMP* including *Wnt* and *TGF-beta* signaling (Jiang, Sun, and Huang 2022; Gong and Si, 2023), was identified in all three RDA-GWAS analyses, and occurred in a window that contained allele frequency changes that were fully parallel (repeated between all three pine stands) according to AF-vapeR (Figure 3.3D). Collectively, these results reinforce the view that polygenic selection is a complex process (Szép et al. 2021; Barton 2022), and while the path from genomic variation to phenotypic variation can take multiple routes, the number of options become more limited at higher levels of biological organization.

Parallelism and genetic architecture of bill morphology

Our morphological analyses presented here and in a concurrent study (Clancey et al., 2023) indicates parallel divergence in bill length between scrub-jays living in pine versus oak habitat. This result is consistent with the findings of Langin et al., (2015), but the results here are based on the measurements of approximately three times more individuals. While the divergence in bill morphology in island scrub-jays is relatively small in absolute terms (~1mm), it mirrors adaptive differences in mainland *Aphelocoma* species, where longer, shallower bills are more efficient at extracting pine seeds from pinecones while shorter, deeper bills are more efficient at hammering open acorns (Peterson 1993; Bardwell et al., 2001; McCormack and Smith, 2008). Indeed, similar relative differences in bill morphology have been shown to have consequential impacts on performance (see Bardwell et al. 2001, Moyer, Peterson, and Clayton, 2002; Clayton et al., 2005). However, we did not find a significant difference in bill depth (Figure 3.1B); which was previously found to exhibit very subtle differences (Langin et al., 2015). Bill morphology also appears to have a polygenic basis based on our findings here and a previous study using a lower density SNP array (Cheek et al., 2022). Several candidate loci within or near genes that we identified in all three RDA-GWAS analyses contained GO terms that were associated with skeletal and cartilage development (e.g. *SMPD3*, *NFIA*). These genes are also involved in bone morphogenic protein (*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 S3.2). This evidence provides additional support that bill morphology in island scrub-jays is highly complex and likely the result of multiple, interacting pathways as seen in other systems (Lundgregan 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 within closely related lineages, regardless of the molecular level examined, due to the increased likelihood that populations are derived from the same ancestral pool of genetic variants (Conte et al, 2012; Ord & Summers, 2015; Bohutinska et al, 2021, Waters and 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 their mainland congener (DeRaad et al., 2022). While we observed some structuring between island scrub-jays living in the east and west regions of Santa Cruz Island according to our ADMIXTURE results (Figure S3.1), 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 3.1C; Langin et al., 2015; Cheek et al., 2022). Thus, the island scrub-jay species is best described as a single population experiencing weak levels of spatial population structure. We would therefore expect that parallelism would be high across molecular levels given the observed lack of population structure and recent shared ancestry (Delaney & Wayne, 2005; McCormack et al., 2011).

Nevertheless, we instead found limited parallelism at the SNP level between island scrub-jays residing in replicate pine-oak ecotones. We observed parallel signals of selection in our genotype-by-environment associations within each ecotone, with individuals sampled in pine and oak habitat clearly separating based on their genotypes after controlling for effects of isolation-by-distance (Figure 3.2A). Yet this repeated pattern of selection between pine and oak habitat exhibited was not observed at the SNP level (Figure 3.2B). We propose that the lack of parallelism in our RDA-GEA candidate SNPs is likely because this analysis captures selection acting on a diversity traits that differ between pine and oak habitats.

While the causative factors driving a lack of genetic parallelism at the SNP level underlying parallel traits remains a topic of discussion (Elmer & Meyer, 2011; Bolnick et al., 2018; Barghi, Hermisson, and Schlötterer 2020), what is clear is that genomic parallelism is not common, even in highly controlled laboratory experiments (e.g. Bailey et al., 2017; Lenski, 2017). This finding suggests that demonstrating genetic parallelism in natural system where the selective landscapes are more complex is even less likely. For example, marine three-spine stickleback have repeatedly colonized and adapted to freshwater habitats which is considered a classic system for the study of parallel evolution (Reid, Bell and Veeramah, 2021), yet varying degrees of genomic parallelism have been found (e.g. Hohenlohe et al., 2010; Jones et al., 2012; Liu et al., 2018; Hohenlohe and Magalhaes 2020; Fang et al., 2020, 2021; Kemppainen et al., 2021; Dahms et al., 2022). Furthermore, parallelism has been found to be more likely with increasingly similar environments (Stuart et al. 2017), suggesting that a lack of genetic parallelism arises because environments that may appear similar differ in ways that alter selection. If selection is very similar and truly repeated among pine oak population pairs in island scrub-jays, then then we would expect that the same genomic regions should repeatedly evolve to drive divergence in at least a few putative candidate SNPs within parallel genomic regions because of processes like shared genetic variation (i.e. population sorting; Lee and Coop 2017, 2019; MacPherson and Nuismer, 2017; Waters & McCullock, 2021). However, in our *AF-vapeR* analysis none of the SNPs flagged by our RDA-GEA analysis were found within any of the parallel windows identified in our three pine-oak population pairs. Thus, despite the expectation that pine and oak habitats are true replicates and impose similar divergent selection, it is more likely there are quantitative environmental differences among the qualitatively similar habits (see Kaeuffer et al., 2012; Bailey et al., 2015 Auld and Brand, 2017; Schiebelhut et al., 2023, for examples).

Environmental variation, for example, is a potential quantitative difference between pine and oak habitats on Santa Cruz Island. Coastal fog, temperature, and topography interact to form a unique natural climate gradient along the east west axis of the island with more arid conditions in the western region (Fischer et al., 2009; Morrison et al., 2011; Gamboa et al., 2022). Indeed, some of the genes that were shared between RDA-GEA analysis included GO annotations related to temperature (e.g. *HTR1B*, *GRM1*) and cold response (e.g. *ZNF423*, *NR1H3*), while several genes related to water homeostasis were unique to different regions (e.g *SCNN1G*, *EXT1*, *EXT2*). Additionally, residual impacts of anthropogenic use and grazing history have significantly impacted the current distribution of woody vegetation on Santa Cruz Island (Pesendorfer et al., 2018). Therefore, it is likely that there are other sources of selection linked to pine and oak habitat, such as precipitation, temperature, and vegetation density and composition, that drives divergence between individuals in pine and oak habitat differently within the same pathways, even at the restricted spatial scale focused on here (250 km^2). Future studies should investigate potential physiological and additional morphological 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).

Conclusion

We found little evidence for parallelism among candidate SNPs associated with bill morphology and environmental variation, but strong evidence for parallelism at the pathway level. Our results support the idea that genetic parallelism in polygenic traits may be best explained by considering their hierarchical or omnigenic basis, where a very large number of SNPs affect a given trait through a smaller number of core pathways. This finding emphasizes the challenge in understanding how genetic variation translates into phenotypic variation.

Figures and tables

Table 3.1: Redundancy analysis (RDA) results testing genetic associations with habitat variation (percent pine and percent oak). P-values and F statistics are reported for global models and predictor terms for all individuals sampled (n=161), as well as separately for individuals from the west (n=66), central (n=42) and eastern regions (n=53).

	All	**West**	**Central**	**East**
P-value	0.109	0.141	0.118	0.189
F	1.052	1.050	1.063	1.062
P-value$_{oak}$	0.063	0.062	0.182	0.160
F	1.089	1.091	1.051	1.087
P-value$_{pine}$	0.384	0.442	0.081	0.329
F	1.015	1.009	1.076	1.037

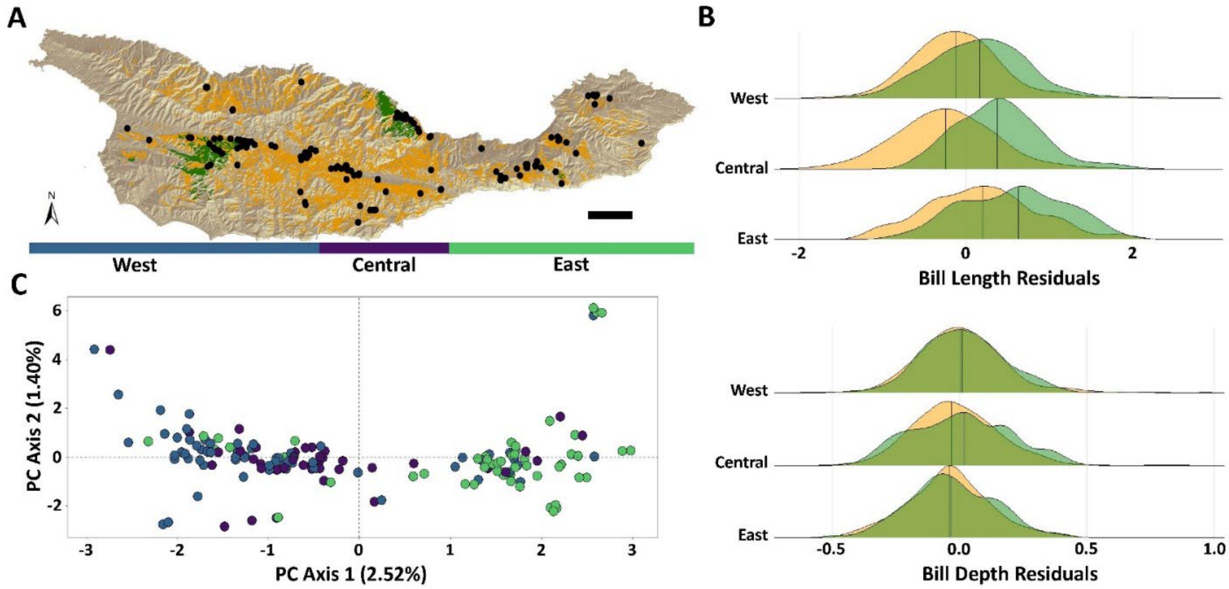

Figure 3.1: Island scrub-jays exhibit repeated habitat-linked phenotypic divergence at a microgeographic scale. (A) Study area of Santa Cruz Island, California, USA, and habitat type (green, pine; orange, oak). Colored bars show the regional breakdown of Santa Cruz Island. Black bar represents 4 kilometers. 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, east pines = 30). 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, 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 (PCA) 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 categorized by which pine stand they are geographically closest to.

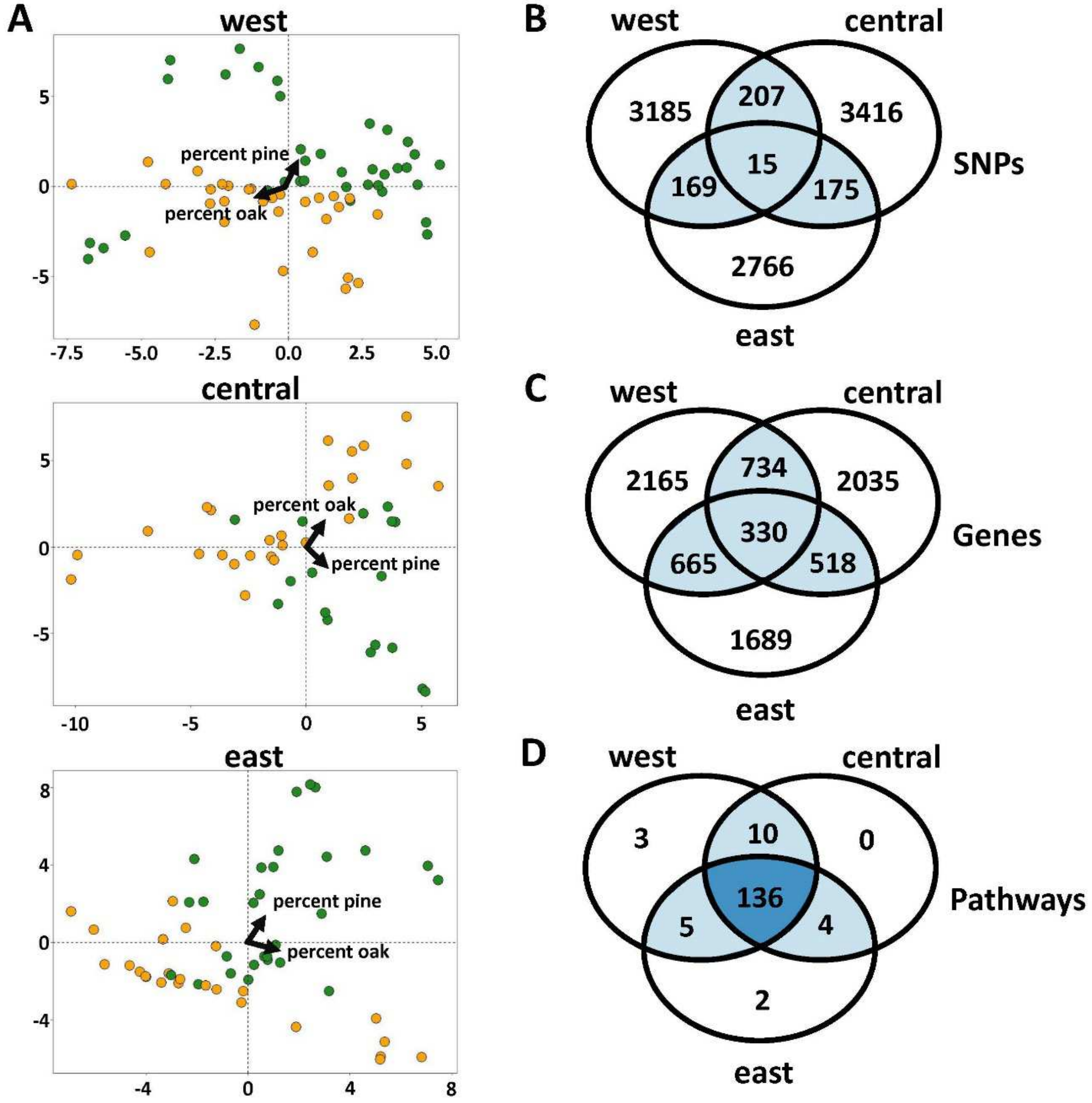

Figure 3.2: Evidence of repeated habitat-linked selection between 161 island scrub-jays sampled in pine and oak habitat. Redundancy analysis showed habitat-linked genetic divergence in the west, central, and east ecotones. Colored points show where individual samples load for RDA axes 1 and 2 shaded by which habitat they were captured in (green, pine; orange, oak) based on a partial RDA 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.

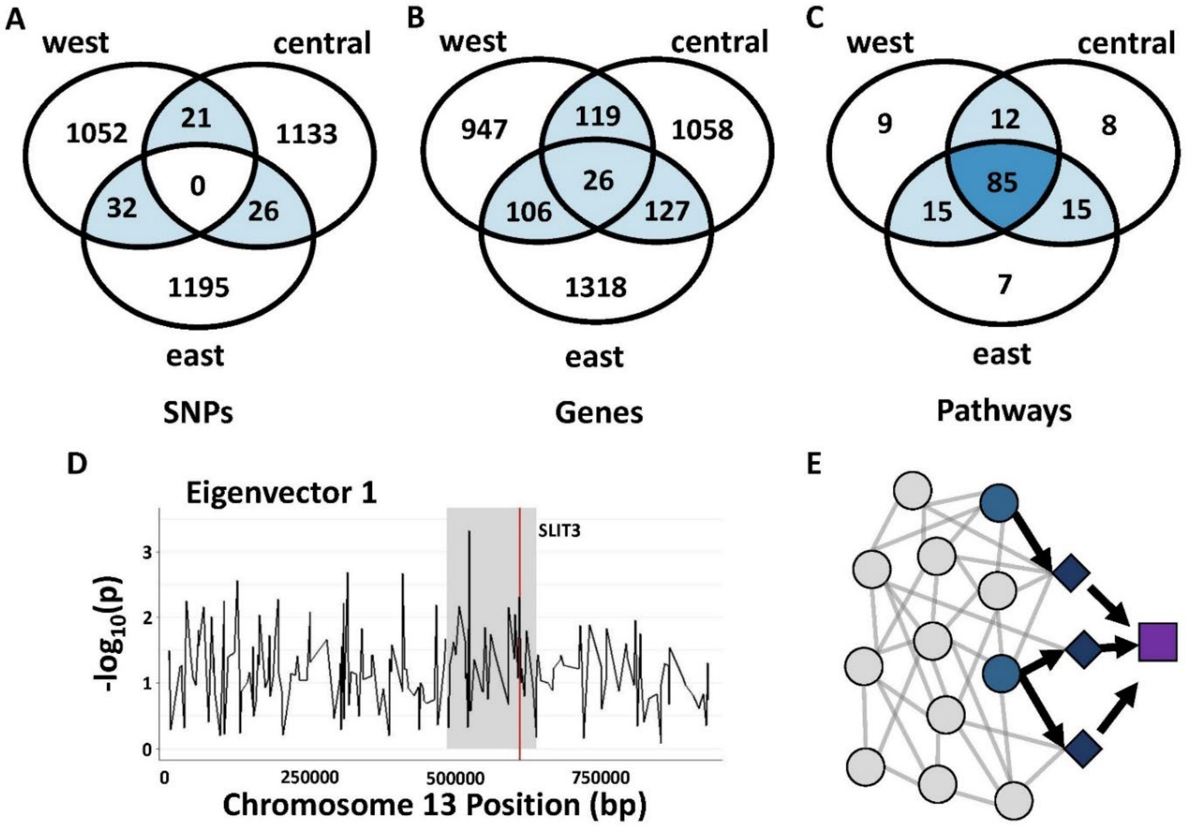

Figure 3.3: Genetic parallelism increases at higher molecular levels in a complex trait. Venn diagrams show that overlap in candidate SNPs (A), genes (B), and pathways (C) associated with variation in bill morphology between island scrub-jays sampled in three pine-oak ecotones increases at higher genetic levels. Genetic parallelism among pine-oak population pairs within chromosome 13 (D) identified as a significant loading on eigenvector 1 within the window (highlighted grey 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 (grey circles), and SNPs within them, may have indirect effects (grey 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 and Austerlitz; 2021).

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CHAPTER 4: MICROGEOGRAPHIC ADAPTATION IN AN ISLAND-ENDEMIC BIRD IS LIKELY FACILITATED BY SEX-BIASED NATAL HABITAT PREFERENCE AND LIMITED DISPERSAL

Summary

Non-random dispersal, where individuals exhibit biased settlement preferences for specific habitats, can have a significant impact on the spatial distribution of genetic variation which, in turn, has important evolutionary implications. Yet relatively few studies have demonstrated how non-random dispersal interacts with other evolutionary processes to impact putatively neutral and adaptive spatial genetic variation in natural systems. Here, we developed a spatially grounded molecular pedigree to test if natal habitat preference or phenotype dependent habitat choice contributes to previously described patterns of microgeographic adaptation in the island scrub-jay (*Aphelocoma insularis*; an endemic resident to Santa Cruz Island, California, USA). Island scrub-jays exhibit a repeated pattern of longer, shallower bills in three geographically distinct pine habitats relative to jays living in adjacent oak habitat, suggesting disruptive selection has led to adaptive phenotypic and genetic differentiation. We find that males exhibit limited dispersal, while female disperse farther away from their natal territories. However, within pine habitat, we found females largely restrict their dispersal to adjacent pine habitats suggesting dispersal is not random with respect to habitat. Analysis of available breeding territories relative to natal sites revealed that natal site habitat composition, but not bill length, predicts breeding territory habitat composition in females who hatched in pine habitat. These results suggest that females in the pine habitats exhibit natal habitat preference which likely contributes to the spatial distribution of adaptive genetic variation in island scrub-jays. Collectively, these results suggest

that patterns of adaptive genetic and phenotypic differentiation typically interpreted as being solely due to the effects of divergent selection should consider how non-random dispersal may contribute to the patterns of adaptive divergence.

Introduction

The role of gene flow in adaptive evolutionary change has been the subject of long-standing debate in evolutionary biology (Mayr, 1963, Slatkin, 1987). Many theoretical models assume gene flow is a random evolutionary process often working against selection by introducing maladaptive alleles (Lenormand, 2002; Kawecki & Ebert, 2004; Räsänen & Hendry, 2008). Yet this paradigm is beginning to shift as patterns of divergence and adaptation are frequently demonstrated in the presence of gene flow at relatively small spatial scales (Tigano & Friesen, 2016). Such cases of “microgeographic” adaptation have become increasingly common in the past decade (Richardson et al., 2014). In many of these cases, evidence of selection and adaptive divergence within the dispersal range of the species is clearly demonstrated (e.g. Skelly, 2004; Charmantier et al., 2016; Urban et al., 2017; Arietta & Skelly, 2021). The prevailing mechanism is assumed to be disruptive selection between populations occupying different environments and is inferred to be sufficiently strong to counteract gene flow (e.g. Boulding et al., 2016; Brousseau et al., 2020; Mikles et al., 2020) or to work in concert with some level of restricted gene flow to facilitate local adaptation (e.g. Weber et al., 2017; Gallego-Garcia et al., 2019). However, relatively few studies have attempted to better understand how patterns of dispersal may on their own generate spatial variation in adaptive genetic and phenotypic variation (but see Edelaar, Siepielski & Clobert, 2008; Clobert et al., 2009; Edelaar & Bolnick, 2012; Nicolaus & Edelaar, 2017; Richter-Boiz et al., 2013; Boussange & Pellissier, 2022; Jahnke et al., 2022). Thus, considering non-random gene flow requires a more comprehensive assessment of how individuals disperse and settle within populations.

Natal dispersal, defined as the permanent movement of an individual from its birthplace to its first reproduction site (Greenwood, 1980, 1982; Ronce, 2007), is a critical mechanism in gene flow and evolutionary dynamics as it impacts demography and genetic diversity among and within populations (Clobert et al., 2009). While dispersal itself does not always result in gene flow, when it is non-random and coupled with habitat choice, dispersal can impact the distribution of alleles across heterogenous landscapes (Cayuela et al., 2018; Bradburd & Ralph, 2019; Battey, Ralph, Kern, 2020). In some cases, patterns that appear to be driven by disruptive selection between different habitats may be generated by individuals selecting habitats non-randomly, which can contribute to the evolution of adaptive traits by decreasing gene flow between habitats or increasing gene flow of individuals to particular environments (Rice 1984; Edelaar, Siepielski & Clobert, 2008; Clobert et al., 2009; Edelaar & Bolnick, 2012; Bolnick & Otto 2013; Nicolaus & Edelaar, 2017). For example, population genomic studies often differentiate between "neutral" and "adaptive" loci when examining the roles of gene flow and selection on genetic variation (Hoban et al., 2016; Ahrens et al., 2018). Yet, in cases where individuals choose to settle in habitats that best match their phenotype, spatial variation in loci that appear to be subject disruptive selection may in fact reflect directed gene flow by different genotypes to those habitats (e.g. Edelaar et al 2008; Edelaar & Bolnick 2012; Nicolaus & Edelaar, 2017).

Non-random dispersal can arise through two potential evolutionary mechanisms; matching habitat choice or natal habitat preference. Matching habitat choice describes a form of dispersal where individuals with a given phenotype preferentially settle in the habitat in which they are best adapted based on an individual's assessment of its capacity to use this habitat (Edelaar, Siepielski and Clobert 2008; Edelaar and Bolnick 2012). One well studied example of matching habitat choice occurs when individuals choose habitats based on the background color to become more cryptic (Merilaita and Stevens 2011; Dreiss et al., 2012; Green et al., 2019; Lowe and Addis 2019; Camacho et al. 2020; Harris et al., 2020). In contrast, natal habitat preference describes when individuals choose breeding habitats that are more similar to the natal habitats they experienced during the early stages of their life-cycle (Davis and Stamps 2004). A preference for the natal habitat may be shaped by experience during time spent in the natal habitat, such that a preference for familiarity develops (Stamps et al., 2009; Camacho, Canal, and Potti, 2016; Merrick and Koprowski, 2016). Either of these non-random dispersal mechanisms can facilitate adaptive phenotypic divergence by reducing the number of maladapted individuals in a given habitat. Measuring dispersal in the context of matching habitat choice and natal habitat preference is therefore critical in our understanding of adaptation, as it has direct implications for the extent to which natural selection alone drives divergence (Eldelaar and Bolnick 2012).

To accurately measure dispersal, individuals must be tracked for a sufficient amount of time to include the locations of birth and first reproduction (Clobert et al., 2001). Several methods can be used to measure the distance between the natal and ultimate breeding environment. Telemetry and capture-mark-recapture methods have commonly been used to track individual movement, but associated logistical and challenges can make such methods prohibitive to fully capture dispersal in long lived species with delayed reproduction or highly mobile organisms (Koenig, Van Vuren & Hooge, 1996; Cayuela et al., 2018). A more recent alternative approach relies on parentage data and spatially grounded pedigree information to estimate post-natal dispersal by calculating the distance between the individual and its parent or another close relative (Bradburd, Coop, & Ralph, 2017; Bode et al., 2018; Bradburd & Ralph, 2019). Such an approach can work particularly well to identify dispersers in continuously distributed populations, but a caveat is that constructing pedigrees using these methods entails an extensive sampling effort to maximize the number of potential parent-offspring relationships as well as data on individual metrics (e.g. age and sex) which are not available in most systems (Huisman, 2017).

In this study we explored how dispersal impacts the spatial distribution of phenotypic and genetic variation in the island scrub-jay (*Aphelocoma insularis*). The jays are non-migratory bird species endemic to a single small island (250 km^2) off the coast of southern California, USA (Delaney & Cheek, 2022). Nearly half of the population (spring population size ~1,700; Sillett et al., 2012; Bakker et al., 2020) has been individually marked over the past decade as part of annual re-sight survey efforts. The jays experience competition for suitable breeding territories and individuals spend 1-7 years as non-breeding “floaters”, until they establish a breeding territory (Caldwell et al., 2013; Delaney & Cheek, 2022). Once established, a breeding male and female pair will jointly defend an approximately three-hectare space year-round for the rest of their lives (Delaney & Cheek, 2022). While the boundaries of territories will shift slightly on an annual basis, it is rare for pairs or individuals to change territories, thus dispersal from the natal territory to the breeding territory can be defined as having a discrete distance (Delaney & Cheek, 2022). These long-term demographic and spatial data present a powerful opportunity to infer the dispersal mechanisms that impact gene flow within a single population. Despite their restricted range, island scrub-jays exhibit fine-scale neutral genetic variation associated with geographic distance consistent with limited dispersal (Langin et al., 2015; Langin et al., 2017a; Cheek et al., 2022). Furthermore, the jays are found in different habitat types (bishop pine forest and oak chaparral) which corresponds to heritable differences in bill morphology (Langin et al., 2015). These patterns are presumed to be adaptive, as they mirror morphological differences in mainland California scrub-jays (*A. californica*) which are associated with differences in foraging efficiency. The long, shallow bills of jays living in pine-juniper forests are more efficient at extracting pine seeds from pinecones relative to the short, deep bills of jays observed in oak woodlands (Peterson, 1993; Bardwell et al., 2001). Adaptive genetic differentiation within pine-oak ecotones at select loci associated with habitat and bill morphology further supports the conclusion that the island scrub-jay exhibits microgeographic adaptation (Cheek et al., 2022). Yet, while selection has been assumed to be the primary mechanism generating the putatively adaptive phenotypic and genetic differences among pine and oak habitats (Langin et al., 2015; Cheek et al., 2022), an untested alternative explanation is that non-random dispersal could also generate these patterns. Indeed, recent simulation models conclude that selection alone is unlikely to explain the observed differences in bill morphology and that non-random dispersal is required to generate and maintain these patterns (Clancey et al. 2024). Like other *Aphelocoma* species (Aguillon et al., 2017), island scrub-jays appear to exhibit female-biased dispersal based on a limited number of direct dispersal observations (Langin et al., 2015). Yet it remains unclear exactly how far males and females disperse across the landscape relative to their natal territory and if jays choose breeding habitats based on their bill morphology or if they exhibit a natal habitat preference.

In this study, we genotyped over 1,400 island scrub-jays to construct a spatially grounded 10-year pedigree of known territory holders based on nesting records and mark - resight data to address two primary research questions: (i) how does dispersal distance vary with respect to sex and breeding habitat, and (ii) to what extent does natal habitat and individual phenotype impact dispersal? We first estimated average dispersal distances between male and female island scrub-jays breeding in both pine and oak habitat. Given our previous results showed a pattern of isolation by distance (Cheek et al., 2022), we predicted that individuals would settle in territories close to their natal site regardless of natal habitat composition or bill morphology and therefore average dispersal will be equal between habitats. Second, we generated a series of generalized linear mixed models to determine whether island scrub-jays exhibit natal habitat preference and/or matching habitat choice. Specifically, we tested alternative hypotheses regarding the effects of natal habitat composition (natal habitat preference) and individual phenotype (matching habitat choice) on the probability of selecting for pine or oak breeding habitat.

Methods

Study System: The Island Scrub-jay

The island scrub-jay is resident to Santa Cruz Island, California, USA, and has been the subject of intense ecological monitoring throughout the central valley and western region since 2008 (Caldwell et al., 2013), with earlier survey efforts taking place through the 1970's and 1990's (Atwood, 1978, 1980; Atwood, Elpers & Collins 1990). Standard population monitoring protocols in both studies have included marking as many individuals possible with a unique combination of up to five colored plastic leg bands and one numbered aluminum U.S. Geological Survey leg band, mapping territory size and location, and estimating home range size, dispersal, and individual survivorship using mark - resight data. A minimum of 15 island scrub-jay territorial pairs have also been monitored in 5 study plots on Santa Cruz Island during the breeding season (February-May) since 2008 to assess annual fecundity and breeding status of individuals (Figure 4.1A; Caldwell et al., 2013). These plots were selected because they encompass a gradient of the island's chaparral habitat and pine forest. Plots in this gradient include dense pine forest (Sauces Canyon), pine forest and oak chaparral ecotone (Buena Vista), relatively open areas dominated by grasslands and sage scrub surrounded by oak chaparral, and continuous oak chaparral with little to no grassland and scrub (Portezuela, Field Station, Coches Prietos Canyon; see Caldwell et al., 2013; Figure 4.1A).

Tissue Sample Collection

Juvenile and adult male and female island scrub-jays were captured using either mist nets or box traps from the western portion of Santa Cruz Island from 2009 to 2021. Morphological measurements were collected from each captured individual using digital calipers to record: (to ±0.01 mm), 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. Wing chord and tail length were measured with a ruler (to ±0.5 mm).

Whole blood samples were extracted 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. 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.

Overview of RAD-Capture Array Development

We used Restriction-site Associated DNA sequencing (RAD-seq) loci generated from 161 individuals sampled across Santa Cruz Island using the PstI restriction enzyme (Cheek Chapter 2) to develop a RAD-capture probe set (Ali et al., 2016). The details of the sample collection, preparation, and data processing of these original RAD loci is described in Cheek et al., (Chapter

2). Using these data, ‘MyBaits®’ baits were designed by Arbor Biosciences (Ann Arbor, MI. USA) to target a total of 7,608 RAD loci. This array included two categories of RAD loci: (i) 6,804 loci spread widely across the genome that were genotyped in more than half of the individuals, had ≤3 non-singleton SNPs, and had a minor allele frequency (MAF) ≥0.33; (ii) 805 loci strongly associated with bill morphology. Each RAD-Capture locus was filtered to retain the SNP with the highest minor allele count (MAC) to minimize potentially confounding effects of linkage disequilibrium. Additional details of the creation of the RAD-capture probe set have been summarized in Cheek et al. (Chapter 2).

RAD libraries produced for the capture arrays were constructed for 1,526 individuals from the western portion of Santa Cruz Island (Figure 4.1B). We chose individuals from this region as this was where we had the greatest temporal and spatial density of samples from pine and oak habitat. Libraries were produced using the BestRAD library preparation protocol with some modifications (Ali et al., 2016). Briefly, DNA was normalized to a final concentration of 75 ng in a 10 µl volume and digested with 6-basepair cutter restriction enzyme PstI (New England Biolabs, NEB). The fragmented DNA was then ligated with PstI specific adapters prepared with biotinylated ends, and 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 Illumina adaptors to the resulting DNA fragments. We size selected using Agencourt® AMPure XP beads for 350-550 bp fragments to select DNA fragments with an average length of 450 bp. Libraries were enriched with PCR, and cleaned again with Agencourt® AMPure XP beads, and assessed final libraries’ fragment size distributions and concentrations on an Agilent Tapestation 4150 and Qubit 2.0 fluorometer. RAD-capture assays were completed following the manufacturer's protocol (Ann Arbor, MI. USA). All but one capture contained two multiplexed RAD libraries. The resulting post-capture libraries were enriched with PCR and sequenced on a single S4 300 Cycle lane of an Illumina NovaSeq 6000 at the University of Oregon Genomics Core Facility (gc3f.uoregon.edu) for paired end 150bp sequencing.

We used Conda v4.8.4 (Anaconda Software Distribution, 2020) environments to manage bioinformatic packages. Next, based on recommendations for reads generated with NovaSeq platforms for low coverage data (Lou & Therkildsen, 2022) we performed a sliding window cut of the 3' end of the reads to remove low quality tails, defined as 4 bases in a row with mean QUAL scores less than 20, using *fastp* v0.23.2 (S. Chen et al., 2018). We checked fastqs for quality using *FastQC* v.0.12.1 (Andrews, 2019) before and after trimming reads. 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; Catchen et al., 2013). We discarded reads with an uncalled base or with low quality scores (Phred score ≤ 10). We removed PCR duplicates using *clone_filter* from *Stacks* version 2.64 (Rochette et al., 2019). We used clean, trimmed, reads to map reads against the California scrub-jay reference genome (GCA_028536675.1; DeRaad et al., 2022, 2023) using *BWA-mem* v. 0.7.17 (Li and Durbin 2009) and used the default parameters in the ref_map.pl in *Stacks* to create a RAD loci catalog. We discarded individuals with high missingness (>70% SNPs per individual) and re-ran populations using a coarse filter: -p 1, -R 0.3, -min_mac 2, retain one random SNP per locus. We further filtered our data with *Plink* v1.90b6.21 (Purcell et al., 2007) using the following settings: retain SNPs with minor allele frequency (MAF) >.4; retain loci with

<1% missing data; remove SNPs that deviate significantly ($p < 1e\text{-}50$) from Hardy–Weinberg. We then used *bcftools insec* of our filtered RAPTURE vcf and our filtered RAD dataset from Cheek et al. (Chapter 2) to compile a list of loci that were shared between the two datasets.

Genetic assignment methods

A population-level pedigree was constructed for island scrub-jays using the R package *sequoia,* which uses a maximum-likelihood framework to identify pairwise relationships between individuals (Huisman, 2017). Island scrub-jays are sexually dimorphic in terms of size, which can be difficult to discern accurately in the field. However, female corvids including scrub-jays produce a rapid, broad-band vocalization referred to as a "rattle" call (Goodwin 1976; Woolfenden and Fitzpatrick 2020; Delaney and Cheek 2022). Therefore, to include sex in our pedigree we sexed individuals using individual or partner rattle call vocalizations observed in the field, or sex assignments from previous studies (Caldwell et al., 2013; Langin et al., 2015; Desrosiers et al., 2021). 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., United Kingdom) following the manufacturers thermal cycling protocol. We estimated age based on plumage (Pitelka 1945; Pyle 1997) and subtracted from the initial year of capture to generate probable birth years for all individuals. For example, jays that were initially banded as adults were estimated at being at least two years old upon capture to provide an estimate of minimum birth year. We estimated our AgePrior using a published pedigree based on microsatellite data (Desrosiers et al., 2021), as well as parent-offspring relationships from banding records of fledglings captured on their natal territory. Once all individuals were matched to a birth year and sex (including unknown), we ran *sequoia* using a heavily pruned dataset of 744 highly informative, independent SNPs (see results). To validate the

SNP pedigree, correlations were estimated between pairwise relatedness estimates based on SNP pedigree and genomic relatedness calculated from *Plink* (Purcell et al., 2007). We used R version 4.3.0 for all analyses (R Core Team, 2023).

Estimation of natal dispersal distance

For the subset of birds marked as juveniles on their natal territory, we measured natal dispersal by calculating the distance between the centroid of their breeding territory (Figure 4.1C) to the centroid of its parent's territory or the location of the nest where it fledged (matured enough to leave the nest). We assumed that once individuals had at least one nest on record they had established a breeding territory whether or not the young fledged. For the subset of breeding individuals without an assigned parent, we identified potential relatives using the GetMaybeRel function from *sequoia* including full and half sibling, grandparent, and avuncular (aunt/uncle) relationships. We assigned breeding individuals to the top relationship according to highest log likelihood. We used the centroid of this close relative's breeding territory or floater locations based on mark - resight data as an estimate of the site from which an individual dispersed. Here, we assumed that individual's relative is geographically close to the location of an individual's true natal site. This estimate of dispersal is potentially biased as it is possible a relative dispersed away from family groups, and we therefore may include the relative's dispersal in addition to the focal individual. To account for these potential biases in our findings, we present distances for breeding individuals in three categories for most liberal to most conservative. 1) Individuals that were assigned to a parent or nest, male or female relative, 2) individuals that were assigned to a parent or male relative, and finally 3) individuals who were assigned to a parent with a known territory or a natal nest. We distinguish between individuals with a male versus a male or female relative because males are known to disperse less than females, thus making male relatives a more conservative measure than females (see also Results below). We use the term “natal” site to refer to the location where an individual is known, or presumed, to have hatched from based on pedigree relationships, and “breeding” site to refer to the location where an individual is known to have established a territory.

We used generalized linear mixed models (GLMM) implemented in the *lme4* R package (Bates et al., 2015) to test for differences in dispersal distances associated with sex, and natal habitat. Dispersal distance values were log transformed to approach normal distribution in the model residuals. We included natal study plot as a random effect to account for repeated measures. We used the *Anova*() function from the *car* package (Fox & Weisberg 2011), and the *emmeans* package (Lenth et al., 2020) to calculate *p*-values and confidence intervals (alpha value = 0.05). Individuals were considered hatched in pine habitat if their natal point was within 300 meters of pine (the diameter of the largest island scrub-jay territories; Caldwell et al., 2013) using a reclassified 2005 vegetation map of Santa Cruz Island (The Nature Conservancy, 2007; Langin et al., 2015; Cheek et al., 2022). We built models for all individuals, and again for individuals who were assigned a parent or nest, or male relative. We did not have sufficient data to apply the same models to the individuals assigned to a parent or nest.

Variation in dispersal in relation to habitat and bill morphology

Because we observed that females assigned to a parent or male relative who hatched in pine habitat dispersed less on average than females born in oak habitat (Figure 4.2B), we explored this difference further by testing if females choose breeding territories based on their natal habitat or their bill morphology. We used a habitat selection function approach (HSF; Fieberg et al., 2021) to iteratively build models to evaluate the role of natal habitat composition and individual phenotype on the probability that an available territory will be used given its habitat composition. HSF, also commonly referred to as resource selection functions (RSF; Manly et al., 2002), are a common methodological tool for examining habitat selection by comparing environmental variables at locations visited or used by an animal to environmental variables at a set of locations assumed to be available to the animal using logistic regression (Boyce and McDonald, 1999; Fieberg et al., 2021). For example, HSF can be used to describe the range of habitats available (e.g. vegetation types, territories, food resources) and then test if any of these habitat features are preferentially used or avoided by an individual (Fieberg et al., 2021). We chose to focus on females who were assigned to a parent or male relative for this analysis as a more conservative dataset to test for natal habitat preference and matching habitat choice. To statistically test for habitat selection, we fitted logistic regression models using the *lme4* R package (Bates et al. 2015). For each model, we included individual female ID as a random effect and used a set of random territories drawn across Santa Cruz Island using Delaunay triangles sized to match 300 meters as "available" habitat. There are no physical barriers to impede island scrub-jay movement on Santa Cruz Island which suggests that these birds could potentially access every territory across the island. Therefore, for the purposes of this analysis we assumed all simulated territories to be available for each dispersing female. In reality, most territories are occupied and the opportunity for dispersing birds to show a preference is dependent on a vacancy, which may explain the delayed breeding in island scrub-jays (see Discussion). We tested several alternative hypotheses that could predict habitat choice, including 1) the similarity of habitat composition between the natal and breeding territory, 2) phenotype (bill length), and 3) the potential quality of the breeding habitat. To test the hypothesis that females choose breeding habitats based on natal habitat preference, we included a model that compared the natal and dispersed habitats of each individual to test if females who hatched in pine preferred breeding habitats with a higher percentage of pine. We measured habitat composition of used and available territories by calculating the percent pine (*%Pine*) and percent oak (*%Oak*) for each simulated territory using a re-classified 2005 vegetation map of Santa Cruz Island (Cheek et al., 2023; Langin et al., 2015; The Nature Conservancy, 2007). We measured natal habitat composition of each individual by calculating the percent pine ($\%Pine_{natal}$) and oak ($\%Oak_{natal}$) within a 300-m radius of each natal point using the same re-classified 2005 vegetation map of Santa Cruz Island. To test the hypothesis that individuals choose breeding habitats based on their phenotype, we first calculated size corrected bill lengths to test if longer billed individuals preferred breeding territories with a higher percentage of pine. Bill length is positively associated with body size (Langin et al., 2015). Therefore, we performed a PCA on measurements of wing chord, tail and tarsus length for all 1,377 island scrub-jays that have been banded to-date as second years, or adults with complete phenotype measurements and extracted values from the first PC axis, which explained 49.98% of the variance, as an index of overall body size across the population. We then performed a linear regression using the *lme4* R package of bill length on this index of body size while treating individual and bander (the researcher who measured the bird) as random effects to account for repeat measures of jays captured multiple times by different teams. We averaged the residuals from this regression for each individual included in this study as a body size-corrected measure of bill length (*BillLength*) in our models. Finally, we tested an alternative hypothesis that females solely choose territories based on overall habitat quality. Habitat-specific abundance during the breeding season for each simulated territory was estimated based on elevation, chaparral density, and canopy height (Sillett et al., 2012; Bakker et al., 2020). We considered this abundance measure a proxy for habitat quality (*Qual*) because nesting quality (an indirect indicator of territory quality) is positively correlated with predicted abundance (Caldwell et al., 2013; Bakker et al., 2020). To find the best model that predicted a female's breeding territory, we selected best fit models for both the median 1,600m buffer, and the maximum 5,000m buffer distance traveled using second order Akaike's information criterion to adjust for small sample sizes (AICc, Burnham and Anderson 2002). We calculated 95% confidence intervals (CI) for top model coefficients using the confint function in the *stats* R package (R Core Team, 2023).

Results

We genotyped 1,526 island scrub-jays (Figure 4.1B) using RAPTURE and obtained a total of 6,287,857,572 reads with an average of 957,715 reads per individual following *clone_filter*. Of the remaining reads, 91.0% mapped to the California scrub-jay reference genome with an average coverage of 12.6x per individual following bwa-mem alignment and genotyping in *gstacks*. Initial processing of individuals with <70% missing data in STACKS resulted in a matrix of 90,432 SNPs and 1,415 individuals. These SNPs were then further filtered in Plink to retain 744 highly informative SNPs with less than 1% missing data that were also retained in our full RAD dataset of 161 individuals. We filtered individuals that were shared between the Rapture libraries and our full RAD dataset to retain the data set with less missing data from the pair. Our final dataset included 1,476 unique island scrub-jays with 2.08% total missing data.

Genetic parentage and relatedness analysis

We identified at least one of the parents for 461 offspring from 276 adults. Of these relationships, 57 offspring were known breeding individuals, 32 of which had known spatial data associated with the location of their parent's breeding territory. There were an additional 5 individuals who were banded as nestlings but without assigned parents in the pedigree, resulting in a total of 37 known breeding individuals with a parent territory and/or nest point of origin. We identified potential relatives including offspring, full and half siblings, grandparents, and aunts or uncles for an additional 96 breeding island scrub-jays. One outlier female who dispersed only 1 meter from a female relative was removed from further analyses. Altogether there was a total of 132 breeding island scrub-jays that we could assign to a parent or relative with accompanying spatial data.

Dispersal distance between sexes

Breeding individuals largely overlapped within according to our three criteria listed above. The raw dispersal distances for

All individuals

Female dispersal distances for individuals assigned to a parent or nest, male or female relative ranged from 81.11 to 7249.94 meters for females (n= 52, standard error= 200.52, median=1669.30; Figure 4.2A) and 54.01 to 7373.55 meters for males (n= 80, standard error= 123.07, median=507.51; Figure 4.2A). Log transformed dispersal distances differed significantly between males and females (*p*-value < 0.001, $\beta_{\text{females}}= 0.789$), but not between males and females who hatched in pine or oak habitat (*p*-value $=0.874$, $\beta_{\text{pine}}= -0.041$; Figure 4.2B; Table 4.1).

Individuals assigned to a parent, nest, or male relative

Female dispersal distances for individuals assigned to a parent, or nest, or male relative ranged from 109.30 to 5244.11 meters (n= 30, standard error= 259.41, median=1782.86) and 54.01 to 2323.00 meters for males (n= 57, standard error= 52.91, median=450.67). We also observed a significant difference in log transformed dispersal distances of females who hatched in pine (*p*-value $=0.025$, $\beta_{\text{pine}}= -0.561$; Figure 4.2C; Table 4.1).

Individuals assigned to a parent or nest

Female dispersal distance for individuals assigned to a parent or natal nest ranged from 422.31 to 5076.16 meters (n= 6, standard error= 643.17, median=2818.30) and 94.26 to 1118.66 meters for males (n= 31, standard error= 41.19, median=424.21). We did not have sufficient data to determine if these dispersal distances differed significantly between sexes born in pine or oak habitat.

Evidence of habitat choice and natal habitat preference in pine females

Our HSF approach found the most support for the hypotheses that island scrub-jay females select breeding territories based on a natal habitat. The two best supported HSF models indicated that pine habitat in a female's natal site ($\%Pine_{natal}$) was the most important predictor of a female selecting a pine territory (*%Pine*; Table 4.2, Figure 4.3A). Parameters of the top model showed a significantly positive relationship in the probability of females who hatched in pine selecting a pine breeding territory ($\%Pine_{natal}$ X *%Pine*; β = 8.17; *p*-value =0.002; CI= [3.73, 14.21]; Figure 4.3B), followed by overall territory quality (*Qual;* β =2.61; *p*-value = 0.02; CI= [-0.21, 4.54]; Figure 4.3B). Parameters in the second ranked model only included the interaction term of natal pine habitat composition and breeding habitat ($\%Pine_{natal}$ X *%Pine*; β = 7.59; *p*-value =0.001; CI= [3.39, 13.25])

We found weaker support for the effect of habitat matching on the probability of selecting a territory. Individual phenotype (*BillLength*) was included in the third top ranked model; however, it had less effect on the probability of selecting a pine territory ($\%Pine_{natal}$ X *BillLength*; β = 1.67; *p*-value =0.587; CI= [-4.45, 7.82]) relative to the interaction of $\%Pine_{natal}$ and *%Pine* (β = 7.58; *p*-value =0.008; CI= [2.62, 14.04]), and habitat quality (*Qual*; β = 2.62; *p*-value =0.025; CI= [-0.19, 4.56]).

Discussion

Theoretical and empirical evidence suggests that adaptive divergence between different environments may be maintained by non-random gene flow (Edelaar and Bolnick 2012; Richardson et al. 2014; Clancey et al., 2024). Yet, studies of adaptive divergence almost exclusively invoke selection and rarely consider the alternative hypothesis that individual genotypes are segregating non-randomly across environments. Island scrub-jays exhibit habitat-linked microgeographic adaptation in bill morphology which is hypothesized to be driven by disruptive selection (Langin et al., 2015; Cheek et al., 2022). Indeed, genotype-environment association studies (GEA) reveal adaptive genetic differentiation between oak and pine habitats, and genome wide association studies (GWAS) finds differences at loci associated with bill morphology across habitats (Cheek et al. 2022). While such results are consistent with disruptive selection across the oak-pine habitats, recent modeling efforts suggest selection alone is unable to generate and maintain the observed divergence in bill morphology (Clancey et al. 2024). Instead, these models conclude that the pattern of microgeographic divergence in bill morphology can only be maintained if there is non-random dispersal of longer billed individuals into pine habitat and shorter-billed individuals into oak habitat (Clancey et al. 2024). The results here support this conclusion, as our spatially grounded molecular pedigree finds evidence for natal habitat preference between breeding individuals living in pine habitat. We found that females disperse significantly further than males (Figure 4.2A), and that females who hatched in pine disperse shorter distances into adjacent pine habitat compared to females who hatched in oak and mover further from their natal sites (Figure 4.2C, Table 4.1). We found evidence that this reduced dispersal in pine females is likely driven by females who hatched in pine preferentially selecting for pine breeding territories (Figure 4.3B; Table 4.2). These findings suggest that non-random dispersal through natal habitat preference operates in conjunction with disruptive selection as a potential driver of microgeographic divergence in a continuously distributed bird species.

Evidence for natal habitat preference

If dispersal was truly random between habitats, then we would expect female island scrub-jays, as the dispersing sex, to disperse approximately the same distance between habitat types. Yet we observed females who hatched in pine dispersing substantially less from their parents and male relatives compared to females who hatched in oak (Figure 4.2C). Furthermore, we found support that pine habitat within a female's natal territory is an important predictor of the selection of a pine breeding territory when considering all territories potentially available on Santa Cruz Island (Figure 4.3A, B; Table 4.3). Scale is an important factor to consider in studies of habitat use (Boyce 2006), so it is possible that the range-wide scale we used for available territories failed to capture all the ecological variables that explain territory use. However, we document multiple cases of individuals travelling several kilometers, presumably over ridgelines and open spaces, to reach their breeding territories (Figure 4.2A). Furthermore, there is little evidence to suggest that habitat patchiness, even towards the island periphery, impedes gene flow (Langin et al., 2017a). Thus, it is unlikely that island scrub-jay females are strictly constrained by geography, as might be the case in other taxa where movement is impeded by environmental features. Given that we do not know how far individual scrub-jays travel to prospect for new territories (Reed et al., 1999; Colbert et al., 2009), additional research exploring the period from when individuals fledge to territory establishment is warranted to determine which scale is most appropriate to measure habitat use.

Natal habitat preference has been observed in other birds and within other *Aphelocoma* jay species (e.g. Piper et al., 2013; Comacho et al., 2016; Davis & Stamps, 2004; Fletcher et al., 2015; Sherer, 2019). This implies that such non-random dispersal effectively reduces gene flow and could play an important role in promoting divergence (Berner & Thibert-Plante, 2015; Bolnick & Otto, 2013: Davis, 2008; Maynard-Smith, 1966) even at limited spatial scales (e.g. Bolnick et al., 2009). A prevailing hypothesis for the adaptive significance of natal habitat preference is that dispersing organisms have higher fitness in familiar environments (Davis & Stamps, 2004). Yet this hypothesis assumes that organisms are actively choosing breeding territories similar to their natal habitat where they have a selective advantage due to their natal experience (Camacho, Canal, and Potti, 2016; Piper et al., 2013). This presumed adaptive potential of natal habitat preference is not always supported in cases where individuals are maladapted (Piper et al., 2013; Comacho et al., 2016; Comacho & Hendry, 2020), or if experience at different life stages impacts individual settlement decisions (Stamps, Krishnan, & Willits, 2009). In the case of the island scrub jay the delayed time required to acquire a territory provides an opportunity for sampling and making adaptive decisions about which territories to choose. Therefore, it is beneficial to consider how alternative mechanisms, such as phenotype, may contribute to individual settlement decisions. Yet, perhaps surprisingly, we found limited evidence to support that bill morphology is associated with the selection of breeding territory.

Phenotype driven habitat matching is a mechanism commonly associated with non-random dispersal and adaptive settlement decisions (Edelaar et al., 2008; Edelaar and Bolnick, 2012; Camacho and Hendry 2020). In the case of island scrub-jays, it is possible the overall strength of matching habitat choice may be limited by factors that we were unable to account for in this study. For example, studies show that the strength of phenotype habitat matching may be highly dependent on age or resource availability (Benkman 2017; Comacho & Hendry, 2020). Matching habitat choice may also be limited due to competition for preferred resources (Comacho et al., 2015; Jacobson, Bubois & Peres-Neto, 2017), or differences in selection pressures between habitats (Bolnick et al., 2009). Nevertheless, the challenge in this system is that longer billed individual characteristic of pine habitat selecting for pine breeding territories would still drive adaptive patterns of genotypic divergence at loci important for bill morphology (Richter-Boix et al., 2013). Island scrub-jay bill morphology changes gradually in relation to distance from pine habitat (Langin et al. 2015), so it is possible that because oak habitat is the dominant vegetation on Santa Cruz Island (Junak 1995), oak females have less opportunity to sample pine habitat and be less likely to be removed from the population by selection. Therefore, matching habitat choice may occur circumstantially or in combination with natal habitat preference rather than as the primary evolutionary mechanism which would be difficult to detect with the data currently available. Experimental manipulations including reciprocal transplant experiments could disentangle the extent of which matching habitat choice occurs relative to natal habitat experience in this system.

Female biased dispersal and male natal site fidelity

Our spatial pedigree informed dispersal estimates showed that female island scrub-jays dispersed significantly further than males (Figure 4.2), an observation that has been reported elsewhere (Langin et al., 2015). The reasons for why male and females differ in their dispersal or the factors that impact an individual or pair's ability to maintain or lose a territory are unclear (but see Desrosiers et al., 2021). One important caveat of this study is that our HSF approach assumes that all "available" territories are unoccupied. Yet high population densities relative to mainland scrub-jay species (Yeaton 1974), body-size differences in territory acquisition (Desrosiers et al., 2021), and saturation of territory holders within suitable habitat (Collins and Corey 1994) suggests there is fierce intraspecific competition among island scrub-jays for breeding territory. Indeed, jays that intrude on territories are aggressively chased and can experience serious injury from territory holders (Delaney & Cheek, 2022). Thus, one potential explanation for why males seem to prefer their natal site is that island scrub-jay parents may be more tolerant of their offspring in or near their territory than unrelated jays. Studies in other corvid and avian species that do not exhibit cooperative breeding behavior suggest that providing a haven for offspring provides extended developmental opportunities that help ensure juvenile survival (Ekman & Griesser, 2002; Holzhaider et al., 2010; Kingma et al., 2021; Uomini et al., 2020). Such parental tolerance in a highly competitive environment could therefore increase an individual male's survival and opportunity to eventually establish a territory.

While both sexes compete for limited breeding habitat (Collins & Corey, 1994), the restricted dispersal observed in males could be driven by differences in territory acquisition between sexes. Studies done on Florida scrub-jays (*Aphelocoma coerulescens*) have documented that males may acquire a breeding territory through inheritance or budding from a parental territory, while such acquisition is rare in females (Fitzpatrick et al., 1999; Woolfenden & Fitzpatrick, 1984). Therefore, we propose that dispersing island scrub-jay females may also compete for the closest available male who can help establish a territory, or access to males that already have a territory.

A complicating factor in this hypothesis is that male island scrub-jays likely play a significant role in which females settle on a given territory. While larger males tend to acquire a territory and breed earlier than smaller males (Desrosiers et al., 2021), it has yet to be determined what factors impact female ascension to breeding. Previous studies have found correlational evidence for assortative mating as longer billed males tend to reproduce with longer billed females (Langin et al., 2015; Clancey et al. 2024). Furthermore, female island scrub-jays produce a distinctive “rattle” call alongside a visual “head bob” display that is associated with pair-bond behavior (Goodwin, 1976; Woolfenden & Fitzpatrick, 2020; Delaney & Cheek, 2022). Females with longer, shallower bills characteristic of pine habitat produce rattle calls that are more rapid and higher frequency than females with short, deep bills characteristic of oak habitat (Langin et al., 2017b). However, it is unclear if island scrub-jay males use the rattle call as an auditory cue to actively choose mates with similar bill morphologies (e.g. Ratcliff & Grant, 1985; Snowberg & Benkman, 2007) or whether the correlation between male and female bill length simply reflects limited dispersal. Given that roughly half of the island scrub-jay population is comprised of non-breeding floaters (Sillett et al., 2012), understanding the factors that impact mate-choice could inform how territories are established by which individuals, and how long it takes for individuals to reach breeding status.

Collectively, our results suggest that the interplay between the similarity of the natal territory and breeding territory, and the availability of an unoccupied territory play a role in how far island scrub-jays disperse. It does not appear that young birds randomly move across the island and simply wait for the closest territory to become available. Instead, island scrub-jays, particularly females, appear to select preferred breeding territories based on natal habitat (e.g. Camacho, Canal and Poti 2013; Milleret et al. 2019; Dawson et al., 2023). Despite putting no restrictions on available territories for females in our HSF models, we still found evidence that pine females exhibit a preference for pine breeding territories. Given that individuals may not breed for up to 7 years of an estimated 16-year lifespan (Caldwell et al., 2013; Delaney & Cheek, 2022), such selectivity in breeding habitat could also partially explain the observed delayed breeding in this non-cooperative species. Regardless, the natal habitat preference we document here provides critical empirical evidence that dispersal is not random with regards to habitat in this highly mobile species and contributes to the patterns of spatial phenotypic and genetic variation in this species.

Figures and tables

Table 4.1: Estimated Marginal Means (emmeans), standard error (SE) and lower (CI low) and upper (CI high) 95% confidence intervals for log transformed dispersal distances in meters by natal habitat for all male (M) and female (F) island scrub-jays hatched in pine or oak habitat, and for the subset of individuals assigned to parents or male relatives.

	Natal Habitat	Sex	emmean	SE	Lower CI	Upper CI	N
all individuals	**Oak**	M	6.37	0.201	5.71	7.03	54
		F	7.16	0.210	6.56	7.76	36
	Pine	M	6.33	0.229	5.66	6.99	26
		F	7.12	0.240	6.48	7.75	16
individuals assigned to parent or male relative	**Oak**	M	6.24	0.184	5.43	7.06	43
		F	7.31	0.200	6.75	7.87	22
	Pine	M	5.68	0.226	5.08	6.29	14
		F	6.75	0.247	6.15	7.35	8

Table 4.2: Habitat Selection Function (HSF) results of selection of models predicting territory use of 22 female island scrub-jays on the basis of habitat quality, natal habitat composition, individual phenotype. We considered all simulated territories across Santa Cruz Island to be available. Column headings give model notation, number of estimable parameters (K), differences in second-order Akaike's information criterion (ΔAIC_c), and AIC_c weights (w_i). Variables considered were percent pine ($\%Pine_{natal}$) and oak ($\%Oak_{natal}$) within a 300-m radius of each natal point, habitat-specific abundance during the breeding season based on elevation, chaparral density, and canopy height as a proxy for habitat quality (*Qual*), the percent pine (*%Pine*) and percent oak (*%Oak*) for each simulated territory, and body size-corrected measure of bill length (*BillLength*). Interactions are denoted with an "X" and additive effects are separated by commas. All models also include female ID as a random effect. Number of females observed = 22.

Model	**K**	**ΔAIC_c**	**w_i**
$\%Pine$ X $\%Pine_{natal}$, *Qual*	6	0.00	0.57
$\%Pine$ X $\%Pine_{natal}$	5	1.40	0.29
$\%Pine$ X $\%Pine_{natal}$, *%Pine* X *BillLength*, *Qual*	8	3.67	0.09
$\%Pine$ X $\%Pine_{natal}$,*%Pine* X *BillLength*	7	5.09	0.05
%Pine X *BillLength*	5	10.36	0.00
Qual	3	27.92	*0*
$\%Oak$ X $\%Oak_{natal}$, *%Oak* X *BillLength*, *Qual*	8	32.89	*0*
$\%Oak$ X $\%Oak_{natal}$	5	34.84	*0*
%Oak X *BillLength*	5	38.08	*0*
$\%Oak$ X $\%Oak_{natal}$, *%Oak* X *BillLength*	7	38.74	*0*

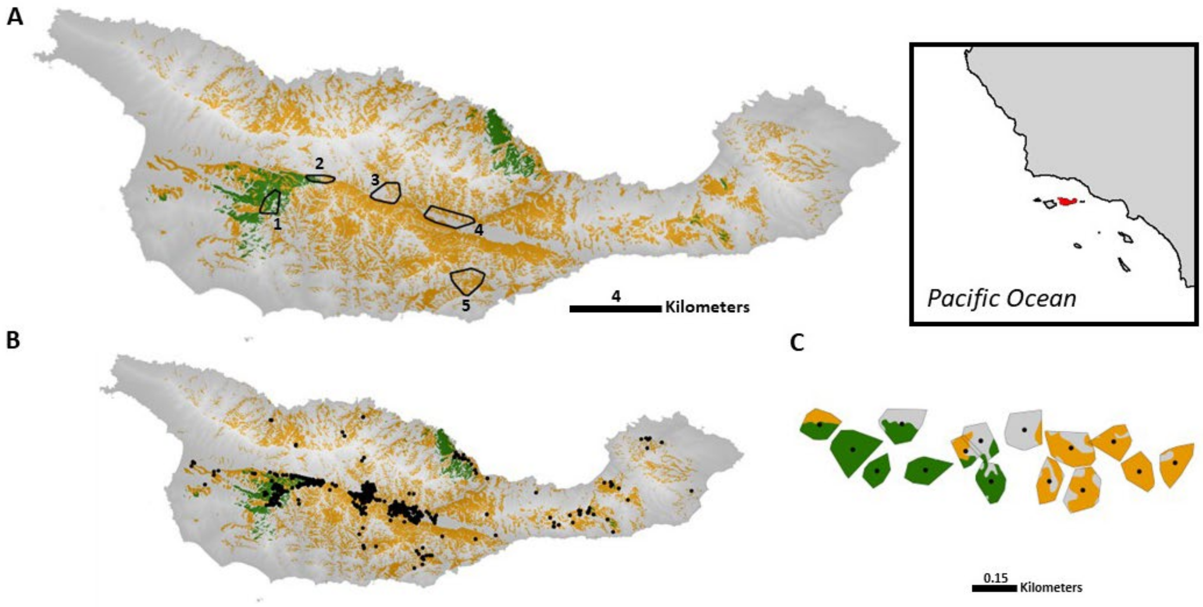

Figure 4.1: (A) Study area of Santa Cruz Island, California, USA, with habitat type (green, pine; orange, oak) shaded. Numbered polygons represent the minimum convex hulls of five study plots included in the nest monitoring surveys from 2008-2023 (1= Sauces Canyon; 2= Buena Vista; 3= Portezuela; 4= Field Station; 5= Coches Prietos Canyon). Inset shows Santa Cruz (in red) relative to the other Channel Islands and mainland California. (B) Sampling localities of individuals genotyped for pedigree inference (black dots; n=1485). (C) Map of breeding territories (polygons with pine and oak habitat shaded) for a representative year (2018) within the Buena Vista ecotone study plot. Black dots represent the centroid of the territory.

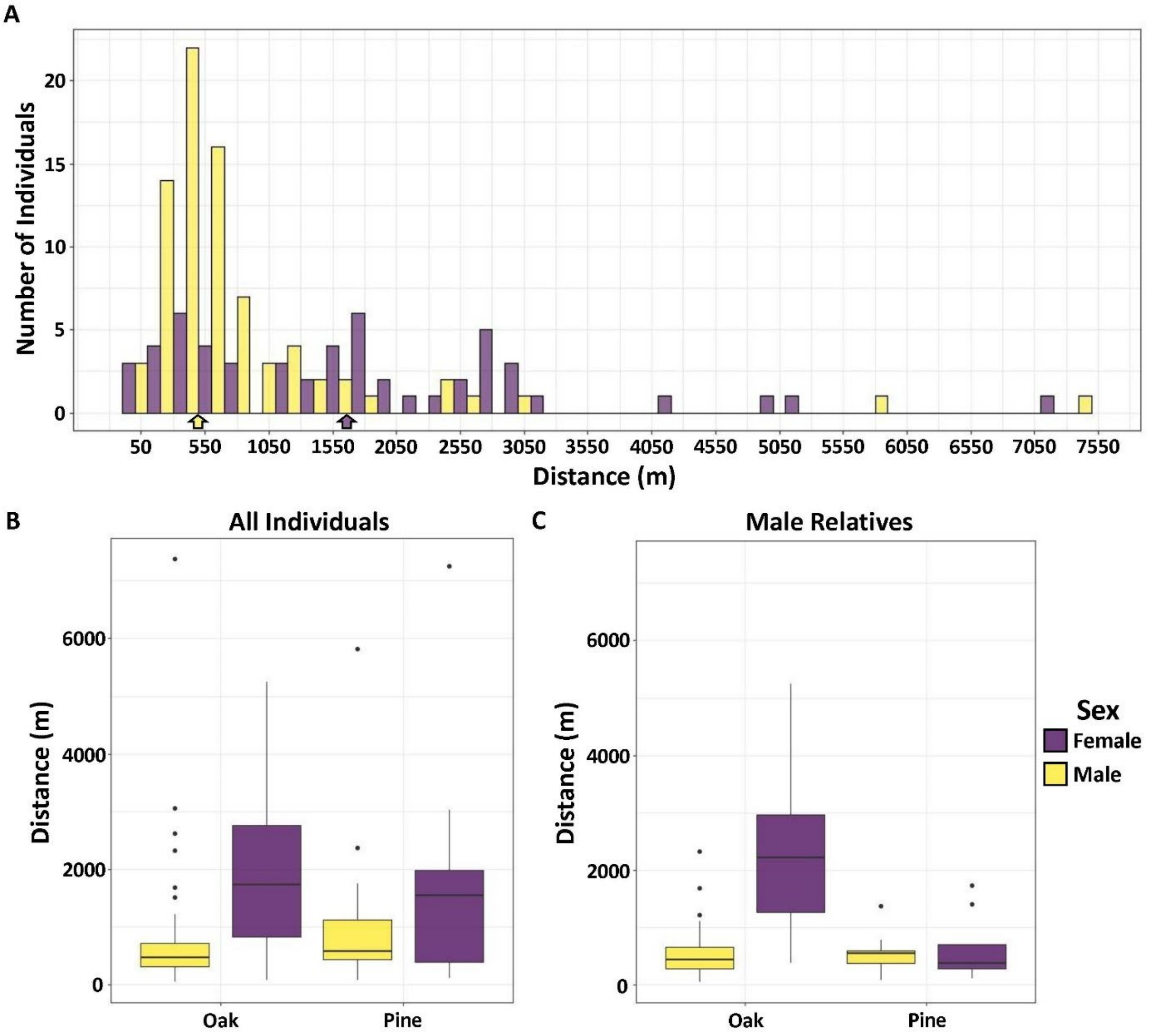

Figure 4.2: (A) Natal dispersal distances for island scrub-jays who bred within 5 nest monitoring study plots between 2008 and 2023 are significantly shorter in males than females (median females = 1669.30m [purple arrow], n=52; median males=507.01m [yellow arrow], n= 80). (B) Boxplots representing natal dispersal distances by natal habitat for all individuals ($\text{males}_{\text{oak}}$ = 54, $\text{females}_{\text{oak}}$=36 , $\text{males}_{\text{pine}}$ = 26, $\text{females}_{\text{pine}}$=16) and (C) individuals assigned to a parent, or nest, or male relative ($\text{males}_{\text{oak}}$ = 43, $\text{females}_{\text{oak}}$=22 ,$\text{males}_{\text{pine}}$ = 14, $\text{females}_{\text{pine}}$=8) show that average natal dispersal distances were less in females assigned to a parent or male relative who hatched in pine (median $\text{females}_{\text{oak}}$ = 2225.35 m; median $\text{females}_{\text{pine}}$=379.17m) .

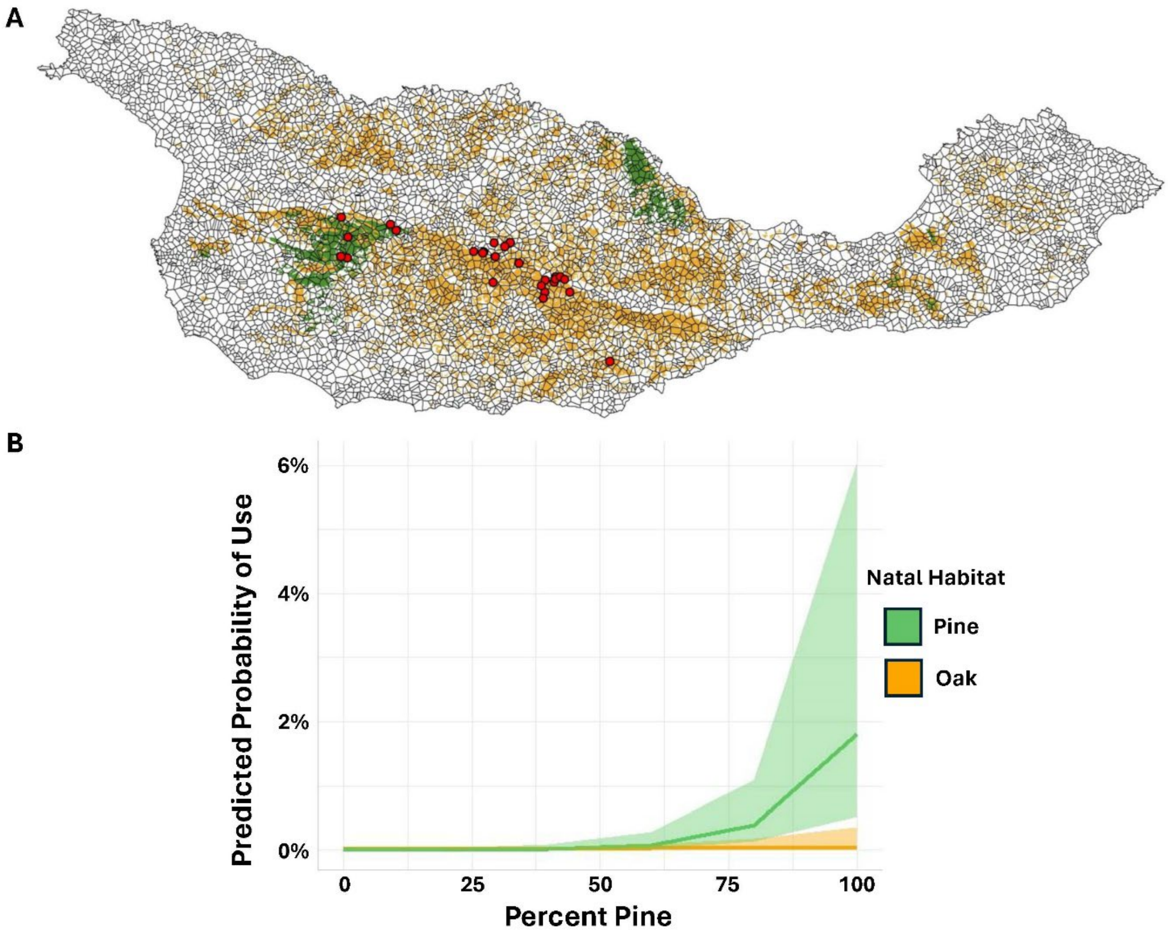

Figure 4.3: A) Santa Cruz Island, CA, with simulated island scrub-jay breeding territories generated by Bakker et al. (2020; black lines) and island scrub jay female natal sites based on pedigree relationships and nest records (red diamonds) with habitat type (green, pine; orange, oak) shaded. B) Percent pine (green line, 95% confidence interval shaded) within a female's natal territory was positively associated with selection of a breeding territory with pine habitat, while a lack of pine habitat (i.e. oak; orange line, 95% confidence interval shaded) was not.

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APPENDIX

Table S2.1: Number of SNPs retained following each filtering step of 123 island scrub-jays (oak=88, pine=35) with less than 24% missing data according to initial Radiator filter tests (Gosselin et al. 2020) and following consecutive filtering steps. Using the interactive module, monomorphic markers were removed automatically before SNPs were filtered by global minor allele count to reduce sequencing errors and assembly artifacts by the number of alternate (minor) alleles. We used MAC=3 because it requires the markers to be genotyped in at least 1 heterozygote and 1 homozygote, or three heterozygous individual alleles, to keep a SNP. Loci were retained if they had a minimum coverage of 6 and maximum coverage of 100. This step removed SNPs genotyped with too low coverage to be accurately called as well as SNPs genotyped with too high coverage that might reflect repetitive regions. We additionally retained markers that were found in at least 70% of individuals and between positions 1-88 (removing the last 7 positions of our reads) due to a higher number of SNPs present in position 89 followed by a limited number of SNPs from position 90-95, suggesting increased error rates due to low sequence quality towards the end of the reads. We then selected the SNP with the highest minor allele count per read to minimize effects of short distance linkage disequilibrium. Finally, SNPs with significant departures from Hardy-Weinberg proportions (*P*-value < 0.0001) were removed. PCAdapt detected a single outlier locus, assuming K=1. After removing this putatively adaptive locus, the final neutral data set contained 3,408 SNPs.

Filtering Step	SNPs count	Filter Parameters	SNPs Removed
Stacks catalog	25815		
Radiator filters			
Filter monomorphic markers	25303	auto	512
Filter samples	25303	[NO]	0
Filter on GLOBAL minor allele count	7821	3	17482
Filter coverage	5947	6 / 100	1875
Filter genotyping	4580	0.3	1367
Filter snp position on the read	4078	1 88	502
Filter snp number	4078	[NO]	0
Filter short LD	3572	MAC	506
Filter long LD	3572	[NO]	0
Detect mixed genomes	3572	[NO]	0
Detect duplicate genomes	3572	[NO]	0
HW pop threshold	3572	1	0
HW *P*-value threshold	3409	0.0001	163

For Tables S2.1-S2.4, visit https://doi.org/10.5061/dryad.8sf7m0cpq for access to the large data sets described.

For Tables S3.1-S3.2, visit https://drive.google.com/drive/folders/17k-PczJK4CpFTTOE4TjeNr1bS5l7-scr?usp=sharing for access to the large data sets described.

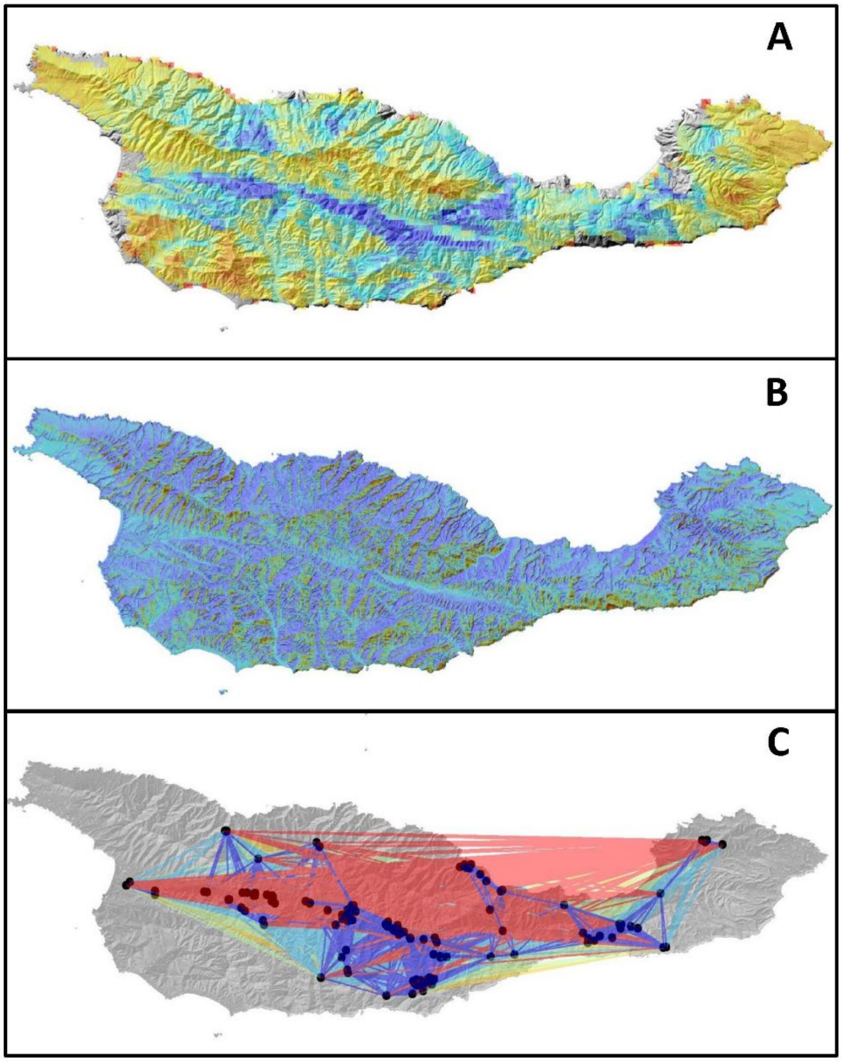

Figure S2.1: Resistance surfaces represent several alternative hypotheses of the effects of landscape variables on island scrub-jay genetic distance (red =areas of high resistance; blue = areas of low resistance) for: (A) Low vegetation density (positive effect on genetic distance), (B) elevation (positive effect on genetic distance), (C) topographic distance (positive effect on genetic distance). Black dots in panel (C) represent sample localities and colored lines represent topographic distance color coded by hypothesized resistance.

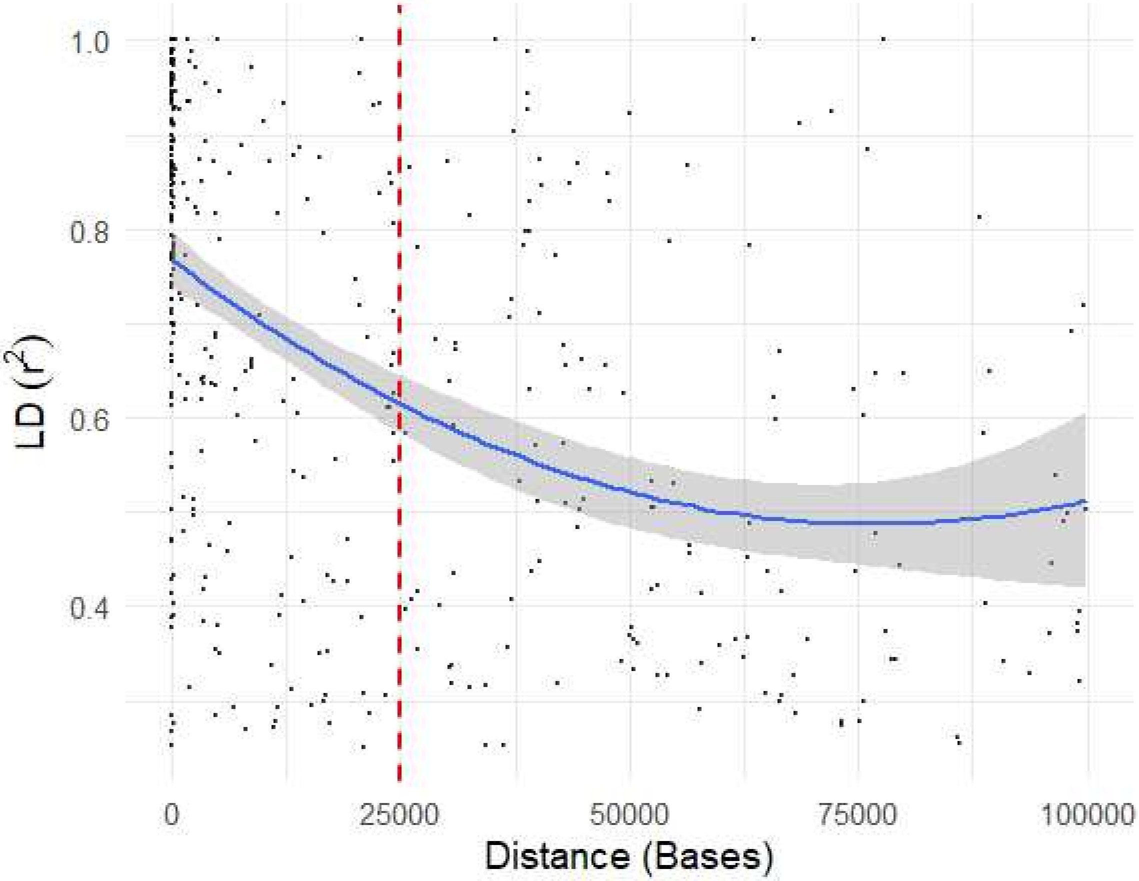

Figure S2.2: Linkage disequilibrium (LD) as a function of distance (base pairs) calculated using VCFtools v0.1.17. Red dashed line corresponds to 25 kb.

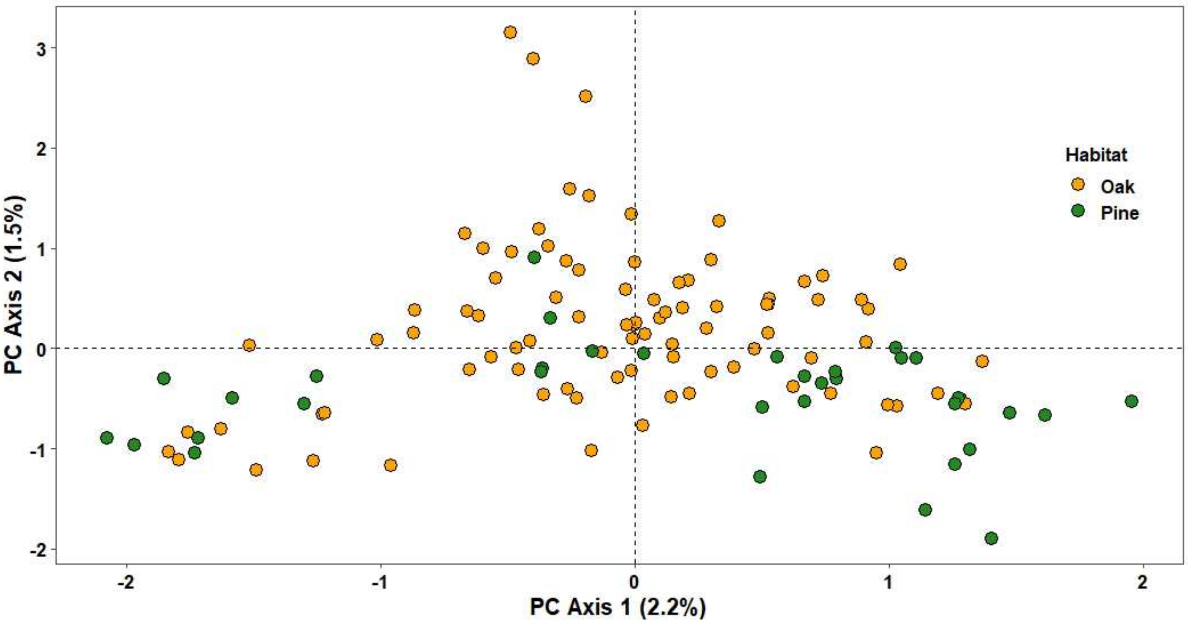

Figure S2.3: Principal component analysis (PCA) showing the genetic relationships of 123 island-scrub jays along the two first PC axes based on 3,342 SNPs. Colored circles correspond to individuals categorized by habitat (oak= orange, pine=green).

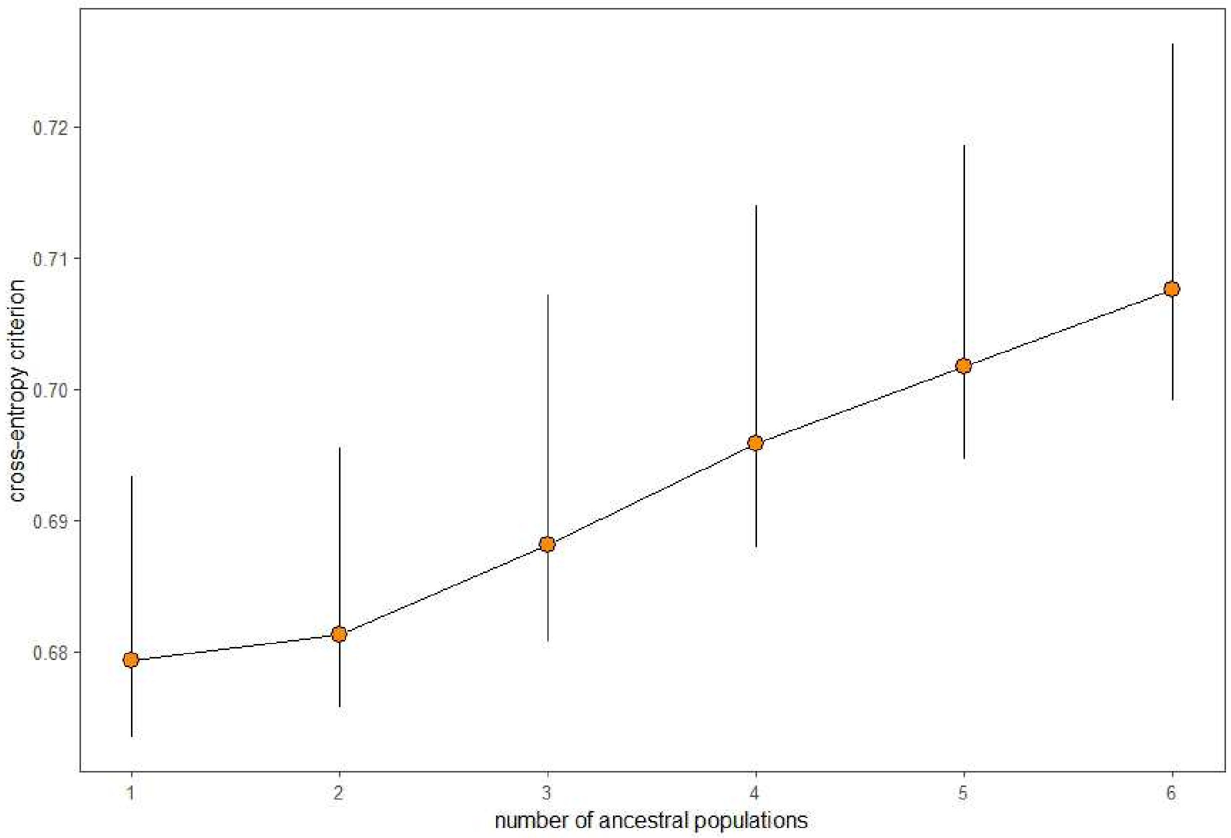

Figure S2.4: Results from population structure analysis using snmf from the LEA package showing the cross-entropy criterion of all runs of K (K= 1 through 6).

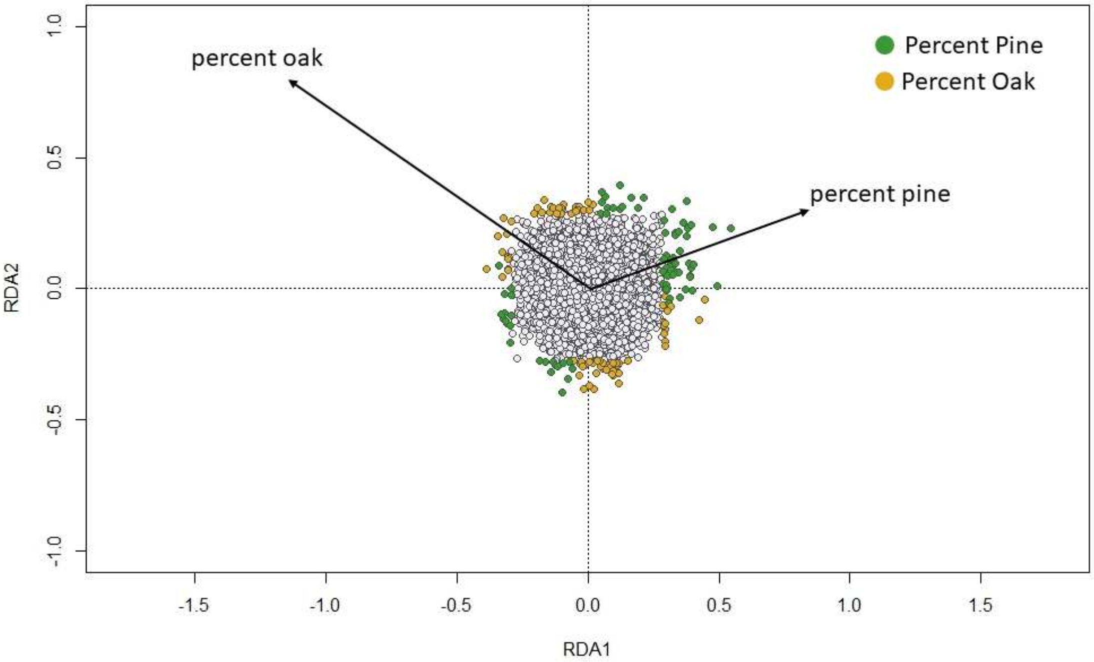

Figure S2.5: Redundancy analysis biplot of 3,345 SNPs using percent pine and percent oak within 300 meters of each sampling location as the predictors (black vectors). Colored SNPs were identified as candidates if they were greater than ±2.5 standard deviations from the mean loading on RDA axis 1 & 2. SNPs that correlated more strongly with pine are shown in green, while SNPs that correlated more strongly with oak are shown in orange. Presumably neutral SNPs are coded as grey.

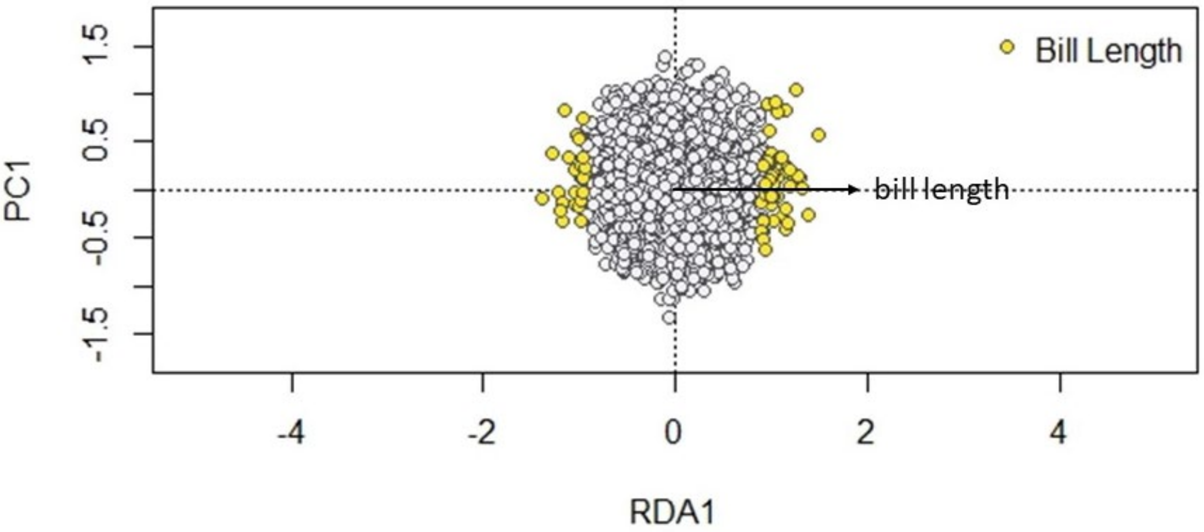

Figure S2.6: Redundancy analysis biplot of 3345 SNPs using body size corrected measures of bill length as the predictor (black vector). SNPs identified as contributing to bill morphology are shown in yellow (±2.5 standard deviations from the mean loading on RDA axis 1).

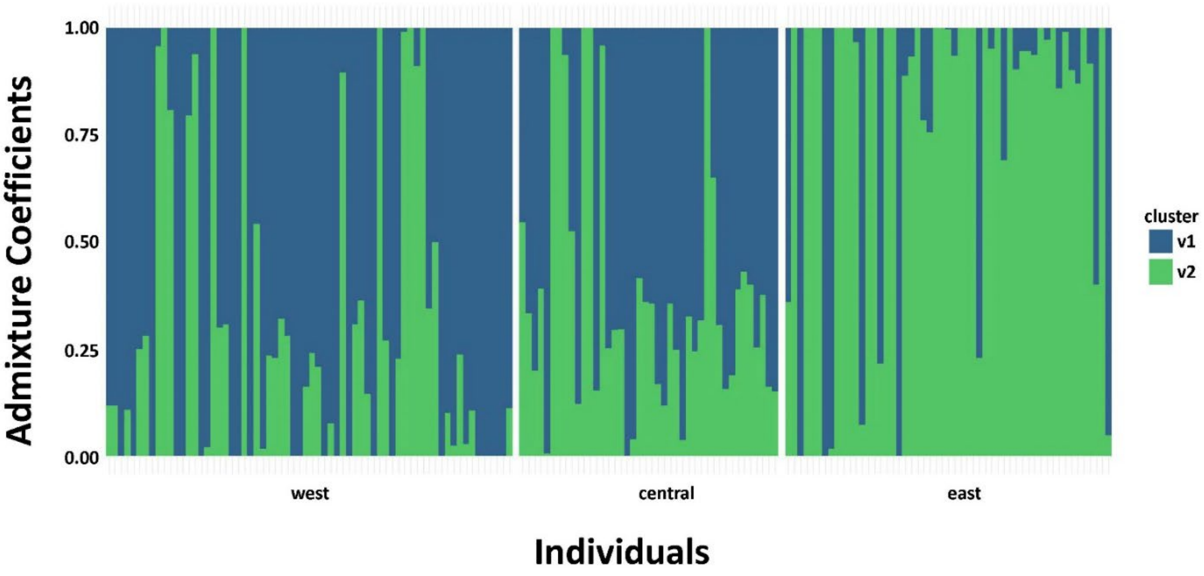

Figure S3.1: Admixture assignment plot for K=2 using 66,503 unimputed SNPs in 161 island scrub-jays arranged by longitude and grouped by pine-oak ecotones. Colored bars represent proportion of ancestry for two (v1=blue and v2=green) hypothetical genetic clusters.

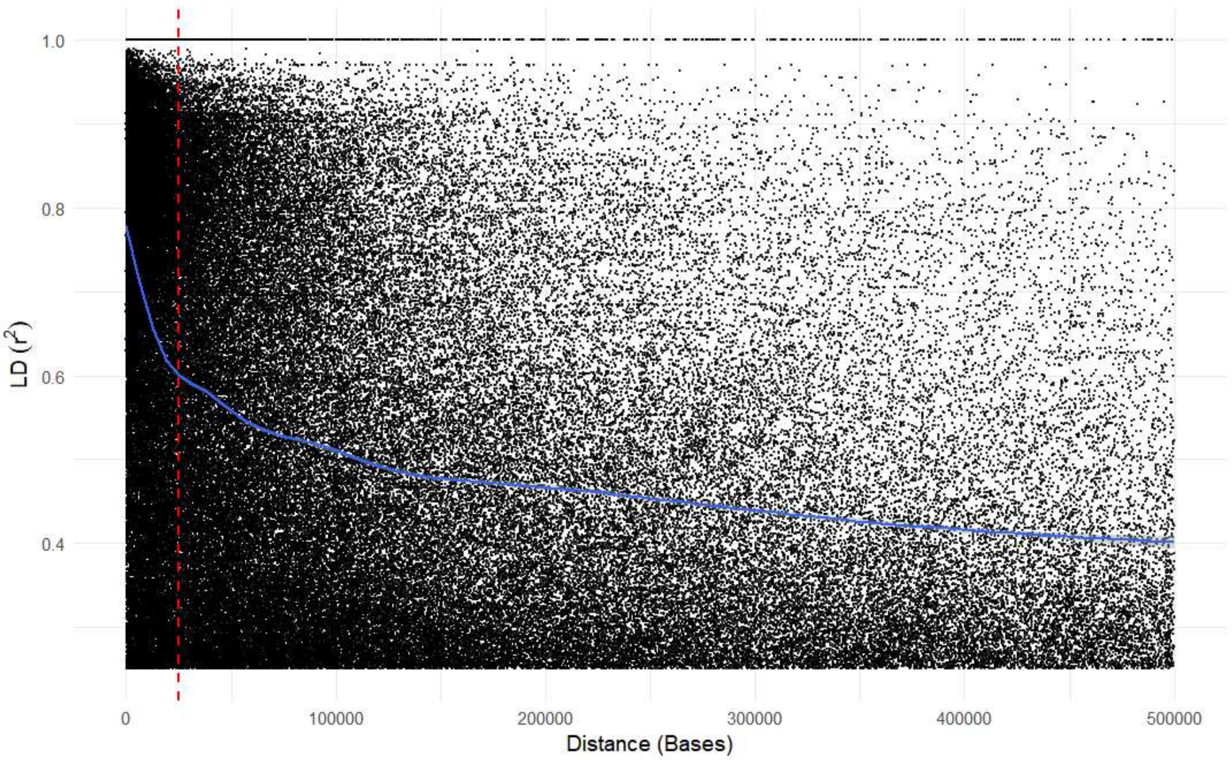

Figure S3.2: Linkage disequilibrium (LD) as a function (blue line) of distance (base pairs) calculated using VCFtools v0.1.16. Red dashed line corresponds to 25 kb.

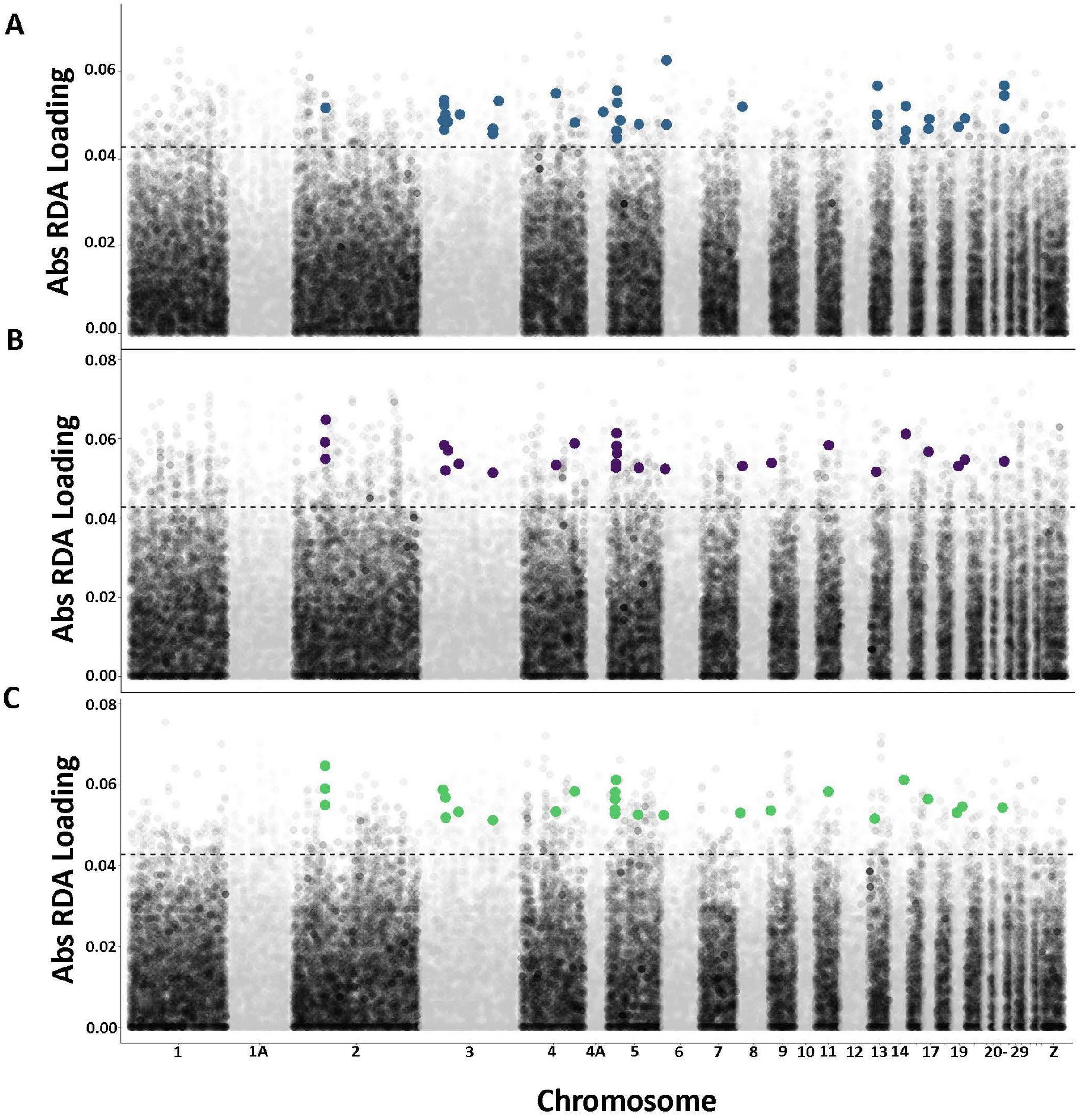

Figure S3.3: Manhattan plots of SNPs identified by genome-wide association (GWA) to be associated with bill length for the west (A), central (B) and east (C) pine-oak ecotones. The significance threshold is indicated by the black dotted line. We ran our GWA analysis for each pine-oak ecotone and colored points are the 26 outlier SNPs identified in all three tests that occurred in or near the same genes.

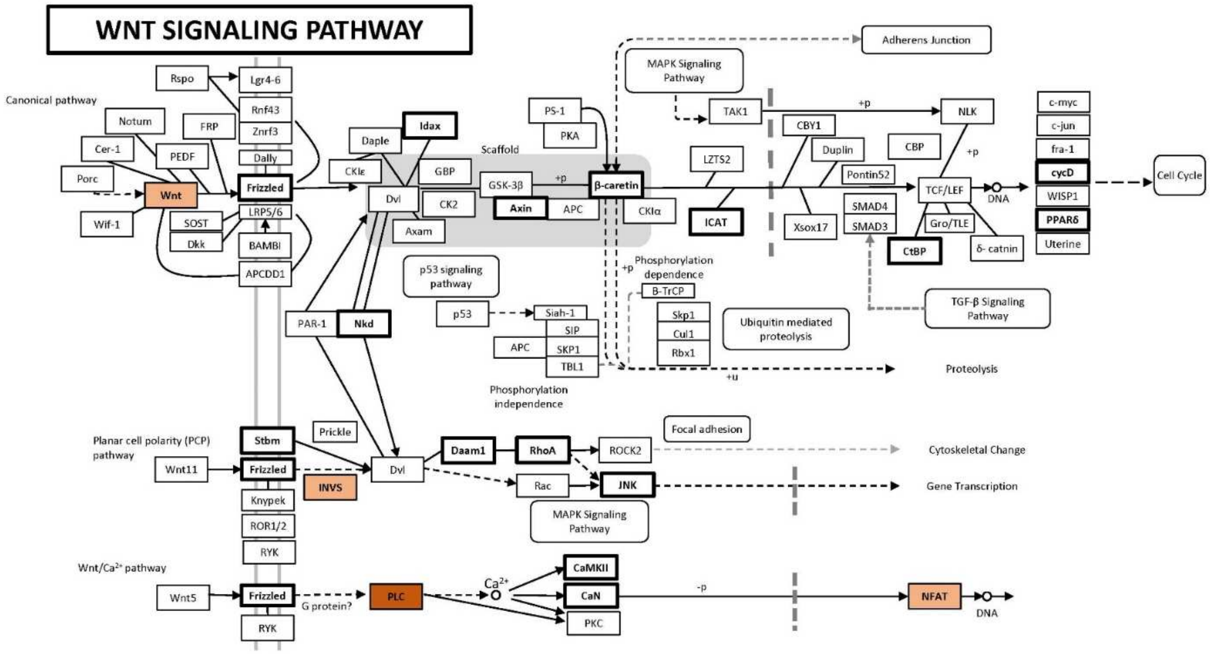

Figure S3.4: Kyoto Encyclopedia of Genes and Genomes (KEGG) diagram representing the Wnt Signaling pathway illustrating the genes that were identified as associated with Island Scrub-jay bill morphology during candidate SNP annotation. Bold genes correspond to genes that were unique to individuals sampled in a single region (west, central, east) of Santa Cruz Island. Highlighted genes were shared between 2 (light highlight) and 3 (dark highlight) regions.