Song dialects, spatial boundaries, and the speciation process in coastal white-crowned sparrows (*Zonotrichia leucophrys*)

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ABSTRACT

Learned bird songs are key mechanisms in speciation, rapidly diversifying into reproductive barriers. Females use song to identify conspecific males, and males use song to mediate intrasexual competition. Given their role in maintaining species boundaries, researchers have long sought to understand when and how *intraspecific* song divergence reduces gene flow between populations. However, the mixed evidence linking song variation to reduced gene flow has left this question unresolved. One complicating factor is the cultural transmission of song. Learned mating signals (e.g., songs) can either impede or facilitate gene flow, highlighting the need to study their intraspecific diversification, in addition to interspecific patterns. In my dissertation, I explored the role of intraspecific song variation in two subspecies of white-crowned sparrow (*Zonotrichia leucophrys*), a model for song learning and cultural evolution. I examined song as a reproductive barrier at multiple stages of speciation: between populations of the Nuttall's subspecies (*Z. l. nuttalli*) (Chapter 1 and 2) and between the Nuttall's and Puget Sound (*Z. l. pugetensis*) subspecies (Chapter 3). In Chapter 1, I found some song differences without associated genetic differences; however, acoustic divergence aligned with a genetic break. To assess this alignment statistically, I implemented boundary statistics in a new R package in Chapter 2, which supported the link between song and genetic divergence in *Z. l. nuttalli*. I posit tcategorical distinctions between dialects are insufficient to reduce gene flow, but high acoustic divergence can act as a reproductive barrier. In Chapter 3, I found that song divergence also reduced gene flow between subspecies, with differences in whistle length—an innate song trait—aligning with a genetic boundary. My dissertation suggests that quantitative acoustic divergence, rather than categorical dialects, and genetically heritable differences in song may determine when intraspecific song variation leads to reproductive barriers.

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INTRODUCTION

Song plays a pivotal role in avian speciation by acting as reproductive barrier between incipient and closely related species (Benites et al., 2015; Boul et al., 2007; Campbell et al., 2019; Edwards et al., 2005; Freeman & Montgomery, 2017; Hebets et al., 2021; Uy et al., 2018). While hybrids between bird species are often viable, species boundaries are typically maintained through pre-zygotic mechanisms, like sexual selection, with bird song functioning as a primary sexual signal used to attract mates and defend territories (P. R. Grant & Grant, 1997; Price & Bouvier, 2002). Birds respond preferentially to conspecific songs, using them to identify potential mates and reproductive competitors, while heterospecific songs hold less relevance in mating contexts (Benites et al., 2015; Brambilla et al., 2008). Behavioral discrimination against heterospecific songs increases with acoustic dissimilarity from conspecific song (Freeman & Montgomery, 2017; Mejías et al., 2021), suggesting the presence of assortative mating and reduced gene flow. However, the evidence that song divergence alone drives reproductive isolation is mixed (Hudson & Price, 2014). Instead, song divergence may act in concert with other mechanisms, such as ecological selection and reduced hybrid fitness, to facilitate speciation (Edwards et al., 2005; Servedio & Boughman, 2017; Uy et al., 2018). Nonetheless, song divergence frequently plays a significant role in maintaining species boundaries, as it reinforces reproductive isolation alongside other evolutionary mechanisms.

The role of song as a reproductive barrier during the speciation process remains unclear, particularly regarding when and how it reduces gene flow. Song may act as a barrier at the earliest stages of population divergence or help maintain separation between incipient species at later stages. To better understand this, it is important to investigate the role of song as a barrier between populations at different stages of differentiation. Behavioral discrimination in response to song variation has been demonstrated both between subspecies (Brambilla et al., 2008; Demko et al., 2019; Lipshutz et al., 2017) and between populations (Danner et al., 2011; Hamao, 2016; Keighley et al., 2019; Searcy et al., 2002). However, such discrimination is not always observed and can be asymmetrical when it does occur (Brooks & Wimberger, 2023; Hamao, 2016). Some studies have shown a correlation between song divergence and genetic divergence between populations (Irwin et al., 2008; Keighley et al., 2019; MacDougall-Shackleton & MacDougall-Shackleton, 2001; Ribot et al., 2012), while others have found no such relationship (Leader et al., 2008; Nelson et al., 2017; Poesel et al., 2017; Wright et al., 2005). Although there is evidence supporting behavioral discrimination at various levels of song divergence, such discrimination is not consistently an effective barrier to gene flow, particularly between populations.

Given the ability of song divergence to drive population divergence, song learning has also been proposed as a mechanism for creating barriers to gene flow. Since learned songs are culturally transmitted, they can diversify rapidly, potentially producing reproductive isolators (Benites et al., 2015; Edwards et al., 2005; Uy et al., 2018). Some evidence suggests that learned songs help maintain species boundaries (Balakrishnan & Sorenson, 2006; B. R. Grant & Grant, 1996), and intraspecific cultural divergence in songs may similarly reduce gene flow between populations (Keighley et al., 2019; Ribot et al., 2012). However, the development of song involves both genetic heritability and cultural transmission, and some song traits are more innate than others. For example, temporal features (timing-related traits) tend to be more genetically heritable, while syllable morphology is more often learned (Araki et al., 2016; Love et al., 2019; Soha & Marler, 2000a). Even in learned songs, the traits involved in song divergence and reduced gene flow may be relatively innate. Therefore, the extent to which culturally transmitted differences in song act as reproductive isolators remains uncertain.

In my dissertation, I investigated the role of song divergence as a barrier to gene flow in white-crowned sparrows (*Zonotrichia leucophrys*) at two levels of genetic differentiation: within subspecies and between subspecies. I focused on two subspecies that inhabit a narrow strip of coastal scrub along the West Coast—the Nuttall's (*Z. l. nuttalli*) and Puget Sound (*Z. l. pugetensis*) white-crowned sparrow. These sparrows have long served as a model system for studying song learning and animal culture, so their song dialects and learning processes exceptionally well documented. Three of the five subspecies, including *Z. l. nuttalli* and *Z. l. pugetensis*, have dialects that have been mapped across much of their range (Baker, 1975; Baker & Thompson, 1985; Baptista, 1977; Cunningham et al., 1987; Hafner & Petersen, 1985; Nelson, 1998; Nelson et al., 2004). These dialects are often acoustically and geographically stable over decades, making white-crowned sparrows an ideal system for studying cultural song divergence as a potential reproductive barrier (Derryberry, 2009; Harbison et al., 1999; Nelson et al., 2004).

While white-crowned sparrows provide an ideal system for studying song divergence, the evidence for song divergence as a reproductive barrier is mixed in this species. Some studies have suggested that dialects reduce gene flow between white-crowned sparrow populations (MacDougall-Shackleton & MacDougall-Shackleton, 2001; Tomback & Baker, 1984), and there is evidence that song divergence can serve as a reproductive barrier between subspecies (Lipshutz et al., 2017). However, other studies have failed to support this conclusion, indicating that song divergence does not always impede gene flow (Brooks & Wimberger, 2023; Nelson et al., 2017; Poesel et al., 2017). These conflicting findings suggest that song divergence may not consistently reduce gene flow in white-crowned sparrows. To address this, I investigated the relationship between song and genetic differentiation across multiple levels of divergence.

In Chapters 1 and 2 of my dissertation, I explored whether song dialects act as a barrier to gene flow within the sedentary subspecies *Z. l. nuttalli*. As the subspecies with the mostly finely structured cultural variation, where song dialects occur densely across small geographic areas, *Z. l. nuttalli* provides an ideal system for investigating how cultural song divergence may promote population structure. In Chapter 1, I conducted an empirical study to assess whether geographic associations exist between song divergence and population structure. I also performed a playback experiment to test for behavioral mechanisms to explain a potential association. Specifically, I tested whether acoustic dissimilarity between dialects influences behavioral song discrimination, which could drive population divergence. In Chapter 2, I developed a new R package to apply boundary statistics to this system. Boundary statistics are used to describe the presence of spatial boundaries in a variable and assess the correlation between the spatial boundaries of two variables across a landscape (Fortin et al., 1996; Jacquez, 1995; Jacquez et al., 2000; Wagner & Fortin, 2005). While these methods are well established, to my knowledge, they have not been available in an accessible platform for researchers. I applied this new R package to analyze genetic population assignments and acoustic song dissimilarity data in *Z. l. nuttalli*. This statistical framework enabled a quantitative investigation of whether song and genetic divergence are spatially correlated. By combining the empirical findings of Chapter 1 with the boundary analyses from Chapter 2, I aimed to determine whether song divergence aligns with genetic divergence between populations, and whether behavioral discrimination based on song may be contributing to this genetic structure.

In Chapter 3, I investigated whether divergence in song traits acts as a reproductive barrier between *Z. l. nuttalli* and *Z. l. pugetensis*. The subspecies diverged allopatrically during the Pleistocene and have maintained both genetic and song divergence, despite ongoing gene flow and the presence of intermediate songs in the secondary contact zone (Baker, 1987; Lipshutz et al., 2017). Current evidence supports the role of song in reproductive isolation between the two subspecies, with males of both responding more aggressively to songs of their own subspecies (Lipshutz et al., 2017). This aggressive response, which is positively correlated with female preference (Derryberry, 2007), suggests a degree of assortative mating. Although the secondary contact zone has been well documented since its initial delineation (Grinnell, 1928), previous studies have not explicitly addressed the mechanisms maintaining the subspecies boundary (Baker et al., 1984; Blanchard, 1941; Corbin, 1981; Lipshutz et al., 2017; Mewaldt et al., 1968; Nelson et al., 2004; Poesel et al., 2017). To explore this, I conducted a clinal analysis to assess whether song traits are maintaining the hybrid zone. By fitting genetic and song trait clines, I tested for an association between song divergence and genetic divergence, providing insight into whether song acts as a barrier to gene flow between subspecies.

The potential for song divergence to serve as a reproductive isolator between species raises the question of whether song also reduces gene flow between intraspecific populations (Uy et al., 2018). Birds frequently discriminate between divergent intraspecific songs, suggesting that song variation may also reduce gene flow within species (Brambilla et al., 2008; Danner et al., 2011; Demko et al., 2019; Hamao, 2016;

Keighley et al., 2019; Lipshutz et al., 2017; Searcy et al., 2002). However, behavioral discrimination and assortative mating do not always create effective barriers to gene flow, especially in the context of song learning and cultural transmission. By studying song as a reproductive isolator at two levels of genetic differentiation in white-crowned sparrows, I aim to clarify the role of culturally transmitted song variation in the speciation process.

CHAPTER ONE

SONG AND GENETIC DIVERGENCE WITHIN A SUBSPECIES OF WHITE-CROWNED SPARROW (*ZONOTRICHIA LEUCOPHRYS NUTTALLI*)

Publication disclosure: This chapter has been published in PLoS ONE under the same title. I am first and corresponding author. I conceptualized the study and chose the methodology, conducted the analyses, and wrote and edited the manuscript. Coauthors: Sara Lipshutz (conceptualization, data curation, funding acquisition, manuscript edits), Jenny Phillips (data curation, manuscript edits), Robb Brumfield (conceptualization, funding acquisition, manuscript edits), and Liz Derryberry (conceptualization, funding acquisition, methodology, supervision, manuscript edits).

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Abstract

Animal culture evolves alongside genomes, and the two modes of inheritance—culture and genes—interact in myriad ways. For example, stable geographic variation in culture can act as a reproductive barrier, thereby facilitating genetic divergence between "cultural populations." White-crowned sparrows (*Zonotrichia leucophrys*) are a well-established model species for bird song learning and cultural evolution, as they have distinct, geographically discrete, and culturally transmitted song types (i.e., song dialects). In this study, we tested the hypothesis that divergence between culturally transmitted songs drives genetic divergence within Nuttall's white-crowned sparrows (*Z. l. nuttalli)*. In accordance with sexual selection theory, we hypothesized that cultural divergence between mating signals both preceded and generated genetic divergence. We characterized the population structure and song variation in the subspecies and found two genetically differentiated populations whose boundary coincides with a major song boundary at Monterey Bay, California. We then conducted a song playback experiment that demonstrated males discriminate between songs based on their degree of divergence from their local dialect. These results support the idea that discrimination against non-local songs is driving genetic divergence between the northern and southern populations. Altogether, this study provides evidence that culturally transmitted bird songs can act as the foundation for speciation by sexual selection.

Introduction

Divergence in sexual signals can act as a reproductive barrier, and it has been theorized as a basis of speciation by sexual selection (Higashi et al., 1999; Mendelson & Safran, 2021; Payne & Krakauer, 1997; Servedio & Boughman, 2017; Sibly et al., 2019). While speciation by sexual selection has been gaining traction as an idea, direct empirical evidence is not always clear (Ritchie, 2007). In support of this hypothesis, behavioral discrimination and mate choice based on divergent sexual signals has been repeatedly demonstrated across a wide variety of taxa (Campbell et al., 2019; Mendelson & Safran, 2021). Such discrimination and the potential for assortative mating have also been demonstrated for culturally transmitted traits (i.e., socially learned and passed through generations) (Creanza et al., 2017, p. 201). Geographic concordance between population structure and sexual signal divergence is also used as evidence that discrimination is producing speciation by sexual selection. However, such evidence becomes less clear as the taxonomic resolution increases. It is possible that sexual trait divergence can only reinforce existing reproductive isolation; if so, one would expect to see areas of genetic divergence with or without sexual trait divergence (Ritchie, 2007). Alternatively, weak population structure—which is predicted in early speciation by sexual selection—is difficult to detect without large sample sizes (Fumagalli, 2013; Panhuis et al., 2001).

Across a wide range of taxa, individuals discriminate between divergent sexual signals (Mendelson & Safran, 2021). Preference for a sexual trait can be genetically linked to the trait itself (Sluijs et al., 2013; Wiley et al., 2012), but in other cases, preferences are learned through processes such as parental imprinting or social learning (P. R. Grant & Grant, 2018; Verzijden et al., 2012). For example, strawberry poison frogs (*Oophaga pumilio*) imprint on and prefer their mothers' coloration (Yang et al., 2019), and female fruit flies (*Drosophila melanogaster*) use social learning to inform their preferences for male coloration (Danchin et al., 2018). In species that learn through social conformity, these preferences can become cultural traditions that are transmitted through generations within a population (Danchin et al., 2018). Thus, learned divergent sexual trait preferences can promote behavioral isolation between closely related taxa, and even intraspecific populations (Servedio & Boughman, 2017).

Such behavioral isolation can drive geographic concordance between populations and sexual signals. For instance, neighboring populations of the Amazonian frog *Physalaemus petersi* that have different male mating call types have lower gene flow, compared to neighboring populations with the same call type, which likely is a result of female preference for the local call type (Boul et al., 2007). If genetic population structure is not detected, speciation by sexual selection is often ruled out (Baker et al., 1984; MacDougall-Shackleton & MacDougall-Shackleton, 2001; Poesel et al., 2017; Zink et al., 1991), but population structure during early speciation by sexual selection can be weak and difficult to detect, while sexual trait variation is apparent (Fumagalli, 2013;

Panhuis et al., 2001). Further, if sexual trait boundaries are leading to genetic breaks (i.e., a discontinuity in a genetic area, or the boundary at which two genetic populations meet), they would be expected to precede them, as well. Genetic population boundaries—when present—would align with the underlying sexual trait boundaries.

Song is a sexual signal commonly theorized to promote speciation in birds (Edwards et al., 2005). Playback experiments have found that birds discriminate between conspecific and heterospecific songs (Benites et al., 2015; Brambilla et al., 2008; Hudson & Shizuka, 2017), with males being the least aggressive toward highly divergent heterospecific songs (Freeman & Montgomery, 2017; Mejías et al., 2021). Within some species, individuals respond more strongly to local conspecific song types, compared to non-local conspecific songs (Baker, 1983; Danner et al., 2011; Hamao, 2016; Lipshutz et al., 2017; Searcy et al., 2002; Williams et al., 2019). However, behavioral responses to song divergence may vary. In some cases, the stronger response to the local dialect only holds for one sex, song type, or aggressive behavior (Danner et al., 2011; Petrinovich & Patterson, 1981). Further, salient aggressive signals may evoke less aggressive responses (e.g., intruder males will leave a territory in response to aggressive songs) (Searcy & Beecher, 2009). Playback studies traditionally test for discrimination between categories of song. But there is evidence that, in some taxa, the strength of behavioral discrimination depends on continuous levels of acoustic dissimilarity (Freeman & Montgomery, 2017; Mejías et al., 2021). In such cases, one would expect the strength of behavioral responses to depend on the extent of acoustic dissimilarity to the local or conspecific song.

Other studies have recovered geographic concordance between intraspecific song variation and population structure. For example, greenish warblers (*Phylloscopus trochiloides*) are a ring species complex around the Tibetan plateau in which two distinct types meet in Siberia; the Siberian types differ strongly in their songs and rarely interbreed, so culturally inherited song divergence appears to be acting as a prezygotic reproductive barrier (Irwin et al., 2008). But other empirical studies do not find evidence of intraspecific population structure aligned with song divergence (Poesel et al., 2017; Wright & Wilkinson, 2001). As signatures of genetic divergence become less perceptible within species, it becomes difficult to elucidate when song divergence can facilitate genetic divergence.

White-crowned sparrows (*Zonotrichia leucophrys*) have long been a model species for cultural evolution, or population-level changes in socially learned behaviors over generations (Marler & Tamura, 1962). The two coastal subspecies have well-described, distinct, and geographically discrete song types, generally referred to as dialects (Baker, 1975; Baker & Thompson, 1985; Baptista, 1977; Cunningham et al., 1987; Hafner & Petersen, 1985). Juvenile white-crowned sparrow males learn songs from nearby adult tutors and are more likely to adopt common dialects (Nelson & Poesel, 2014). While juveniles can learn multiple songs, adult males typically choose one song type to sing throughout adulthood, preferring to retain the song most similar to their neighbors (Nelson & Poesel, 2009). This conformity bias leads to the formation of dialect regions, or discrete geographic areas in which most males sing highly similar songs. White-crowned sparrow dialects are categorized using their trill notes, which are highly similar between most males in a dialect area, but distinct between dialects. White-crowned sparrow dialects can remain stable and recognizable for decades, in spite of minor changes in song traits (Derryberry, 2009; Harbison et al., 1999; Nelson et al., 2004).

Evidence for speciation by sexual selection is mixed in white-crowned sparrows, despite the presence of discrete and spatiotemporally stable song dialects (Heinemann, 1981). Between subspecies, Lipshutz et al. (2017) found that song is a barrier to gene flow between Puget Sound (*Z. l. pugetensis*) and Nuttall's (*Z. l. nuttalli*) white-crowned sparrows along the West Coast. While the barrier is porous, song divergence appears to be slowing admixture during secondary contact. However, these two subspecies also differ in song syntax and migratory behavior (*Z. l. pugetensis* is migratory, while *Z. l. nuttalli* is sedentary), indicating that song divergence may not be the only reproductive barrier between them. Within subspecies, there is support both for female preference for local dialects (Baker, 1983; Tomback & Baker, 1984) and concordance between population structure and dialect regions (MacDougall-Shackleton & MacDougall-Shackleton, 2001). Other studies have found no relationship between song dialects and population structure (Nelson et al., 2017; Poesel et al., 2017). This may be due to dispersal across dialects or could be a sign that the populations are too early in the process of speciation by sexual selection to detect population structure from neutral markers (Panhuis et al., 2001).

In this study, we test the hypothesis that song divergence is restricting gene flow through assortative mating within continuously distributed populations of *Z. l. nuttalli*. Assortative mating can arise directly via female preferences for the local song dialect or indirectly via reduction in a male's ability to hold a territory with a non-local song, thus limiting his ability to attract a mate (Andersson, 1994). *Z. l. nuttalli* are sedentary with finely structured cultural populations (i.e., each song dialect is sung over a small area). The more limited dispersal and exposure to other song dialects in *Z. l. nuttalli* make them a better candidate for speciation by sexual selection, compared to the other four *Z. leucophrys* subspecies, which are all migratory (Nelson et al., 1995, 2001). Even low levels of dispersal between populations can prevent genetic divergence, so the relatively low dispersal of *Z. l. nuttalli* is more conducive to genetic drift between cultural populations (Baker & Mewaldt, 1978; Petrinovich & Patterson, 1982).

We first examined geographic concordance between population structure and song variation. If song divergence is facilitating genetic divergence, songs would diverge first, and the resulting population structure would align with the underlying song variation. In the early stages of reproductive isolation, this may appear as multiple song boundaries but little to no population structure. Therefore, we predicted the presence of more song boundaries than genetic boundaries, but that existing genetic boundaries would align with song boundaries.

We then experimentally evaluated behavioral responses to song divergence. Using a song playback experiment, we tested whether the strength of behavioral discrimination is due to continuous song divergence from the local dialect. Discrimination studies generally use treatments in which categories of foreign song can represent species or population identity (Benites et al., 2015; Brambilla et al., 2008; Lipshutz et al., 2017), levels of acoustic dissimilarity to the focal males' song (Freeman & Montgomery, 2017; Mejías et al., 2021), or some combination of the two (Lipshutz et al., 2017; Mejías et al., 2021). To account for the potentially confounding effects of the singer's genetic population, we included two non-local treatments: non-local song from the same genetic population and non-local song from another genetic population. The two non-local treatments are similar in their acoustic dissimilarity to the local song but differ in the singer's genetic identity relative to the focal male. While our primary prediction is that the strength of behavioral discrimination depends on acoustic dissimilarity, the comparison of these treatments can test whether focal males are discriminating based on the singer's genetic identity.

Male territorial behaviors are easier to assay in the field than females, and male aggression is positively correlated with female preference in white-crowned sparrows (Derryberry, 2007; Yang et al., 2019). However, in a territorial context, male and female *Z. l. nuttalli* respond differently to local and non-local dialects. While both sexes responded weakly to *Z. l. pugetensis* song and a distant dialect, females respond more strongly to the adjacent dialect across all aggressive behaviors, while males sang more in response to the local dialect and trilled and fluttered more in response to the adjacent dialect (Petrinovich & Patterson, 1981). Additionally, male aggression itself is a measure of signal efficacy, as the facilitation of male-male competition is an important function of song (Derryberry, 2007). A signal stimulus that elicits a stronger aggressive response is typically interpreted as reflecting greater stimulus salience (Derryberry, 2007; Podos, 2007).

Methods

Genetic sampling and sequencing

Genetic samples of *Z. l. nuttalli*, *Z. l. pugetensis*, and *Z. l. nuttalli* x *pugetensis* hybrids from 22 locations used in Lipshutz et al. (2017) were reanalyzed for this paper, along with additional samples collected from 11 locations in the hybrid zone, three additional locations south of the original sampling, and 12 individuals from three outgroups (Gambel's white-crowned sparrows (*Z. l. gambeli*), mountain white-crowned sparrows (*Z. l. oriantha*), and golden-crowned sparrows (*Zonotrichia atricapilla*)). The entire dataset was used to delimit the full breeding range of *Z. l. nuttalli*, since the subspecies is admixing with *Z. l. pugetensis*; we defined the breeding range as the localities in which our discriminant analysis of principal components (DAPC) assigned multiple individuals to the *Z. l. nutalli* cluster. In total, we used 285 samples collected along the West Coast (**Figure 1.1**). 215 blood samples were collected during banding in 2004, 2005, and 2014. Dispersal is limited in the subspecies so there should be little, if any, change in population structure over the 10-year sampling period (Baker & Mewaldt, 1978; Petrinovich & Patterson, 1982). In addition, 63 tissue samples were collected from vouchered specimens housed at the LSU Museum of Natural Science. Seven Genotype by Sequencing (GBS) samples were extracted from tissue loans from the University of California Berkeley Museum of Vertebrate Zoology (3 samples) and University of Washington Burke Museum of Natural History and Culture (4 samples).

Genetic samples were sequenced using GBS at the Institute of Genomic Diversity at Cornell University in Ithaca, NY, USA. Libraries were prepared following Elshire et al. (Elshire et al., 2011), using the restriction enzyme PstI (CTGCAG) and a unique barcode for each sample on the plate. The first two GBS plates were analyzed in Lipshutz et al. (2017), and a third plate of samples was extracted and sequenced following the same methods at the same facility. All three plates were processed and analyzed together in this study. We demultiplexed the GBS reads (i.e., sorted sequence reads into a separate file for each sample) for all three plates using the *process_radtags* function in STACKS (Catchen et al., 2013), dropped samples with less than 500,000 reads, and called SNPs using the STACKS *ref_map* pipeline and a white-crowned sparrow draft genome as the reference. We filtered out SNPs with less than five percent minor allele frequency and more than 80 percent missing data using vcftools (Danecek et al., 2011). After data filtering, 263 individuals (251 focal samples and all 12 outgroup samples) and 26977 SNPs were retained in the dataset.

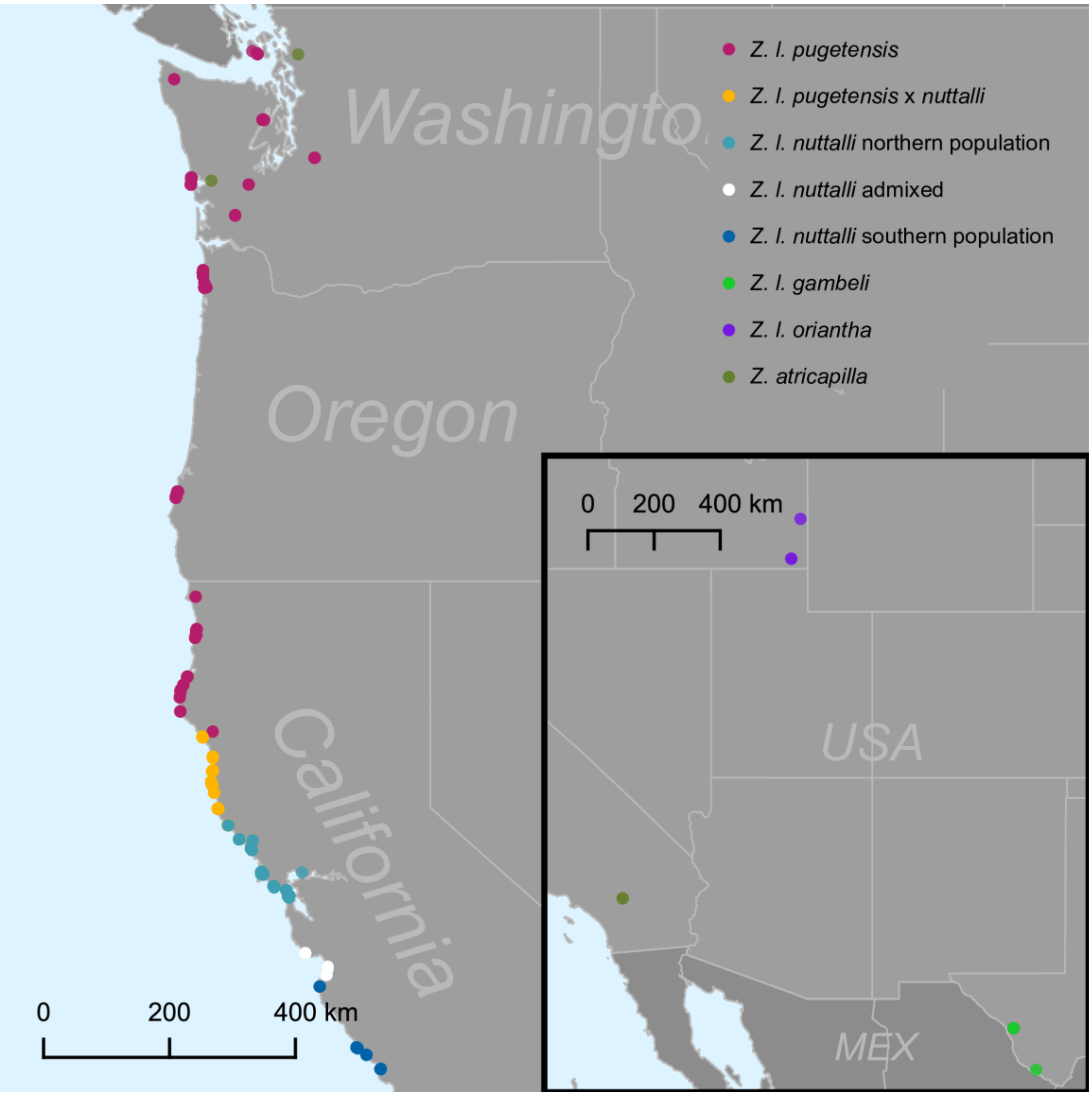


Figure 1.1. Map of all genetic samples before data filtering. Classification of *Z. l. nuttalli*, *Z. l. pugetensis*, and their hybrids is based on DAPC analysis of SNP data.

Population structure

We first calculated pairwise Fst between sampling localities using the R package hierfstat (Goudet, 2005). The focal subspecies of this study, *Z. l. nuttalli*, hybridizes with another coastal subspecies, *Z. l. pugetensis*. We analyzed the same samples used by Lipshutz et al. (2017) with the addition of more samples from the putative hybrid zone. We first ran fastSTRUCTURE (Raj et al., 2014) on all of the *Z. l. nuttalli*, *Z. l. pugetensis*, and *Z. l. nuttalli* x *pugetensis* samples. We ran the analysis using multiple numbers of clusters, k = 2 through k = 6, with 10 independent replicates for each k value, and estimated the optimal value of k using the chooseK.py script. We then averaged the results of the replicates of the optimal k value.

Clustering analyses like fastSTRUCTURE often recover two groups representing only the deepest divergence (Janes et al., 2017). To estimate finer resolution population structure within the subspecies, we ran the analysis using only the *Z. l. nuttalli* samples identified by DAPC. We ran a DAPC using the adegenet R package (Jombart, 2008) without the outgroup individuals. The snapclust function (maximum likelihood clustering) in adegenet found that k = 1 had the lowest BIC value, but the find.clusters function (K-means clustering) supported k = 2, with low assignment consistency for hybrids between replicates. This suggested support for two clusters but with unstable assignment for hybrids, so we ran a DAPC with k = 2 and hybridization. Parental groups and hybrids were assigned by snapclust, which we initialized with a priori subspecies assignments. Putative hybrids assigned to *Z. l. nuttalli* in the DAPC were included in subsequent analyses.

With the *Z. l. nuttalli* samples, we ran fastSTRUCTURE with k = 2 to k = 5 and used the chooseK.py script to estimate the optimal number of clusters. Using the optimal number of clusters identified by chooseK.py, we ran 100 fastSTRUCTURE replicates and averaged the 25 replicates that maximized the marginal likelihood. We repeated this process within each identified *Z. l. nuttalli* cluster until no further hierarchical population structure was found.

We located the geographic boundary between the two *Z. l. nuttalli* genetic populations by using ordinary kriging to spatially interpolate the fastSTRUCTURE output with the R package gstat (Pebesma & Wesseling, 1998). We started by fitting a semivariogram on the admixture coefficients, or estimated amount of heritage from each ancestral population, of the samples. We then produced a regular grid of 10,000 points within California's coastal scrub habitat (Goudey & Smith, 1994) (bounded by the latitudes of the genetic samples), interpolated the ancestry coefficient at each grid point, and rasterized the point grid. Some predicted values were slightly above one or below zero, so they were truncated to one or zero to keep all values as proportions.

Song sampling

We used two sets of song recordings for different analyses. The first set (i.e., song dissimilarity dataset) included 175 songs from 82 *Z. l. nuttalli* males, representing 18 song dialects (i.e. 18 trill note types from different localities) recorded between 2010 and 2022 (**Figure 1.2**). Song dialects and genetic populations have both been stable for at least four decades, making the disparity in sampling times trivial (Corbin, 1981; Derryberry, 2009). Songs were recorded during the breeding season within the *Z. l. nutalli* breeding range, as determined by the DAPC described earlier. Songs were pulled from the Derryberry Lab recording repository (153 songs from 72 males) and Xeno-Canto (22 songs from 10 males). We clipped songs from the Xeno-Canto recordings using Audacity 3.2.1. For all the songs, we removed low-frequency background noise in Audacity 3.2.1 using a high-pass filter between 1.5-2.5 kHz with a 48 dB/octave spectral rolloff.

The second set of songs (i.e., song trait dataset) was comprised of 113 songs from 79 *Z. l. nuttalli* males; all the males in this dataset were also included in the GBS sequencing dataset. The songs were recorded between 2004 and 2014. We measured the durations of the whole song, introductory whistle, complex notes (mean duration), trill section, and individual trill notes (mean duration); bandwidth of the whole song, complex notes, and trill; maximum and minimum frequencies of the song, complex notes, and trill; trill rate; and dominant frequency of the introductory whistle (**Figure 1.3**). Measurements of song traits were taken in SIGNAL v5 (Beeman, 1998), using spectrograms with a 256-

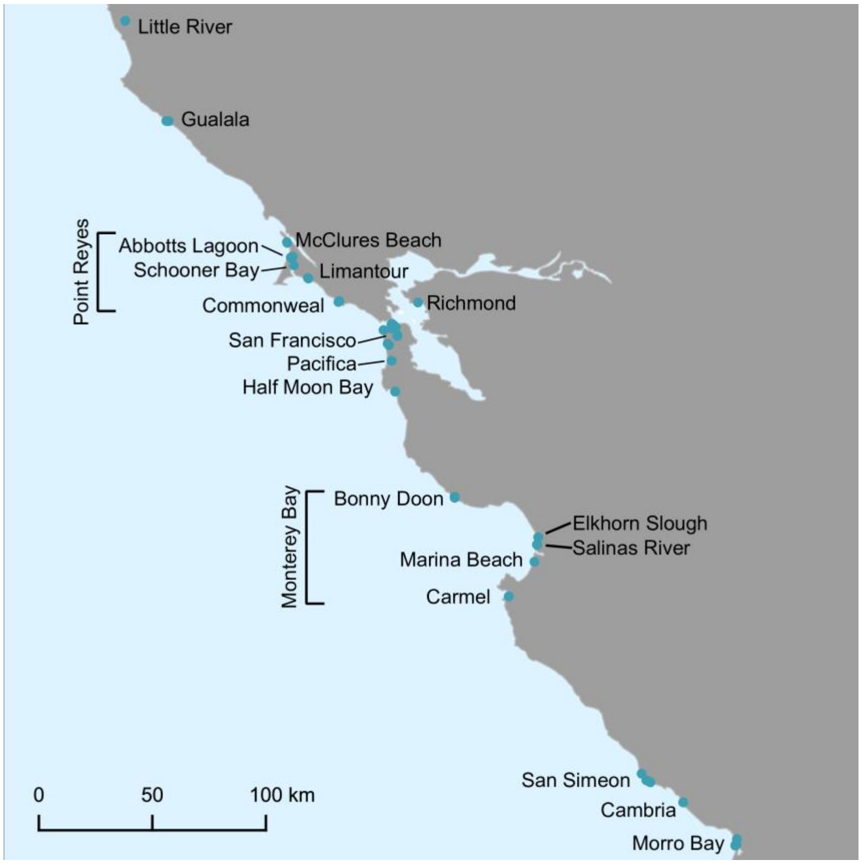


Figure 1.2. Map of song recording localities included in the song dissimilarity dataset. Dataset includes 175 songs (18 dialects) from 82 males of *Z. l. nuttalli*.

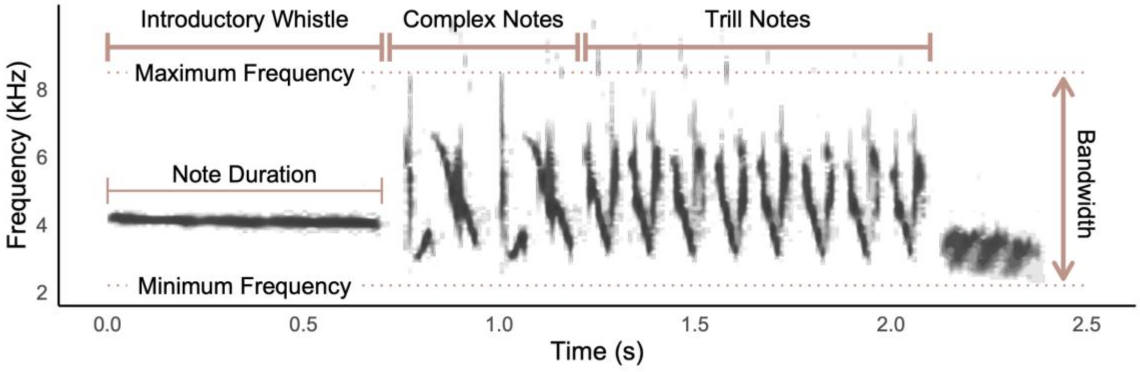


Figure 1.3. White-crowned sparrow song sections and song traits measured for PCA.

point transform and frequency resolution of 97.7 Hz and 10.2 ms time resolution. The means of each measurement were calculated for each male.

Song analyses

With the 175 songs in the song dissimilarity dataset (**Figure 1.2**), we calculated the dissimilarity between every pair of songs using the R package warbleR (Araya-Salas & Smith-Vidaurre, 2017). We first tracked the contours of the dominant frequency for each song; frequencies below 2 kHz and above 8 kHz were filtered out, and the fundamental frequency was sampled 200 times for each song, with a window length of 5000, 90% window overlap, 0% amplitude threshold for frequency detection, and frequency smoothing of 0.1 kHz. Using the frequency time series, we produced a pairwise dissimilarity matrix with a dynamic time warping (DTW) algorithm.

Given that song recordings from the repositories differ in their recording protocol, audio equipment, and audio quality, we wanted to ensure that this variation did not influence our analyses. Using pairwise dissimilarities from the DTW matrix, we determined whether the two source repositories produced artificial clusters in the songs. We ordinated the songs onto two axes using multidimensional scaling, then grouped the songs with k-means clustering and a maximum of two clusters. If the repositories are producing artificial clusters, songs from the same repository should cluster together.

We then found geographic boundaries between songs using the R package GeoOrigins (Hulme-Beaman et al., 2020) with a 95% confidence level for provenancing and Spearman’s correlation coefficient. Using sample coordinates and pairwise dissimilarity data, GeoOrigins estimates geographic areas in which sample values are highly correlated with neighboring samples. For each sample, boundary elements are defined as grid cells at which a minimum correlation coefficient threshold is crossed; grid cells that represent boundary elements for multiple samples are more intense geographic boundaries (Hulme-Beaman et al., 2020). The placement and apparent strength of some boundaries may be partially an artifact of geographic sampling bias. For example, large gaps in sampling might produce a boundary in the gap, and “strong” boundaries might appear near heavily sampled areas. To assess the strength of the major boundaries, we compared the average song dissimilarity of each song to (1) songs from the same side of the boundary and (2) the opposite side of the boundary. We ran D'Agnostino tests for normality using the moments R package (Komsta & Novomestky, 2022) and found that the datasets are all non-normal. Therefore, we ran one-tailed paired Wilcoxon signed-rank tests at each boundary to compare the same-side and across-boundary song dissimilarity.

GeoOrigins recovered a song boundary coincident with the genetic boundary (see Results below), so we performed a principal components analysis (PCA) to determine whether that boundary represents consistent song trait differences between the genetic populations. All measurements were scaled in the PCA, and only significant principal components (PCs), as determined using the Broken Stick method, were retained. For each retained PC, we ran D'Agnostino tests for normality and F-tests to compare variances for all genetic groups—the two genetic populations and the admixed group (i.e., from the area with genetic contributions from both populations). Each PC had non-normal groups or unequal variances, so we ran Kruskal-Wallis tests to compare the genetic groups (Ostertagová et al., 2014). We then ran Conover-Iman post hoc tests with Bonferroni corrections on PCs with significant Kruskal-Wallis tests.

Playback experiment

We ran a playback experiment at six sites. Two sites each were located within the (1) northern *Z. l. nuttalli* population (Abbotts Lagoon and Commonweal, in Point Reyes National Seashore), (2) southern *Z. l. nuttalli* population (Morro Bay and San Simeon State Parks), and (3) admixture area between the populations (Moss Landing and Bonny Doon State Beaches) (**Figure A-1**). Song stimuli were drawn from recordings produced between 2004 and 2020.

Focal males were presented with a sequence of three stimulus periods within a 24-minute trial. Each stimulus period represented one treatment: (1) a local song, (2) a non-local song from the same genetic population, and (3) a non-local song from the other Z. l. nuttalli population. All admixed males heard a local song and a non-local admixed song, but 10 males heard non-local songs from the northern population, and 7 heard non-local songs from the southern population. Treatment order was randomized for each male to compensate for order effects, and stimulus sequences were prepared in advance, so that we scored the behaviors while blind to the treatments. Neighbors were tested on different days so that they did not become habituated to the stimuli during tests on neighboring males. To avoid pseudo-replication (McGregor, 2000), every male heard a unique set of stimuli, and every song was used only once.

We placed a speaker (black Ultimate Ears Wonderboom 3) in a male's territory, near a perch on which we observed him singing, to broadcast the stimuli. Starting from the speaker, we rolled out two ropes flagged at 2m, 4m, 8m, and 16m; the ropes were laid out at roughly 90°-180° from one another, depending on the vegetation structure of the male's territory. Song amplitude was normalized and broadcast at a level typical of free-living males (80–82 dB sound pressure level at 1m). The stimulus periods were each three minutes long, with one song played repeatedly at a natural rate of six songs per minute. Each stimulus period was separated by a six-minute "cooldown" period. Focal male behavior was observed during the three-minute playback periods and for three minutes after each playback period. We measured four response variables: mean distance from the speaker during the (1) playback period and the (2) post-playback period, (3) number of flights over the speaker during the playback period and (4) number of songs during the playback period. Distance from the speaker was estimated in meters every 10 seconds within distance intervals (0-2m, 2-4m, 4-8m, 8-16m, and >16m), using the flagged ropes as references for these intervals. The middle distance within the interval was used as the value (e.g., 1 m for the nearest interval). These mean distance for a period was calculated across all estimates within the period.

We ran a PCA on the four behavioral variables, all scaled, to extract an aggression metric. We used PC1 as our aggressive response variable, since higher values of PC1 indicate increased aggressive behavior (e.g., increased song rate, smaller distance to speaker). We then calculated behavioral discrimination between songs as the difference in aggression between the local and non-local treatments (discrimination = $PC1_{local} - PC1_{foreign}$), leaving two data points for each male: discrimination against each non-local song. For each male tested, we calculated song dissimilarity between the local stimulus and each foreign stimulus using a dynamic time warping algorithm, following the methods under the song analyses section. We then normalized song dissimilarity using z-scores, so the magnitude of variation would more closely match the Fst values. Two out of 64 focal males we tested were not present for all three trials. Specifically, they did not show up until after the first song stimulus was over. It is difficult to differentiate between a lack of motivation and a lack of perception in these cases, so they were removed from the statistical analysis.

If the treatments differed in both their acoustic dissimilarity and genetic identity (i.e., all the males from the other genetic population also had more dissimilar songs), the genetic divergence would confound the potential effect of song divergence on behavioral discrimination. Therefore, we aimed to decouple genetic population and acoustic dissimilarity in this experiment. We selected song stimuli to ensure that the non-local treatments contained similar levels of acoustic dissimilarity to the local song treatment. The treatments differed in genetic divergence from the focal male but not song divergence. Rather, similar levels acoustic dissimilarity from the local dialect were present within each treatment, which we confirmed with a t-test. To determine whether songs from the other genetic population were less salient signals, regardless of acoustic dissimilarity, we ran an ANOVA comparing the aggression metric (PC1) across the three treatments. Irrespective of whether males discriminate between categories of song, our focus is to understand how males respond to continuous variation in song that exists in both treatments.

We then tested whether acoustic dissimilarity or Fst could predict the aggression of focal males toward the song stimuli. Pairwise Fst values were calculated for each site based on the GBS data, not these experimental males. For 11 focal males, one of the treatments was missing an Fst value; the foreign song stimulus was drawn from a population without genetic data. Data points for those 11 males were initially removed from the analysis. We first ran a linear mixed model (LMM) to test whether song dissimilarity is correlated with Fst or geographic distance (reduced dataset: $n_{males} = 51$), using the R package lmerTest (Kuznetsova et al., 2017) with focal male and dialect comparison as random effects. For the dialect comparison, each factor was the combination of the focal male's locality and the dialect of the song stimulus (e.g., focal males from Abbotts Lagoon that responded to a Morro Bay stimulus song, or focal males from Moss Landing that responded to a San Francisco stimulus song). We used dialect comparison as a random effect to account for non-independent genetic and song sampling, since both sample types were drawn from the same localities.

Since the two predictors are uncorrelated, we then ran an LMM with behavioral discrimination as the response variable, using only focal male as the random effect, as the song stimuli are unique to each trial (reduced dataset: $n_{males} = 51$). We compared the corrected Akaike Information Criterion (AICc) values of LMMs with different fixed effects—song dissimilarity, Fst, or both—using the dredge function in the MuMIn package [76], then ran the LMM with the best (i.e., lowest) AICc value. Because the best model included only song dissimilarity as a fixed effect, we included the males with the missing Fst values in the final model ($n_{males} = 62$).

Results

Population structure

fastSTRUCTURE identified k = 2 as the optimal k value for *Z. l. pugetensis* and *Z. l. nuttalli* samples, and as in Lipshutz et al. (2017), the two subspecies were found to be genetically distinct but admixing (**Figure A-2**). The DAPC reassigned 17 putative hybrids to *Z. l. nuttalli* and 35 to *Z. l. pugetensis* (**Figure A-3**). The individuals reassigned by the DAPC to the parental clusters were at the edges of hybrid zone, effectively narrowing the putative hybrid zone. fastSTRUCTURE recovered two *Z. l. nuttalli* clusters, northern and southern (**Figure 1.4a**). Further runs on subsets of *Z. l. nuttalli* samples found no additional population structure. Admixed individuals were found around Monterey Bay, from Bonny Doon to Marina Beach (**Figure 1.4b**). Carmel, on the southern part of Monterey Bay, had relatively high pairwise Fst values across population comparisons (**Figure A-4**).

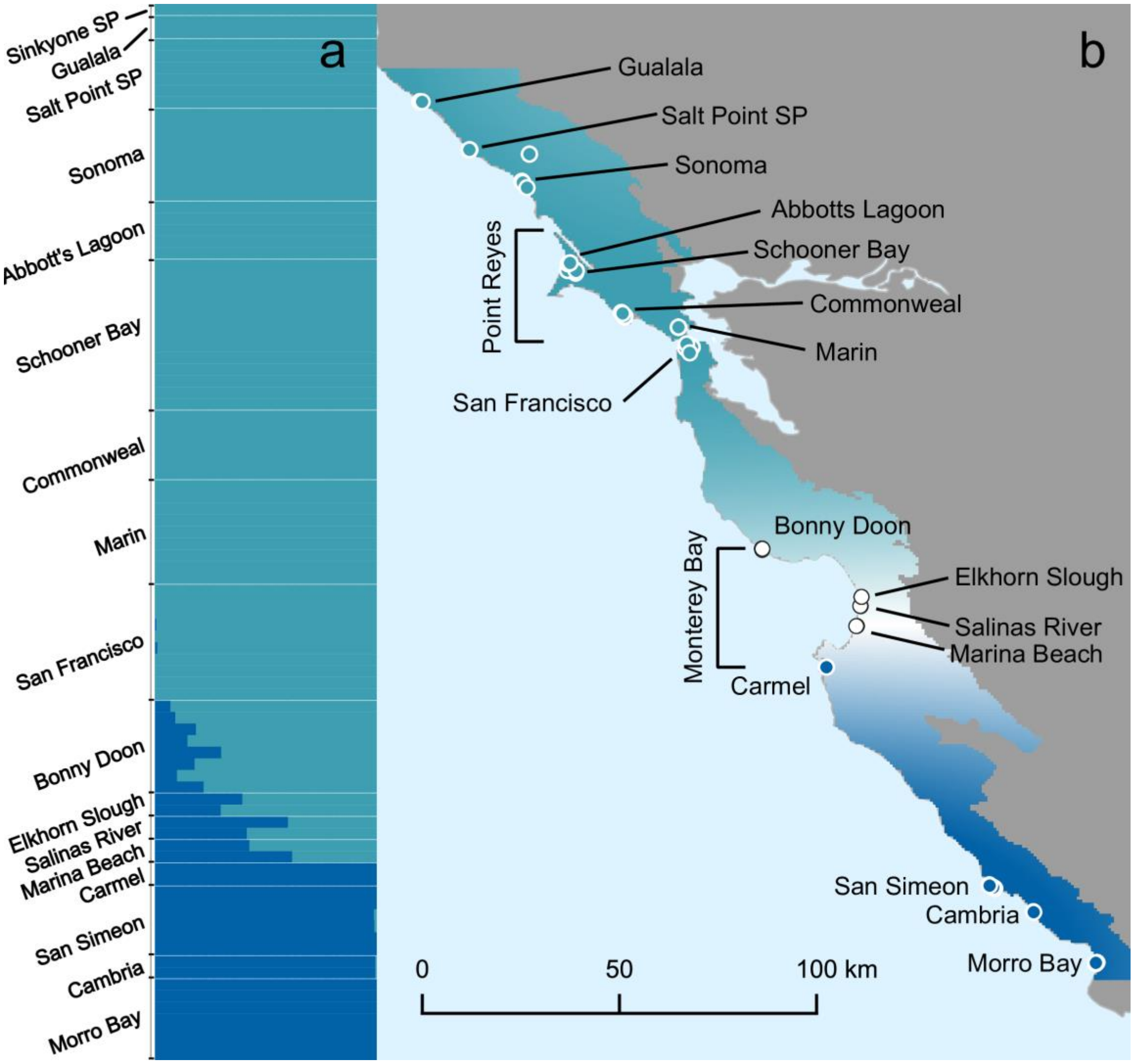


Figure 1.4. Population structure of *Z. l. nuttalli*. Light blue indicates the northern population and dark blue the southern population. (A) fastSTRUCTURE assignment probabilities, sorted north to south. (B) Interpolated assignment probabilities to northern and southern *Z. l. nuttalli* populations, with the white band at Monterey Bay representing admixture.

Song analyses

We recovered three major song boundaries at Point Reyes, San Francisco, and Monterey Bay using GeoOrigins (**Figure 1.5**). We found no evidence that variation in recording protocol or audio quality between repositories produced artificial clustering in the songs (**Figure A-5**); these boundaries do not appear to be artifacts of sampling from multiple repositories. These boundaries roughly delineate four cultural populations, which is notably fewer than the 18 sampled song dialects. Using paired one-tailed Wilcoxon signed-rank tests at each boundary, we found significantly higher song dissimilarity to songs across the boundary, relative to dissimilarity to songs on the same side of the boundary (Monterey Bay, $n = 143$, effect size = 8.56, $V = 8670$, $p < 0.001$; San Francisco, $n = 117$, effect size = 3.15, $V = 5719$, $p < 0.001$; Point Reyes, $n = 166$, effect size = 6.90, $V = 9673$, $p < 0.001$; $\alpha = 0.05$). In other words, songs from the same side of a boundary were, on average, significantly more similar to each other than to songs from the other side of the boundary. This comparison was the most stark at the Monterey Bay boundary (**Figure 1.5**).

The first four principal components of the song trait PCA were found to be statistically significant using the Broken Stick method. PC1 explained 21.43% of the variance, PC2 explained 20.78%, PC3 explained 13.70%, and PC4 explained 10.95% (Appendix 1) PC1, PC2, and PC4 had significant differences between some genetic groups within Z. l. nuttalli (Kruskal-Wallis tests: $X^2 = 21.49$, $X^2 = 56.1$, and $X^2 = 14.0$, respectively; $df = 2$, $p < 0.001$, $\alpha = 0.05$ for all tests). The northern and southern populations were significantly different from each other along all three of these PCs (PC1: $Z = -4.11$, PC2: $Z = -6.02$, and PC4: $Z = 3.73$; $p_{adj} < 0.001$, $\alpha = 0.05$ for all tests) (12.6). The admixed group was significantly different from both the northern and southern populations along PC1 (admix-north: $Z = -2.51$, $p_{adj} = 0.0212$, admix-south: $Z = -5.37$, $p_{adj} < 0.001$) (**Figure 1.6a**), only the northern population along PC2 ($Z = 9.66$, $p_{adj} < 0.001$) (**Figure 1.6b**), and only the southern population along PC4 ($Z = 3.87$, $p_{adj} < 0.001$) (**Figure 1.6c**).

PC1 was strongly and positively loaded with the bandwidth for the overall song and trill notes; maximum frequency of the overall song, complex notes, and trill notes;

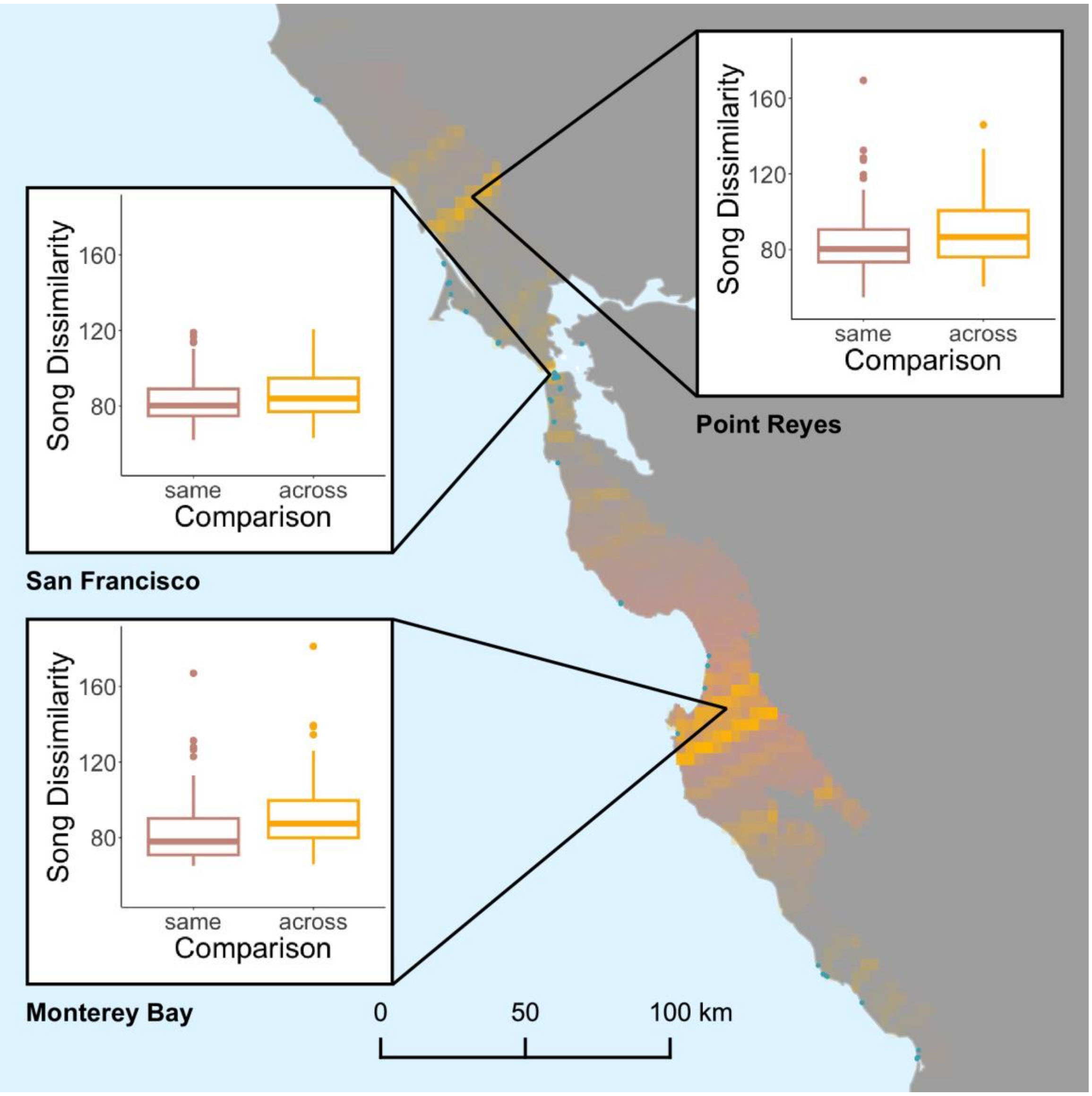


Figure 1.5. Song boundaries and acoustic dissimilarity comparisons at boundaries. Points are sampled songs, and yellow cells represent boundary elements, with darker yellow indicating more intense boundaries. Pink shading shows admixture at the genetic boundary, with the darkest pink indicating an assignment probability of 0.5. Insets show comparisons of mean across-boundary and same-side acoustic dissimilarity.

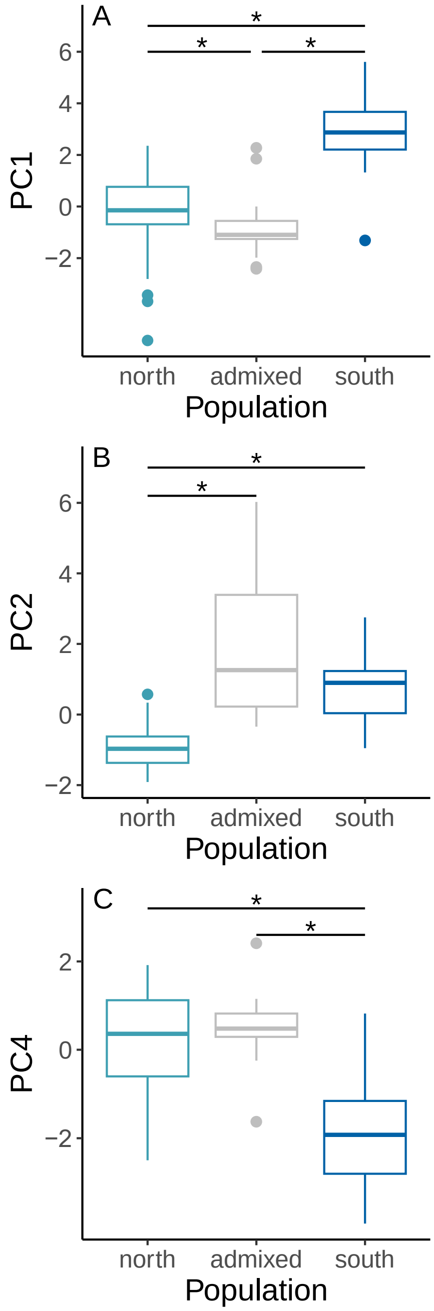


Figure 1.6. Song trait principal components by genetic population. (A) PC1, for which high values indicate higher song maximum frequencies and longer trill notes; (B) PC2, for which high values indicate higher minimum frequencies and longer complex notes; and (C) PC4, for which higher values correspond to higher minimum trill frequencies. Each PC is shown separated by genetic Z. l. nuttalli group. Asterisks indicate statistically significant differences between the groups.

and trill note length. It is negatively loaded with trill rate. PC2 has strong positive loadings for complex note length, trill rate, and overall minimum frequency, and is negatively loaded with the minimum frequency of complex notes and maximum frequency of trill notes. PC4 has strong positive loadings for minimum and maximum complex note frequencies and trill minimum frequency; it is strongly and negatively loaded with complex note length (**Figure A-6**). In short, higher values of PC1 are associated with longer and higher frequency notes with higher bandwidth, which are inversely related to trill rate; higher values of PC2 are associated with disparate traits, though largely minimum and maximum frequencies; and like PC1, higher values of PC4 are associated with higher frequencies and shorter notes.

Playback experiment

We first ran a PCA to collapse the four behaviors into a single metric for aggression. We used PC1, which explains 53.11% of variance in the behavioral response, as the aggression score. Higher values of PC1 represent more songs and flights and proximity to the speaker (**Figure A-7**). Therefore, a high PC1 is indicative of high aggression. Our ANOVA found no significant difference between responses to the three categorical stimulus treatments (F = 0.008, p = 0.992). The non-local song treatments overlapped in their degree of acoustic dissimilarity to the local song treatment (paired t-test: t = 0.080794, df = 61, p = 0.9359). Therefore, this analysis does not account for the continuous variation in song divergence that we hypothesize is driving male discrimination.

To assess the effects of continuous song dissimilarity, calculated using a dynamic time warping algorithm, on behavioral discrimination in males, we ran an LMM. In our full model, local males' behavioral discrimination between local and foreign songs was predicted by Fst and song dissimilarity. When the models were competed against each other, the model with the lowest AICc retained only song dissimilarity as a fixed effect. The LMM with the reduced dataset (only focal males with associated Fst values, n_{males} = 51) recovered a positive but nonsignificant trend between discrimination and song dissimilarity (effect size = 0.182, se = 0.107, t = 1.71, p = 0.0907). When we added the males with missing pairwise Fst values back into this model, we found a significant positive relationship between discrimination and song dissimilarity ($n_{males} = 62$**,** effect size = 0.255, se = 0.0912, t = 2.79, p = 0.00625, α = 0.05) (**Figure 1.7**).

In the case that acoustic dissimilarity is a function of genetic or geographic distance, the effect of acoustic dissimilarity would be confounded by these variables. But based on an LMM, acoustic dissimilarity between songs was not correlated with either predictor (Fst: effect size = -0.560, se = -0.41, t = -1.37, p = 0.185; geographic distance: effect size = 1.32×10^{-7}, se = 8.18×10^{-8}, t = -1.65, p = 0.104). In other words, differences in song between populations is not explained by the genetic distance or the geographic distance between those populations. Therefore, the relationship between acoustic dissimilarity and behavioral discrimination appears to be independent of genetic and geographic distance.

Discussion

Culturally inherited mating signals mediate assortative mating across taxa (Creanza et al., 2017; Danner et al., 2011; Lipshutz et al., 2017; Ribot et al., 2012). Conspecific songs are more salient signals for attracting females and holding territories (i.e., male competition), so interspecific song divergence can reduce gene flow across taxa (Brambilla et al., 2008; Campbell et al., 2019; Edwards et al., 2005; Freeman & Montgomery, 2017; Higashi et al., 1999; Hudson & Shizuka, 2017; Mejías et al., 2021; Payne & Krakauer, 1997). There is also evidence that sexual selection against divergent mating signals can produce reproductive isolation between subspecies (Lipshutz et al., 2017; Toews, 2017). But the ability of song to reduce gene flow within subspecies or populations has remained unresolved. Here, we investigated whether song has the potential to reduce gene flow within the subspecies *Z. l. nuttalli*. Our results are consistent with early stages of speciation by sexual selection.

As predicted under sexual selection theory (Panhuis et al., 2001), white-crowned sparrows show more “cultural population structure” than genetic population structure, along with concordant cultural and genetic boundaries. We identified three major song

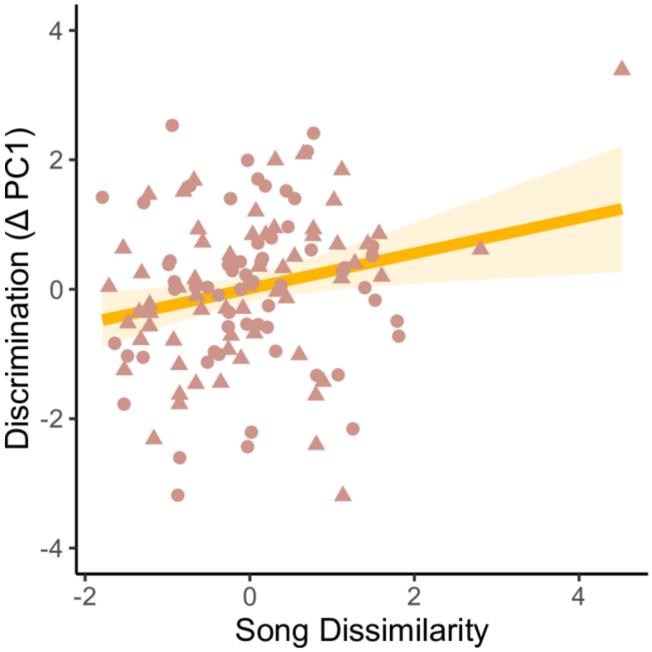


Figure 1.7. Correlation between behavioral discrimination and song dissimilarity. Each male is represented by two points: discrimination toward a non-local song (circles) from the same population and toward a song from the other population (triangles).

boundaries in Point Reyes, San Francisco, and Monterey Bay. We also recovered one genetic boundary separating northern and southern genetic populations within *Z. l. nuttalli*. These boundaries are consistent with predictions that sexual trait divergence arises first in the speciation process, producing an initial disparity between cultural and genetic variation (Janicke et al., 2019). It is important to note that the different number of boundaries for song and genetic data could be a function of the different statistical tools used; however, it is unlikely that fastSTRUCTURE missed additional genetic boundaries given the low genetic differentiation among populations, except at Monterey Bay. The different number of boundaries could also be due, in part, to the genetic data collection method we used. For example, future genome resequencing could uncover geographically structured genetic variation we did not detect using GBS.

The genetic boundary is concordant with the song boundary at Monterey Bay, which is the most prominent song boundary in terms of acoustic dissimilarity. Lipshutz et al. (2017) also found geographic concordance between song and genetic divergence in white-crowned sparrows, and that males discriminate between songs from their own subspecies and the other subspecies. Together, these studies suggest that song divergence can act as a reproductive barrier in white-crowned sparrows at two stages of genetic divergence: between the subspecies *Z. l. nuttalli* and *Z. l. pugetensis*, and between populations within *Z. l. nuttalli*. We posit that these two points of concordance indicate that culturally inherited song divergence can produce reproductive barriers through sexual selection, though we suggest further studies examining geographic concordance between genetic and song boundaries—especially those that focus on learned songs and multiple stages of speciation.

Given the presence of 18 discrete geographically structured song dialects in our dataset, as determined by visual inspection of spectrograms, it was unsurprising that GeoOrigins identified multiple song boundaries. The recovered boundaries do not separate all *Z. l. nuttalli* dialects but group some neighboring dialects together. Given the wide latitudinal range of the songs, the sampling density or resolution of the analysis may not have been fine enough to separate all the dialects. Alternatively, these larger groupings may represent "superdialects," similar to the Puget Sound subspecies (Baker &

Thompson, 1985). Unlike *Z. l. pugetensis*, the dialects in *Z. l. nuttalli* generally do not vary syntactically. Whether the grouping is due to low resolution in the analysis or the presence of inter-dialect similarity, these results are consistent with early speciation by sexual selection since they establish more cultural populations than genetic populations. For our playback experiment, we interpreted a weaker male response as an indicator of lower signal salience of the stimulus (i.e., songs that elicited less aggression are less salient in the context of territorial defense). Under this interpretation, our results suggest that songs that are more divergent from those of the focal male are less salient; male responses to foreign stimuli were lower relative to their responses to the local dialect. Males singing such songs should be less able to acquire and maintain a territory in the same song neighborhood, which should reduce gene flow between areas with divergent songs.

However, it is possible to interpret the strength of response in the opposite direction. A weaker male response would instead indicate higher signal salience of the stimulus (i.e., songs that elicited less aggression are more salient signals in the context of territorial defense, for instance in cases of de-escalation (Searcy & Beecher, 2009)). With this interpretation, our playback results suggest that songs that are more divergent from the focal male's song are in fact more salient, so males singing highly divergent songs could successfully compete for territories against males singing the local dialect. If this is the case, song divergence would not restrict gene flow, and we would not expect to find concordant genetic and song boundaries. Regardless of whether highly salient signals elicit stronger or weaker male responses, the difference in responses between stimuli suggests differing levels of salience (Searcy & Beecher, 2009).

The genetic differentiation at Monterey Bay might be maintained by acoustic dissimilarity through sexual selection and assortative mating. Together, the playback results and the genetic and song boundary overlap suggest songs that are highly dissimilar from the local dialect may be less salient mating signals. Studies focusing on the behavioral discrimination between songs generally compare categorical treatments, but these treatments either represent different genetic identities, levels of acoustic dissimilarity, or both (Freeman & Montgomery, 2017; Mejías et al., 2021). Here, the two non-local song treatments represented different genetic identities but similar ranges of acoustic dissimilarity from the local song dialect. The results of our playback experiment demonstrate that males responded similarly to the non-local treatments, suggesting that they did not discriminate between songs based on the genetic identity of the singer. Instead, males discriminated more strongly as the non-local song became more acoustically dissimilar from the local song. While the recovered effect of acoustic dissimilarity is weak, these are contiguous populations. These results suggest the early signatures of behavioral discrimination between song dialects based on acoustic divergence, even between continuous populations of a single subspecies.

Songs that elicit a weaker response from males—in this case, songs that are more acoustically dissimilar to the local dialect—may be less salient territorial defense signals, thereby limiting mating opportunity (Andersson, 1994). Moreover, female preference is positively correlated with male aggression in white-crowned sparrows (Derryberry, 2007). Therefore, females may base their mating preferences on acoustic dissimilarity, further driving assortative mating based on acoustic dissimilarity.

We posit that the greater song divergence at Monterey Bay has produced sufficiently strong sexual selection to drive concordant genetic divergence. The song boundary at Monterey Bay is stronger than the others; it has the greatest acoustic dissimilarity to songs from the other side of the song boundary, relative to songs from the same side of the boundary. Why we did not observe genetic differentiation across the Point Reyes and San Francisco remains unclear, though. The other two song boundaries may be younger or less spatiotemporally stable than the Monterey Bay song boundary. We know the genetic break between northern and southern populations has been present at Monterey Bay for at least 40 years (Corbin, 1981), which is approximately 40 generations for this species. Unfortunately, longitudinal data that would allow us to examine the spatiotemporal stability of the song transitions were unavailable.

Alternatively, the zone of admixture could represent secondary contact after the *Z. l. nuttalli* populations diverged in allopatry. In this case, song divergence would be acting to maintain genetic divergence between the northern and southern populations. Monterey Bay is a site of taxonomic turnover for marine (Briggs & Bowen, 2012) and terrestrial

(Calsbeek et al., 2003; Feldman & Spicer, 2006; Kuchta et al., 2009; Myers et al., 2013; Reilly et al., 2015) organisms. A marine embayment at what is now Monterey Bay is thought to have persisted approximately from 18 million years ago until 600,000 years ago; this marine embayment is a commonly posited mechanism for vicariance in terrestrial taxa (Calsbeek et al., 2003; Feldman & Spicer, 2006; Reilly et al., 2015). *Z. l. nuttalli* populations, which now occur continuously around Monterey Bay, may have been fragmented during this time. But genetic divergence between *Z. l. nuttalli* and *Z. l. pugetensis*—which we found to be deeper than the genetic break within *Z. l. nuttalli*—likely occurred during the last glacial maximum (20,000 years ago), so both divergences may be more recent than the marine embayment (Lipshutz et al., 2017). For some other taxa, genetic breaks at Monterey Bay are also not consistent with the marine embayment period, suggesting the presence of other mechanisms for vicariance around the bay (Kuchta et al., 2009; Matocq, 2002). Therefore, we cannot rule out allopatry as the original driver of genetic divergence at Monterey Bay.

In terms of which aspects of song are acting as a reproductive barrier, we did not directly examine whether differences in particular song traits—rather than overall song dissimilarity—are acting as barrier to gene flow at Monterey Bay. White-crowned sparrow females use song traits to recognize potential mates (Soha & Marler, 2000b), assess male quality (Derryberry, 2007; Luther et al., 2016; Phillips & Derryberry, 2017a, 2017b; Podos, 1997), and differentiate between local and non-local songs (Luther et al., 2016; Marler & Tamura, 1962; Phillips & Derryberry, 2017b, 2017a; Podos, 1997). Vocal performance is a function of trill rate and trill bandwidth in white-crowned sparrows; high performance songs have higher trill rates and bandwidth (Derryberry, 2007; Luther et al., 2016; Phillips & Derryberry, 2017b, 2017a; Podos, 1997). Importantly, large trill bandwidth, not low trill minimum frequency itself, indicates high male vocal performance (Luther et al., 2016). The northern and southern population differ in minimum and maximum frequency, average trill note length, and trill minimum frequency, but not any bandwidth measures or trill frequency. While these traits vary with habitat structure (Derryberry, 2009; Phillips et al., 2020), they have not been shown to directly influence signal salience. Vocal performance does not appear to differ between populations, but females may be using population-level song trait differences to discriminate against non-local songs.

Our results generally support the hypothesis that sexual selection based on the degree of song divergence is reducing gene flow between the northern and southern *Z. l. nuttalli* populations. We posit that song divergence may have driven this genetic divergence, but with these data, we cannot rule out paleoecological changes that could have resulted in a genetic break at Monterey Bay. Regardless, culturally inherited song divergence appears to be maintaining the genetic break, laying the groundwork for speciation by sexual selection within a subspecies. This study and other recent studies support the ability of cultural dissimilarity to reduce gene flow between populations through assortative mating (Jonker et al., 2013; Porter & Benkman, 2019; Ribot et al., 2012; Yeh, 2019), highlighting the influence of gene-culture coevolution on biological evolution.

CHAPTER TWO

BOUNDARYSTATS: AN R PACKAGE TO CALCULATE BOUNDARY OVERLAP STATISTICS

Publication disclosure: This manuscript has been submitted to the R Journal for publication. The version included here the manuscript initially submitted to the journal. I am the sole author on this manuscript.

Abstract

Ecologists and epidemiologists frequently rely on spatially distributed data. Studies in these fields may concern geographic boundaries, as environmental variation can determine the spatial distribution of organismal traits or diseases. In such cases, environmental boundaries produce coincident geographic boundaries in, for example, disease prevalence. Boundary analysis can be used to investigate the co-occurrence of organismal trait or disease boundaries and underlying environmental boundaries. Boundary and boundary overlap statistics test for the presence of significant geographic boundaries and spatial associations between the boundaries of two variables. There currently exists one implementation of boundary overlap statistics, though only on Windows and ESRI ArcView, limiting the availability of boundary overlap statistics to researchers. I have created BoundaryStats—an R package available on CRAN—that implements boundary and boundary overlap statistics. BoundaryStats is the first open-source, cross-platform implementation of these statistical methods making the statistics more widely accessible to researchers.

Introduction

Geographic boundaries are an intrinsic feature of spatial ecology and epidemiology, as the relationships between organismal traits or disease prevalence with an underlying environmental variable often produce coincident geographic boundaries. Boundaries are areas in which spatially distributed variables (e.g., bird plumage coloration, disease prevalence, annual rainfall) rapidly change over a narrow space. They can also represent edges or discontinuities (e.g., neighborhood edges, ecotype boundaries). Boundary zones themselves may be of interest; for example, the temporal boundary dynamics between ecotypes can provide insight into the factors that produce mosaic landscapes (Bowman et al., 2023).

Boundary analysis involves the assessment of whether significant geographic boundaries are present (Jacquez, 2010; Tarroso et al., 2014) and whether the boundaries of multiple variables are spatially correlated (Jacquez et al., 2000). Such analysis includes two categories of statistical test: boundary statistics (i.e., tests for the presence of cohesive boundaries) and boundary overlap statistics (i.e. tests for spatial association between boundaries). BoundaryStats runs two boundary statistics tests and three boundary overlap statistics, as initially described in Jacquez 1995 and Fortin et al. 1996. The boundary statistics are (1) the length of the longest boundary and (2) the number of cohesive boundaries on the landscape (Fortin et al., 1996). The boundary overlap statistics are (1) the amount of direct overlap between boundaries in variables A and B, (2) the mean minimum distance between boundaries in A and B, and (3) the mean minimum distance from boundaries in A to boundaries in B (Fortin et al., 1996; Jacquez, 1995).

While other spatial statistics account for complications like spatial autocorrelation and environmental heterogeneity (Wagner & Fortin, 2005), boundary analysis can uniquely leverage geographic discontinuities to answer spatial questions. By identifying significant cohesive boundaries, researchers can delineate relevant geographic sampling units (e.g., populations as conservation units for a species or human communities with increased disease risk) (Jacquez, 2010). Associations between the spatial boundaries of two variables can be useful in assessing the extent to which an underlying landscape variable drives the spatial distribution of a dependent variable. For example, ecologists are often interested in whether landscape-level ecological boundaries limit gene flow, thereby producing population structure; if the putative ecological boundary is limiting gene flow, one would expect concordant geographic boundaries in the landscape variable and population structure (Tarroso et al., 2014; Wagner & Fortin, 2013). Landscape boundaries can similarly limit the distribution of taxonomically similar species (Polakowska et al., 2012). In an epidemiological context, this may look like neighborhood effects on public health outcomes, including COVID-19 infection risk (Hong et al., 2021; Van Ham et al., 2012) or spatial relationships between high pollutant density and increased disease risk (Adimalla et al., 2020; Waller et al., 1992).

Currently, there is at least one tool that has implemented boundary overlap statistics: GEM, which was released as an extension of ESRI ArcView and a standalone Windows package (Jacquez et al., 2000). GEM is not available as a cross-platform, free, and open-source software, thereby limiting its accessibility to researchers. R is a common, well-supported, and cross-platform language for statistical analysis. BoundaryStats implements boundary and boundary overlap statistics in R. It is available to download on the Comprehensive R Archive Network (CRAN), making the tools more accessible for researchers, especially in epidemiology and spatial ecology.

Boundary definitions

In this framework, we classify raster cells into a pseudobinary: boundary elements (1), non-boundary cells (0), or missing data (NA). For categorical variables, the algorithm for identifying boundary elements is simple: if any of a cell's neighbors belongs to a different category, the cell classified as a boundary element. For quantitative variables, cells with the highest boundary intensity values, with a threshold set by the user, are classified as boundary elements. Boundaries are defined here as subgraphs of boundary elements, or contiguous cells that are all marked as boundary elements (**Figure 2.1**). For the purpose of defining subgraphs, cells are considered neighbors using the queen criterion (i.e., eight neighboring cells, including diagonally touching cells).

Boundary intensities for landscape-level variables can be calculated in a number of ways, including lattice- and triangulation-wombling (Fortin et al., 1996; Jacquez, 1995; St-Louis et al., 2004; Strydom & Poisot, 2023), fuzzy set modeling (Jacquez, 1995), Monmonier's algorithm (Manni et al., 2004), spatial Bayesian clustering (Caye et al., 2016; Safner et al., 2011), agglomeration of inner lines (Wei & Larsen, 2019), and removal of outer lines (Wei & Larsen, 2019). For quantitative variables, BoundaryStats will accept raster objects with the spatial variable directly or boundary values calculated from these or other methods. If given boundary intensity values, boundary elements will be classified directly using the top percent of values. The default proportion of values is 0.2, though this threshold can be changed by the user. When given the variable directly, BoundaryStats will use the Sobel-Feldman operator to calculate the boundary intensity.

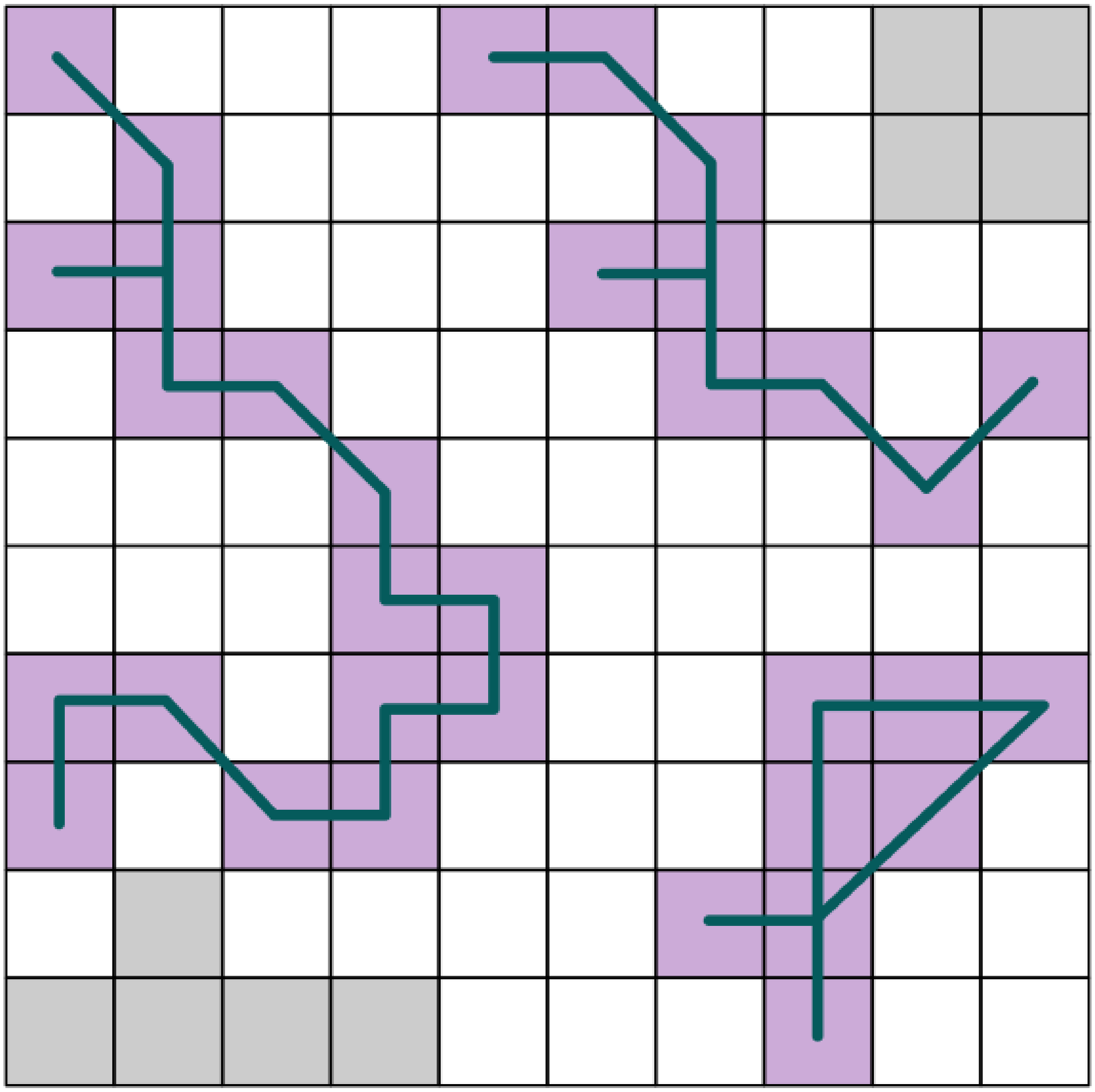


Figure 2.1. Example boundary subgraphs. Gray cells are missing values, white cells are non-boundary cells, and purple cells are boundary elements. Subgraphs are each represented using a line connecting all the boundary element cells that comprise them.

In accepting either the variables or boundary intensities, there is flexibility for users to define boundaries using relevant metrics for their data.

The Sobel-Feldman operator is commonly used for edge detection in computer vision applications. It approximates the magnitude of the partial derivative (i.e., rate of change) across each cell using the following kernels:

$$G_x = \begin{matrix} 1 & 0 & -1 \\ 2 & 0 & -2 \\ 1 & 0 & -1 \end{matrix} * A \qquad \text{and} \qquad G_y = \begin{matrix} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{matrix} * A$$

where A is the input raster cell, and G_x and G_y are the rates of variable change in the horizontal or vertical directions, respectively. The boundary intensity value is the overall rate of change:

$$G = \sqrt{{G_x}^2 + {G_y}^2}$$

Statistics

BoundaryStats runs two boundary statistics and three boundary overlap statistics, as initially described in Jacquez 1995 and Fortin et al. 1996. Below, I describe these five statistics:

Number of subgraphs

The first boundary statistic is the number of subgraphs. The number of subgraphs describes the number of boundaries on the landscape for a variable. In a raster of boundary elements, it is the number of unique subgraphs (three subgraphs each in **Figure 2.2a** and **Figure 2.2b**).

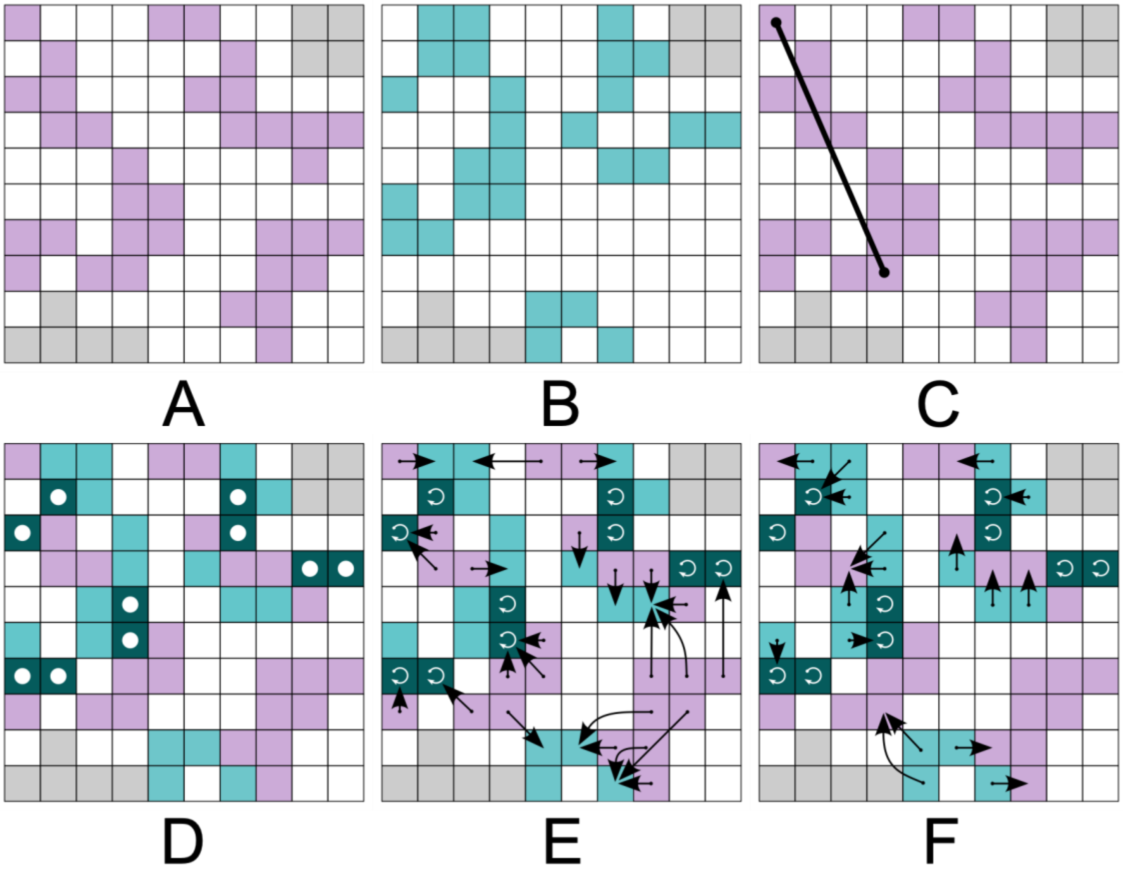


Figure 2.2. Example boundaries and statistics. (A) and (B) are boundary elements for hypothetical variables A and B. White cells are non-boundaries, gray are missing values, and purple or teal are boundary elements. (C) Length of the longest subgraph. (D) Produced by overlapping cells in A and B. Dark blue cells, highlighted by white dots, are where the boundary elements overlap. (E) For every boundary element for variable A, the nearest boundary element for variable B. Circular arrows indicate distance to self. (F) For every boundary element for B, the nearest boundary element for A.

Longest subgraph

The other boundary statistic included here is the length (in meters) of the longest subgraph, or boundary. The function calculates the longest length across each subgraph, then converts the length to distance based on the cell resolution and the projection of the raster. The length of the longest subgraph is then retained (**Figure 2.2c**).

Direct overlap

The direct overlap statistic, O_d, is a count of the number of overlapping boundary elements of two variables (**Figure 2.2d**).

Mean minimum distance between boundaries

This statistic describes the spatial proximity between boundaries of variables x and y, as defined by the mean distance to the nearest boundary element of the other variable. Spatial relationships between boundaries may not result in direct overlap, so this statistic accounts for potential correlations in non-overlapping boundaries. For each boundary element in variable x, the function calculates the distance to the nearest boundary element in variable y, then repeats the inverse for each boundary element in variable y (**Figure 2.2e** and **Figure 2.2f**). It then takes the mean of these minimum distances across boundary elements in x and y:

$$O_{xy} = \frac{\sum_{i=1}^{N_x} \min(d_i) + \sum_{j=1}^{N_y} \min\left(d_j\right)}{N_x + N_y}$$

where *i* and *j* are boundary elements for variables *x* and *y*, respectively; *min(d)* is the minimum distance between a boundary element for one variable to a boundary element in the other variable; and *N* is the number of boundary elements for the variable.

Mean minimum distance from boundary x to boundary y

This statistic describes the mean distance from boundary elements in x to the nearest boundary element in y. It is an indicator for whether the boundaries in variable x depend on variable y. The reciprocal nature of the previous statistic implies some reciprocity of effect, as opposed to the unidirectionality implicit here. For each boundary element in the raster for x, the function calculates the distance to the nearest boundary element of variable y, then takes the mean across all boundary elements in x (**Figure 2.2e**):

$$O_x = \frac{\sum_{i=1}^{N_x} \min(d_i)}{N_x}$$

Neutral models

In addition to calculating each statistic, BoundaryStats uses iterations of a neutral landscape model to determine whether the boundaries in the input landscape differ from a random landscape. Users select a neutral landscape model and number of iterations of that model to produce a null distribution of each statistic, based on the selected model and the structure of the input landscape. BoundaryStats implements three neutral landscape models: stochastic landscapes, Gaussian random fields, and modified random clusters.

The simplest neutral landscape model is complete stochasticity. It takes all the cell values from the input raster and assigns each value to a random cell. Each cell in the simulated raster is assigned a value from the original dataset, with no replacement of values. It will ignore cells with missing data (e.g., for terrestrial data along a coastline, it will not draw from or assign new values to ocean cells). The simulated raster has the exact same values as the original raster, but values are randomly placed with no spatial autocorrelation.

The next neutral landscape model simulates a Gaussian random field with the same degree of spatial autocorrelation as the input raster. It is suited for continuous or discrete quantitative variables. This method calculates the local Moran's I across the original raster and builds local indication of spatial association (LISA) clusters (Anselin, 1995). Each cluster circumscribes an area with significant local spatial autocorrelation. The maximum distances across each LISA cluster are extracted, then the median is taken across clusters; the resulting value represents the average range of significant spatial autocorrelation. The function then simulates a Gaussian random field with the same range of spatial autocorrelation, extent, and resolution as the input raster. If the spatial autocorrelation range is too large for the spatial extent, this parameter is reduced by 10% and another Gaussian random field is attempted; this step is repeated until a simulated raster is successfully produced. If there are missing data cells in the original landscape (e.g., a coastline or lake is present, but the data are terrestrial), the corresponding cells in the simulated raster will be set as missing data (NA).

The modified random cluster model is an implementation of the method described by Saura and Martínez-Millan for simulating neutral landscapes for categorical variables (Saura & Martınez-Millan, 2000). The first step is a percolated raster (**Figure 2.3a**). Each cell is assigned a value $0 \leq x \leq 1$ from a uniform distribution, and cells with values above a threshold probability p are marked. p is defined by the user, and higher values of p result in larger cluster sizes in the final simulated raster. Next, contiguous sets of marked cells are grouped into clusters, using the rook criterion (i.e., neighbors are the four edge-touching neighbors) (**Figure 2.3b**). Clusters are then assigned a category (**Figure 2.3c**). Categories from the input raster are chosen one at a time, and random clusters are assigned to that category. When the proportion of that category in the simulated raster reaches the proportion in the original raster, clusters are then assigned to the next category, until all the clusters are assigned. In the last step, the unmarked cells are categorized based on the most frequent category among their neighbors using the queen criterion (**Figure 2.3d**). If there is a tie between two categories, one of the tied categories is picked at random. If all neighbors are unassigned, a random category is picked; probabilities for each category are based on their proportions in the input raster. The simulated raster is then cropped, so that the simulated raster has missing data in the same areas as the input raster.

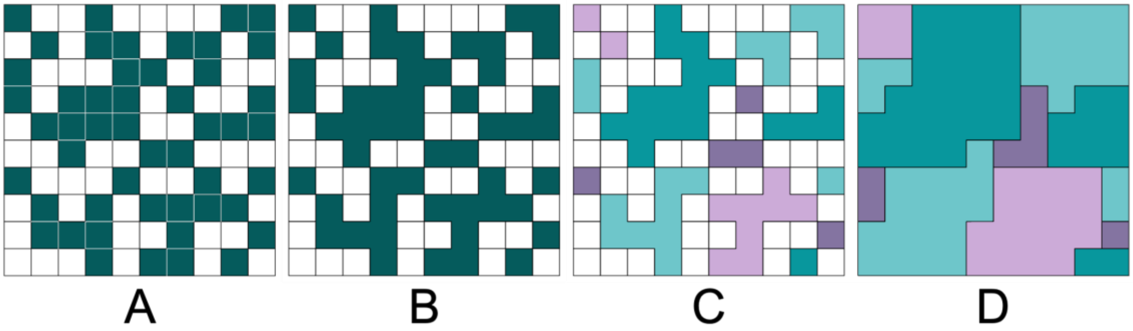


Figure 2.3. Modified random cluster procedure, adapted from Saura and Martínez-Millan 2000. (A) Percolated raster with p = 0.5. (B) Marked cells merged into cluster values estimated using GeoOrigins (Hulme-Beaman et al., 2020), based on the acoustic dissimilarity and spatial relationship between recorded songs. The genetic data are spatially interpolated admixture coefficients. The admixture coefficients of samples were estimated in fastSTRUCTURE (Raj et al., 2014) and interpolated using local kriging.

Implementation and example

Data in this example are from Luo et al. (2024), in which the authors hypothesized that song divergence is facilitating genetic divergence in white-crowned sparrows through speciation by sexual selection. The data below are song boundaries and genetic admixture interpolations from the study. The song data are boundary intensity values estimated using GeoOrigins (Hulme-Beaman et al., 2020), based on the acoustic dissimilarity and spatial relationship between recorded songs. The genetic data are spatially interpolated admixture coefficients. The admixture coefficients of samples were estimated in fastSTRUCTURE (Raj et al., 2014) and interpolated using local kriging.

Read in data

Read in raster data to terra (Hijmans, 2023) SpatRaster objects (**Figure 2.4**). The two objects need the same projection, extent, and resolution.

```
> library(terra)
> library(magrittr)

> songs <- rast('2010_2022_song_boundaries.asc')
> genetic <- rast('genetic_interpolation.asc') %>%
  resample(., songs)
> songs <- crop(songs, genetic) %>%
  mask(., genetic)
```

Calculate spatial boundaries for variables

If the variable is categorical, use the *categorical_boundary* function. For continuous variables, use the *define_boundary*, which applies a proportion threshold for the highest boundary intensity values (default = 0.2). Boundary intensity can be calculated however the user chooses; if the input raster for a continuous variable already contains boundary intensities, the argument *convert* in *define_boundaries* should be set to FALSE (default). For a raster with the variable, one can set *convert* to TRUE to use the

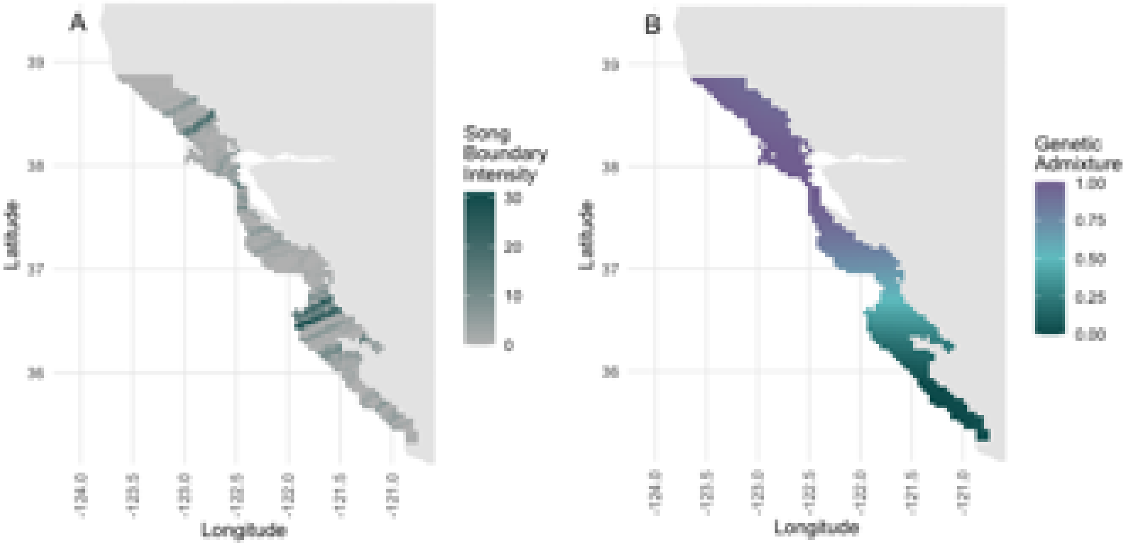


Figure 2.4. Maps of (A) song boundary intensity and (B) genetic admixture between two populations.

Sobel-Feldman operator to calculate boundary intensities.

Both variables in this example are continuous, so I will use *define_boundary*. The song raster already contains boundary intensity values, from which boundary elements can be directly determined, so I can use the default of FALSE for *convert*. But the genetic data are spatially interpolated from the admixture coefficients of sampled white-crowned sparrows, so boundary intensity needs to be calculated from this variable (*convert* = TRUE).

```
> library(BoundaryStats)
> song_boundaries <- define_boundary(songs, threshold = 0.1)
> genetic_boundaries <- define_boundary(genetic, threshold, = 0.1, convert = TRUE)
```

Plot boundary overlap

This optional step is to visualize where the boundaries of the two variables are overlapping using *plot_boundary* (**Figure 2.5**). The function is a wrapper function for ggplot (Wickham, 2016), and the colors and trait names can optionally be customized.

Create null distributions for statistics

For both boundary statistics, use *boundary_null_distrib*. For the three overlap statistics, use *overlap_null_distrib*. Both functions simulate random iterations of a raster based on the specified neutral landscape model and input data. Statistics are calculated for each iteration. Custom null probability distributions are calculated based on the iterations.

The functions take the SpatRaster object(s) containing the landscape variable, a neutral landscape model, whether the variable is categorical, and the number of iterations. For *overlap_null_distrib*, separate models can be specified for the two variables, and the first raster is assumed to depend on the second raster. The argument *rand_both* specifies whether y should be modeled in each iteration; since some hypotheses assume variable x depends on an underlying distribution of boundaries in variable y, users can choose to

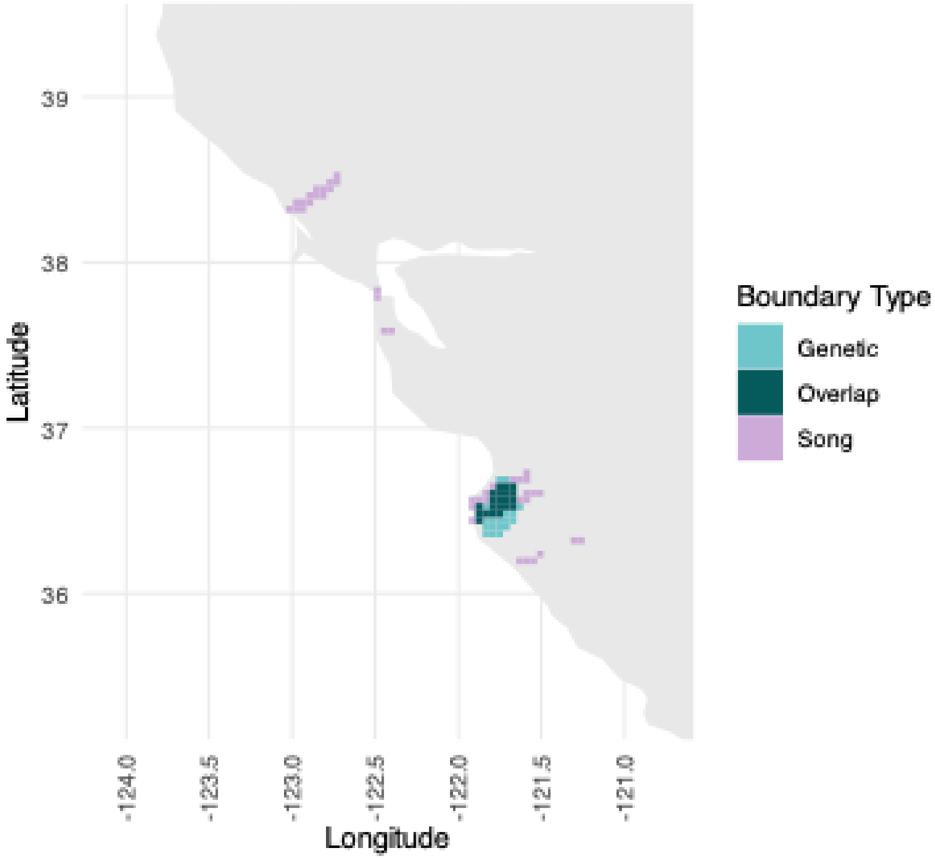


Figure 2.5. Output of the *plot_boundary* function.

keep boundaries in y static for each iteration. For this example, the genetic boundary is hypothesized to depend on song boundaries. Therefore, the SpatRaster object containing the genetic admixture interpolation is the first argument, and I keep variable y static (*rand_both* = FALSE).

```
> song_boundary_null <- boundary_null_distrib(songs, convert = FALSE, cat = FALSE,
n_iterations = 1000, threshold = 0.1, model = 'random')
> genetic_boundary_null <- boundary_null_distrib(genetic, convert = TRUE, cat =
FALSE, n_iterations = 1000, threshold = 0.1, model = 'gaussian')
> boundary_overlap_null <- overlap_null_distrib(genetic, songs, rand_both = FALSE,
n_iterations = 1000, x_convert = TRUE, threshold = 0.1, x_model = 'gaussian')
```

<u>Run statistical tests</u>

Both boundary statistics functions require only the raster with boundary elements for the variable and the matching null distribution object, produced by *boundary_null_distrib*

```
> n_subgraph(song_boundaries, song_boundary_null)
 n subgraphs            p-value
6.0000000               0.006404757
> max_subgraph(song_boundaries, song_boundary_null)
length of longest boundary          p-value
0.4951836                           0.0000000
> n_subgraph(genetic_boundaries, genetic_boundary_null)
n subgraphs    p-value
1              1.739e-07
> max_subgraph(genetic_boundaries, genetic_boundary_null)
length of longest boundary          p-value
0.40399140                          0.03884196
```

The boundary overlap statistics similarly takes the boundary element raster and null distribution as arguments. In this case, it requires two boundary element SpatRaster objects, one for each variable. The order of the variables should match that used in *overlap_null_distrib*. In this case, genetic boundaries first, then song boundaries.

```
> Odirect(genetic_boundaries, song_boundaries, boundary_overlap_null)
n overlapping boundary elements              p-value
21                                           0
> Ox(genetic_boundaries, song_boundaries, boundary_overlap_null)
average minimum distance (x depends on y)          p-value
3.053541e+03                                       1.706287e-05
> Oxy(genetic_boundaries, song_boundaries, boundary_overlap_null)
average minimum distance                p-value
4.083163e+04                            5.529067e-02
```

Interpretation of example data output

When analyzing the data from Luo et al. (2024), the boundary statistics for both genetic and song data were significant. These results suggest the presence of a cohesive genetic boundary and cohesive song boundaries. Results from the boundary overlap statistics show significant direct overlap between the genetic and song boundaries and spatial proximity from the genetic boundary to a putative song boundary, while the reciprocal minimum distance between genetic and song boundaries is not significant. These suggest that the relationship between genetic and song boundaries is unidirectional (i.e., the genetic boundaries are dependent on song boundaries). While boundary overlap statistics can only demonstrate a correlation between boundaries, the results support the hypothesis that song boundaries are facilitating a coincident genetic boundary.

Summary

BoundaryStats implements five boundary and boundary overlap statistics that can be used for boundary analysis. Boundary analyses can be used across many contexts that make use of spatially distributed data. For example, spatial ecologists and epidemiologists can use boundary overlap statistics to assess whether environmental variables are influencing the distribution of organismal traits or disease occurrences. Environmental influences can, in some cases, be detected through the co-occurrence and coincidence of geographic boundaries; environmental boundaries may produce boundaries in the variables of interest. As such, this new open-source, cross-platform implementation will make boundary statistical methods more widely accessible to researchers.

CHAPTER THREE

SONG AND GENETIC CLINES IN A SECONDARY CONTACT ZONE BETWEEN TWO WHITE-CROWNED SPARROW (*ZONOTRICHIA LEUCOPHRYS*) SUBSPECIES

Publication disclosure: This chapter will be submitted to Molecular Ecology and Evolution for publication. I am first author and corresponding author. I conceptualized the study and chose the methodology in conjunction my advisor, conducted the analyses, and wrote and edited the manuscript. Coauthors: Sara Lipshutz (data curation, funding acquisition, editing of manuscript), Jenny Phillips (data curation, editing of manuscript), Robb Brumfield (funding acquisition, editing of manuscript), and Liz Derryberry (conceptualization, funding acquisition, methodology, supervision, editing of manuscript).

Abstract

Bird songs can act as reproductive barriers within species, reducing gene flow and potentially promoting speciation through sexual selection. In this study, we examine the role of song divergence in a secondary contact zone between two subspecies of white-crowned sparrows (*Zonotrichia leucophrys nuttalli* and *Z. l. pugetensis*). We aim to determine (1) whether the secondary contact zone functions as a tension zone, (2) which divergent song traits are potential reproductive barrier in the zone, and (3) the extent to which cultural transmission of song affects the role of song as a reproductive barrier. Using clinal analyses of genetic, song, and morphological data, we explore the structure of the secondary contact zone. The hybrid index cline, which reflects the degree of genetic admixture between subspecies, is significantly narrower than expected under neutral diffusion, suggesting that this secondary contact zone is a tension zone. Morphological traits, while differing between subspecies, were not clinal. They instead exhibited a pattern of isolation by distance. Instead, we found that the introductory whistle of the song forms a cline that is both coincident and concordant with the hybrid index cline. Thus, it may act as a pre-zygotic reproductive barrier that is maintaining the subspecies boundary at this tension zone. The interaction of song divergence and a possible migratory divide may be working together to reinforce reproductive isolation in this system.

Introduction

Acoustic mating signals play a role in maintaining species boundaries by acting as pre-zygotic reproductive barriers, even when hybrids are viable (Price & Bouvier, 2002). Given their power in maintaining species boundaries, divergence in acoustic mating signals between populations can reduce gene flow through assortative mating. Between species, females respond preferentially to conspecific signals, thereby reducing the rate of heterospecific mating (blue-winged and golden-winged warblers, Gill & Murray, 1972; Bermuda white-eyed vireos, Mejías et al., 2021; Hawaiian crickets, Shaw & Lesnick, 2009; reviewed in Wilkins et al., 2013). Within some species, individuals respond more strongly to local than to foreign conspecific mating signals (Baker, 1983; Boul et al., 2007; Podos, 2007). Such intraspecific discrimination between signals suggests the potential for assortative mating, which could reduce intraspecific gene flow. However, assortative mating—and sexual selection more broadly—does not consistently promote intraspecific population divergence (Kopp et al., 2018; Maan & Seehausen, 2011; Servedio & Boughman, 2017). For instance, divergent sexual selection promotes population divergence when preferences represent local adaptations, but phenotypic matching based on a trait that is unimodal in a population hinders population divergence (Servedio & Boughman, 2017). Understanding how and when acoustic signals reduce gene flow is key to uncovering their role in the speciation process, particularly within hybrid zones, where barriers to gene flow are tested.

The ontogeny of acoustic mating signals may affect the rate at which signals divergence and become reproductive isolators. Learned acoustic mating signals—such as bird song—have the potential to diversify rapidly and accelerate population divergence (Edwards et al., 2005; Mason et al., 2017; Verzijden et al., 2012). On the other hand, if individuals from different populations can learn the same signals, then learning can erode signal differences or population structure between populations in contact (Kenyon et al., 2011; Love & Goller, 2021; Qvarnström et al., 2006). Consistent with these divergent possible outcomes, cultural differences in song are associated with population structure in some species (Keighley et al., 2019; Luo et al., 2024; Ribot et al., 2012), but completely disassociated in others (González & Ornelas, 2014; Kenyon et al., 2017; Poesel et al., 2017; Wright & Wilkinson, 2001). Given the mixed evidence for learned signals in the maintenance of reproductive barriers, it is important to examine the specific conditions under which learned songs promote or hinder population divergence.

The genetic heritability of song traits may determine their efficacy as reproductive barriers. Even in learned songs, some song traits are innate while others are less so, though both genetic encoding and cultural transmission contribute to each trait (Araki et al., 2016; Love et al., 2019; Soha & Marler, 2000a). The relative contributions of genetic heritability and cultural transmission for a given song trait may determine the degree to which that trait can reduce gene flow; a genetically heritable component of song can more readily respond to selection against hybridization. By investigating both genetic and learned components of song in hybrid zones, we can better understand the mechanisms that sustain reproductive isolation.

Hybrid zones are natural laboratories that offer an opportunity to assess how song divergence functions as a reproductive barrier, particularly in the context of gene flow and selection. Hybrid zones are geographic areas in which divergent taxa meet and interbreed, and the outcomes of interbreeding can highlight factors that either promote reproductive isolation or allow gene flow to occur (Hewitt, 1988; Uy et al., 2018). Under neutral diffusion (i.e., no barrier to gene flow), introgression widens the hybrid zone over time until genetic differentiation between taxa dissolves (Barton & Gale, 1993). In contrast, tension zones are hybrid zones in which parental genotypes disperse into hybrid zones, but selection against hybrids penalizes hybridization (Barton & Hewitt, 1985). Thus, genetic differentiation between taxa can be maintained at hybrid zones without the complete cessation of gene flow (P. R. Grant & Grant, 1997; Ottenburghs et al., 2017). Tension zones are characterized by stable clines (i.e., areas in which genotypes or phenotypes transition from one parental state to the other) that do not decay over time (Barton & Hewitt, 1985). In a stable tension zone, traits that share the center (coincidence) and width (concordance) of the genetic cline may be under selection and act as reproductive barriers between taxa (Baldassarre et al., 2014; Scordato et al., 2017; Walsh et al., 2016). Divergent song traits that are aligned with a genetic cline may be acting as a barrier to gene flow.

Song is often one of multiple traits that can reduce gene flow in tension zones (Uy et al., 2018). For example, migratory behaviors that differ between species or populations can also reduce gene flow; interspecific mating may be hindered through offset breeding seasons (Ruegg et al., 2012), or hybrids may be selected against due to intermediate migratory behaviors (Blain et al., 2024; Delmore & Irwin, 2014). Similarly, hybrid morphological traits can be selected against through sexual selection (e.g., male plumage coloration as sexual signals, Turbek et al., 2021) or natural selection (ecological speciation of morphometry along an elevational gradient, Caro et al., 2013). In such cases, migratory or morphological traits may reproductively isolate taxa at a tension zone, either without the influence of song divergence, or in conjunction with divergent songs.

In this study, we characterize the hybrid zone between Nuttall's (*Zonotrichia leucophrys nuttalli*) and Puget Sound (*Z. l. pugetensis*) white-crowned sparrows, exploring the role of song divergence in maintaining subspecies boundaries. The subspecies are in secondary contact, making their hybrid zone a secondary contact zone that may either dissolve or be maintained by selection against hybrids. We ask (1) whether the secondary contact zone is a tension zone, (2) whether song divergence is associated with this contact zone, (3) and to what degree learning affects the efficacy of song traits a reproductive barriers. We conduct clinal analyses using reduced genome sequencing, songs, and morphology to characterize this secondary contact zone. The genetic clines, including a hybrid index cline, will be used to establish the genetic basis of the hybrid zone. Using this genetic basis, we then compare the clines of song and morphological traits to assess whether they are associated with the hybrid zone. We hypothesize that sexual selection based on song divergence is maintaining a tension zone between subspecies. Based on the characteristics of a tension zone, we predict that the genetic basis of the secondary contact zone is represented by a steep cline, narrower than a cline produced without a barrier to gene flow. We further predict that song traits are clinal, and these clines are coincident and concordant with the genetic cline. If song traits associated with the genetic cline tend to be more culturally transmitted than innate, then culturally transmitted song differences could be a barrier to gene flow between the subspecies.

Methods

Study system

White-crowned sparrows are a songbird that inhabits large portions of North America. Three of the five subspecies have culturally inherited song dialects, or discrete song types that are each (1) sung by most males in an area and (2) transmitted over generations with great accuracy. *Z. l. nuttalli* and *Z. l. pugetensis* are two of the three subspecies that have song dialects. Both subspecies inhabit a thin strip of coastal scrub along the West Coast, and *Z. l. nuttalli* is sedentary, while *Z. l. pugetensis* is a short-distance migrant. *Z. l. nuttalli* is distributed from central to northern California, and from there *Z. l. pugetensis* extends northward to southern British Columbia. In the original delineation of the two subspecies, Grinnell (1928) placed the zone of intergradation along the coast of Mendocino County. Subsequent studies have integrated morphological (Banks, 1964), allozyme (Corbin, 1981) and behavioral variation (Baker, 1987; Blanchard, 1941; Lipshutz et al., 2017; Mewaldt et al., 1968) to characterize the hybrid zone. The successive lines of evidence largely corroborate one another, still placing the hybrid zone along Mendocino County.

Genetic sampling

We reanalyzed genetic samples of *Z. l. nuttalli*, *Z. l. pugetensis*, and hybrids used in Lipshutz et al. (2017) and Luo et al. (2024). A total of 273 samples were collected from 36 localities along the West Coast (**Figure 3.1a**). Of these, 215 were blood samples collected in 2004, 2005, and 2014, and the remaining samples were tissues obtained from vouchered museum specimens (LSU Museum of Natural Science, the University of California Berkeley Museum of Vertebrate Zoology, and the University of Washington Burke Museum of Natural History and Culture).

Genetic samples were sequenced using GBS at the Institute of Genomic Diversity at Cornell University in Ithaca, NY, USA. Libraries were prepared following Elshire et al. (2011), using the restriction enzyme PstI (CTGCAG) and a unique barcode for each sample on the plate. The first two GBS plates were sequenced together and originally analyzed in Lipshutz et al. (2017). A third plate was sequenced with the same methods and facilities, were analyzed together in Luo et al. (2024). In Luo et al. (2024), reads were demultiplexed using *process_radtags* in STACKS (Catchen et al., 2013). Samples with less than 500,000 reads were dropped before calling SNPs with STACKS *ref_map*. Individuals with greater than 80 percent missing data were removed, as were SNPs with less than five percent minor allele frequency or more than 15 percent missing data using vcftools (Danecek et al., 2011). For this study, we further filtered SNPs based on linkage disequilibrium using PLINK2.0 (Chang et al., 2015; Purcell & Chang, 2015), with a 500kb window size and 0.2 overlap. After filtering, we retained 251 individuals and 4283 loci.

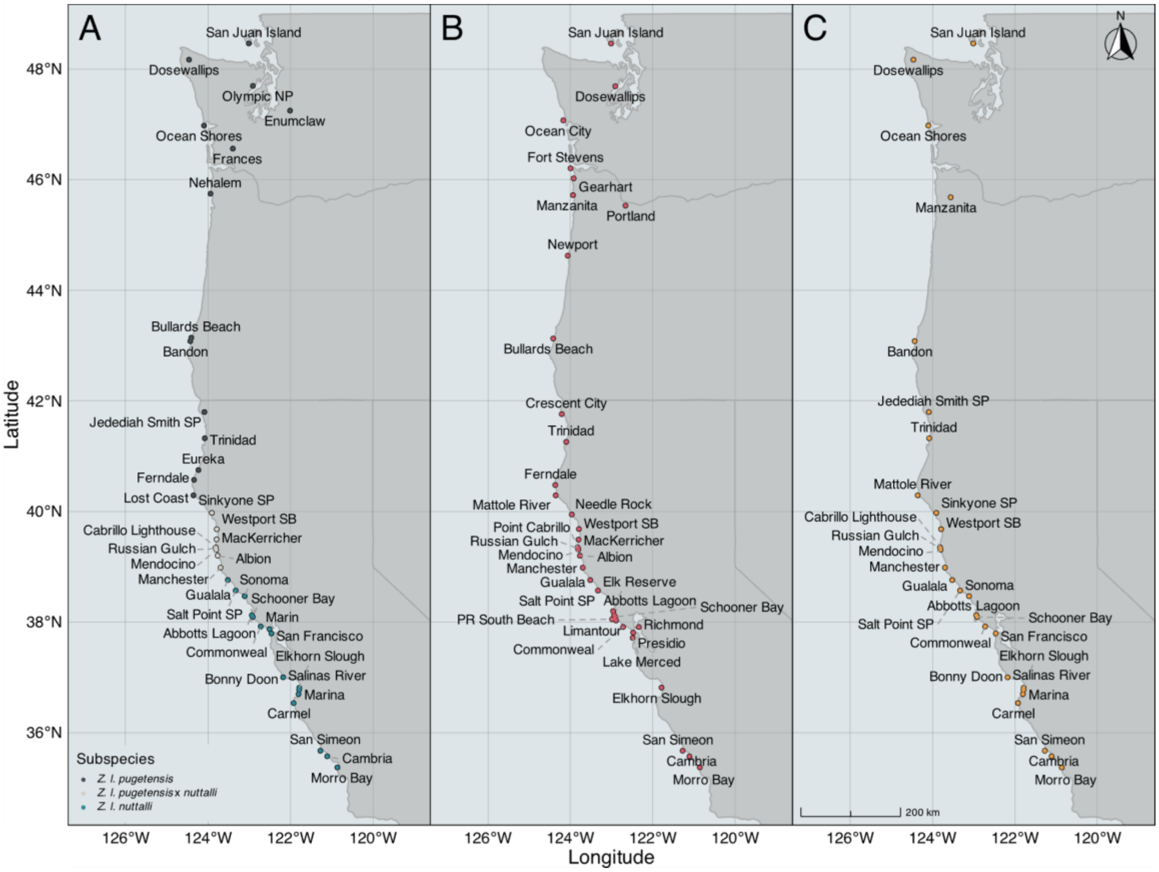


Figure 3.1. Sampling localities for genetic, song, and morphological data. (A) Genetic sampling, with localities colored based on subspecies or hybrid status. (B) Song sampling localities. (C) Morphological sampling localities.

Song sampling

We measured songs recorded along the coast from northern Washington State to Central California. Songs were recorded from 324 males, spanning 38 dialects, including a total of 1,227 songs from 2004 and 2023 (**Figure 3.1b**). For each song, we measured the length of the song, intro whistle, complex notes (mean duration), trill, and trill notes (mean duration); frequency bandwidth of the song, complex notes, and trill; minimum and maximum frequencies of the song, complex notes, and trill; trill rate; dominant frequency of whistle; entropy of the song; and peak frequency (**Figure 3.2).** Some males were represented by multiple songs, so we took the mean trait values for each male. Songs were segmented using Chipper (Searfoss et al., 2020) and syllable types were annotated using a custom GUI. Song traits were analyzed using warbleR (Araya-Salas & Smith-Vidaurre, 2017), which allowed for consistent trait measurement across the dataset.

To ensure that temporal variation in songs did not influence our results, we compared whistle lengths from historical recordings (1970—1971) by Luis Baptista with contemporary recordings (2004—2023). If the song traits are consistent between the two datasets, then this would suggest that whistle lengths have not changed significantly enough to explain our findings.

Morphological sampling

We used morphological data from birds that were banded in 2004, 2005, 2010, and 2014 (**Figure 3.1c**). 340 birds were measured, but not all measurements were taken for all birds. The mass, wing chord, bill depth, bill width, and bill length were measured.

Geographic clines

We used the R package HZAR (Derryberry et al., 2014) to build geographic clines for the genetic data, song traits, and morphological traits. To represent the genetic basis of the hybrid zone, we built SNP frequency clines and a hybrid index cline. Clines were built based on geographic distance from San Juan Island (SJI). While some localities were not represented in all three datasets, SJI was the northernmost locality in

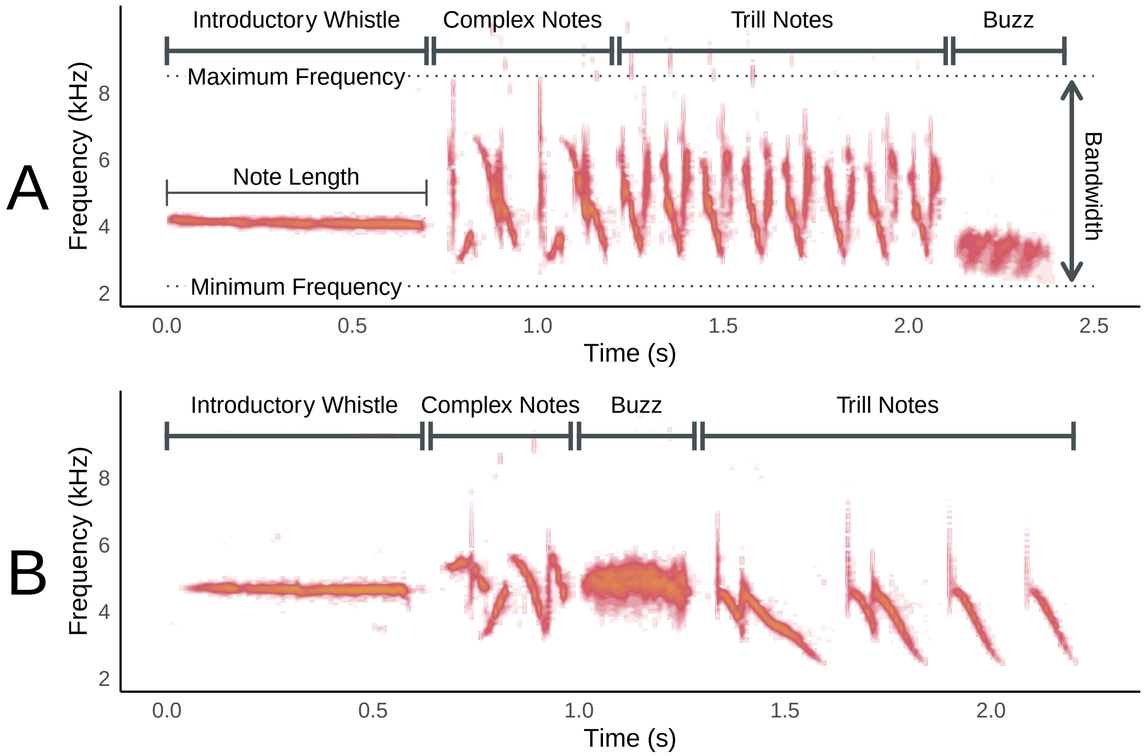


Figure 3.2. Song traits measured from songs. Figure shows dialect from (A) Abbotts Lagoon, California, and (B) Fort Stevens State Park, Oregon.

all three datasets. We calculated Euclidean distances to SJI using the *distm* function from the R package geosphere, then converted the distances from meters to kilometers (Hijmans, 2022).

For the SNP frequency clines, we calculated the allele frequency for each locus at each sampling locality using PLINK2.0. If any of the alleles had clines that increased in frequency from north to south, we switched the allele used in HZAR, so that all the loci were represented by alleles that decreased in frequency, moving southward from SJI. For each locus, we fit frequency cline models using all five tail fitting options all three scaling options, along with a null model (i.e., 16 total models per locus).

We used fastSTRUCTURE (Raj et al., 2014) to estimate Q values, which represent a hybrid index for clinal analysis. The hybrid index cline represents the amount of admixture across the sampling range and is therefore the genetic basis of the secondary contact zone. Luo et al. (2024) found that the optimal number of clusters across these subspecies is two, so we ran 10 iterations of fastSTRUCTURE with k = 2, then averaged the hybrid indices across the 10 iterations. We fit phenotypic clinal models using all five tail fitting options and a null model, for a total of six models. HZAR does not have a default null model for phenotypic clines, so we used a null model in which each locality's mean and variance was equal to the overall mean and variance.

Next, we fit clines to song and morphological traits, using the same phenotypic clinal models as the hybrid index cline. If a locality had no measurements of a trait, it was dropped from the clinal models for that trait. We also built clinal models for song and morphological principal components (PCs). We ran two principal components analyses (PCA): one with the song traits and one with the morphological traits. PCs were retained based on the broken stick method, implemented in the *bsDimension* function from the PCDimension package (Coombes & Wang, 2022).

For each cline, models were compared using the AICc, and the model with the lowest AICc was chosen independently for each cline. If the null model was the best model (i.e., the trait was non-clinal), the SNP or trait was dropped from further analysis. Since previous work suggests body mass may be clinal, we also ran the clinal analysis for body mass (Mewaldt et al., 1968). For the retained traits, we ran three independent chains of the best model, using default MCMC parameters (10,000 burn-in and 1,000,000 MCMC iterations) and randomized parameter seeds. We then checked the chains for convergence of cline center and width values.

Of all the song traits, only the length of the introductory whistle was clinal, but the songs were recorded over two decades (2004 to 2023). We therefore assessed whether the clinal pattern was produced by the temporal spread or timing of the recordings by comparing them to recordings at the same localities from 1970-1971. Fewer localities are represented in the historical songs, but we used historical songs to confirm that whistle lengths are geographically stable over time.

Cline comparisons

We then assessed the coincidence and concordance of clines of individual SNPs and the introductory whistle length to the hybrid index cline (i.e., the Q-value cline). We calculated the AICc of the best clinal model for the hybrid index after the MCMC chains converged, then calculated the AICc of a constrained form of the model. In the constrained form, either the center or width of the hybrid index cline was constrained to the center or width of the cline being compared (e.g., the hybrid index clinal model constrained to the center of the whistle length cline, to test whether the two clines are concordant). If the difference in AICc between the constrained and unconstrained models was greater than 2, the two clines are either non-coincident or discordant.

Similarly, we compared SNP clines to one another by comparing the AICc of each SNP cline to a constrained model with the center or width of another SNP cline. In this manner, we produced a matrix of pairwise comparisons of coincidence and concordance. These comparison matrices are not symmetrical across the diagonal line, as in some cases, the constrained model for one SNP has a $\Delta\text{AIC} > 2$, but the reciprocal constrained model does not. For example, cline A constrained to the center of cline B may be significantly different from cline A, but cline B constrained to the center of cline A may not be significantly different from cline B.

Neutral diffusion

If whistle length is acting as a reproductive barrier between subspecies, we would predict that gene flow would be relatively restricted with a narrower geographic cline, when compared to gene flow under neutral diffusion. Under neutral diffusion, the width of the cline (w) is predicted to be:

$$w = 2.51\sigma\sqrt{t}$$

with σ being the standard deviation of natal dispersal distance and t being the number of generations since contact (Barton & Gale, 1993). The model assumes that the tails are fixed, which is satisfied by the hybrid index cline.

Individuals begin mating in their second year (i.e., the breeding season following their hatch year), so we use a generation length of one year. The two subspecies have been in secondary contact for about 2,000 years (Lipshutz et al., 2017). We therefore assume that approximately 2,000 generations have occurred since the formation of this hybrid zone.

We used both conservative and lenient estimates for natal dispersal, with larger a variance in dispersal distance producing a wider cline under the neutral diffusion model. Natal dispersal represents the lifetime dispersal of white-crowned sparrows, as males disperse after their hatch year and generally defend the same territory or a nearby territory each breeding season (Petrinovich & Patterson, 1982). *Z. l. oriantha*, a long-distance migratory subspecies, has longer natal dispersal distances, relative to the sedentary *Z. l. nuttalli* (Morton, 1992). While the natal dispersal of *Z. l. pugetensis* has not been estimated to our knowledge, we expect that they are also greater than *Z. l. nuttalli*. To account for the likely difference in natal dispersal, we calculated the clinal width under neutral diffusion using estimates of natal dispersal from both *Z. l. oriantha* (Morton, 1992) and *Z. l. nuttalli* (Baker & Mewaldt, 1978).

We then tested whether the width of the hybrid index cline is narrower than the width predicted under neutral diffusion. As with the comparison of the overall genetic and whistle length clines, we compared the AICc of the best clinal model and a constrained model. In this case, we constrained the width of the cline to the predicted widths of the neutral diffusion model.

Isolation by distance

For the genetic data, morphological traits, and non-clinal song traits, we tested for isolation by distance using Mantel tests. To test for genetic isolation-by-distance (IBD), we first calculated pairwise Fst between all localities base using the SNPs and the R package hierfstat (Goudet, 2005). We then ran a Mantel test (*gl.ibd*) in the dartR package (Mijangos et al., 2022). For each non-clinal song trait and morphological trait, we calculated a matrix of pairwise Euclidean distances between the localities using the *dist* function in R. We took the locality-wide average of each trait to calculate inter-locality trait distances. If there were no measurements of a trait at a given locality (e.g., the local song dialect has no complex notes), we dropped that locality from the distance matrix. We then ran a Mantel test on each trait using the *mantel* function in the vegan package (Oksanen et al., 2022).

Results

Clines

We characterized the basis of the hybrid zone using genetic data. We found that 12 SNPs were clinal, and the best model varied across SNPs. None of the SNPs were fixed to alternative alleles on both sides, but all were fixed on one side. While the center and width of the SNP clines varied, the average center was 955 km from SJI, and the average width was 838 km (**Figure 3.3a**). We used fastrSTRUCTURE to calculate a hybrid index based on two populations, as previous works has found support for two genetic groups (Lipshutz et al., 2017; Luo et al., 2024). We found that the hybrid index was clinal, and the best model had mirrored exponential tail estimates. The hybrid index cline was centered 992 km south of SJI and had a width of 39 km (**Figure 3.3b**).

For the song traits, only the length of the introductory whistle was clinal, and the model with the lowest AICc had a left exponential tail. The center of the whistle length cline was at 996 km from SJI, and the width was 44 km (**Figure 3.4**). Songs recorded by

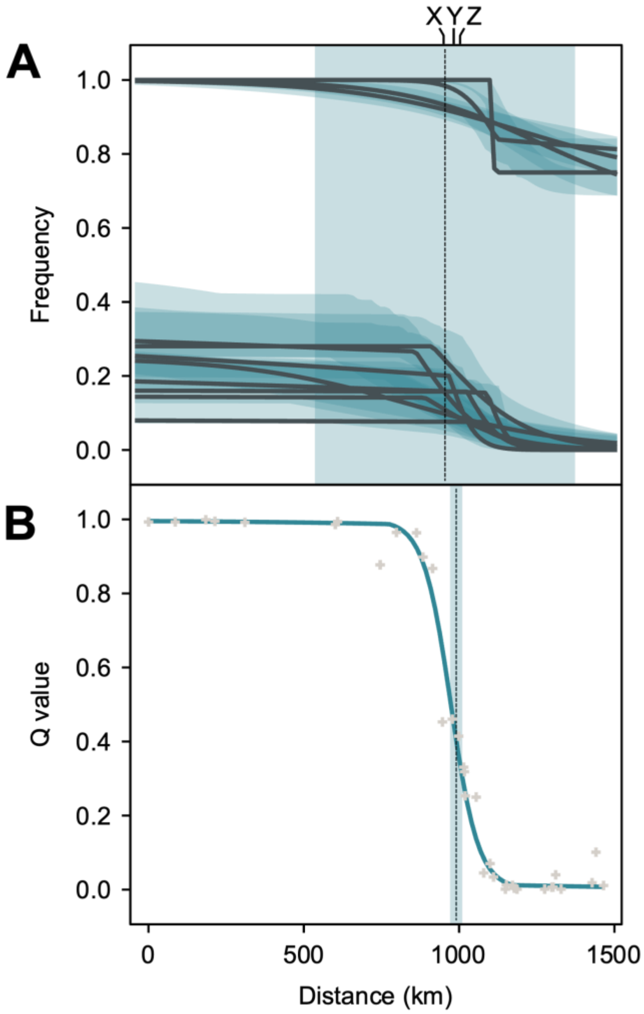


Figure 3.3. Genetic clines. (a) Individual SNP clines, with confidence intervals in light blue around the clines. The vertical line indicates the average center across all SNP clines, and the blue range surrounding it is the average width. (b) Hybrid index cline. Each cross shows the average hybrid index for individuals at a locality. The vertical line indicates the center of the cline, and the width is indicated by the blue range surrounding the center. Distance from San Juan Island is shown on the x-axis, and the three localities nearest the centers are indicated with letters at the top. X = Sinkyone State Park (946 km), Y = Westport State Beach (978 km), and Z = MacKerricher State Park (998 km).

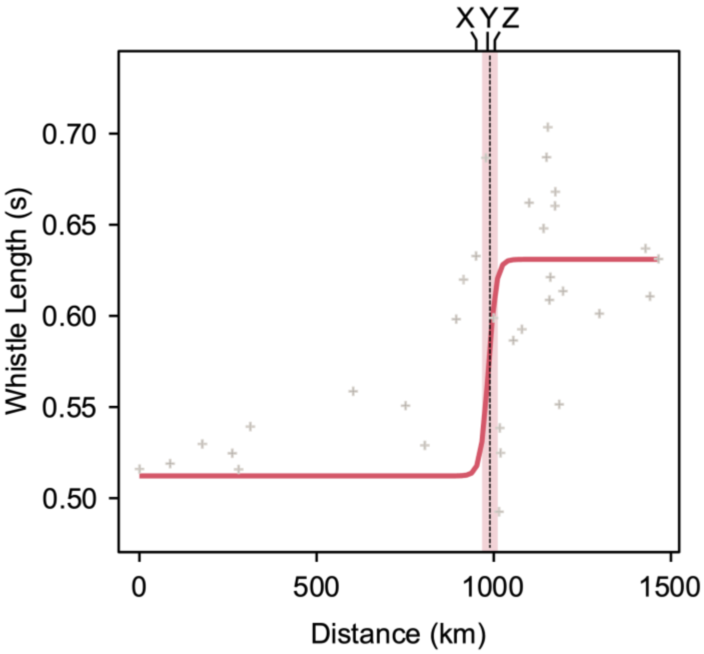


Figure 3.4. Trait clines. Each cross point represents the average whistle length at the locality. Distance from San Juan Island is shown on the x-axis, and the three localities nearest the clinal center are indicated with letters at the top. X = Sinkyone State Park (946 km); the closest song locality is Needle Rock (949 km). Y = Westport State Beach (978 km). Z = MacKerricher State Park (998 km). The vertical lines indicate the center of the cline, and the colored band surrounding it indicates the cline width.

Baptista from 1970 to 1971 represent fewer localities, and we did not recover a clinal pattern with the historical songs. But whistle lengths in the historical dataset are consistent with the contemporary recordings, with a similar increase in length southward from SJI (**Figure A-8**). Only the first song PC was significant, based on the broken stick method, and it was not clinal.

None of the morphological traits were clinal based on AICc comparisons (i.e., the null model had the lowest AICc for all traits). We retained the first two morphological PCs, but neither were clinal.

Cline comparisons

When comparing clines, the whistle length cline was both coincident and concordant with the hybrid index cline, as there was no significant difference in the center or width of the clines (ΔAIC = 0.271 and ΔAIC = 0.635, respectively) (**Figure 3.5**). However, the individual SNP clines showed less alignment, both with each other and with the overall genetic cline. These SNP clines were generally wider, with centers offset from the overall genetic cline (**Figure 3.5**). While the average SNP center is relatively close to the center of the overall genetic cline—37.5 km north—it is still not coincident (ΔAIC = 3.70). The average width of the SNP clines is about 21 times greater than the overall genetic cline, making it discordant, as well (ΔAIC = 74.5). Amongst themselves, the SNP clines are mostly non-coincident but were more likely to be concordant. In other words, the SNP clines largely did not share centers with other SNPs, but many were similar in width (**Figure 3.6**).

Neutral diffusion

The hybrid zone is narrower than predicted under neutral diffusion, regardless of the parameter estimates used in the neutral diffusion model (**Figure 3.7**). We use the width of the hybrid index cline, or the reciprocal of the maximum slope of the cline, to represent the empirical width of the hybrid zone. We used multiple estimates of natal dispersal to account for differences in dispersal between the subspecies, resulting in considerable variation in predicted widths of the secondary contact zone. Based on the

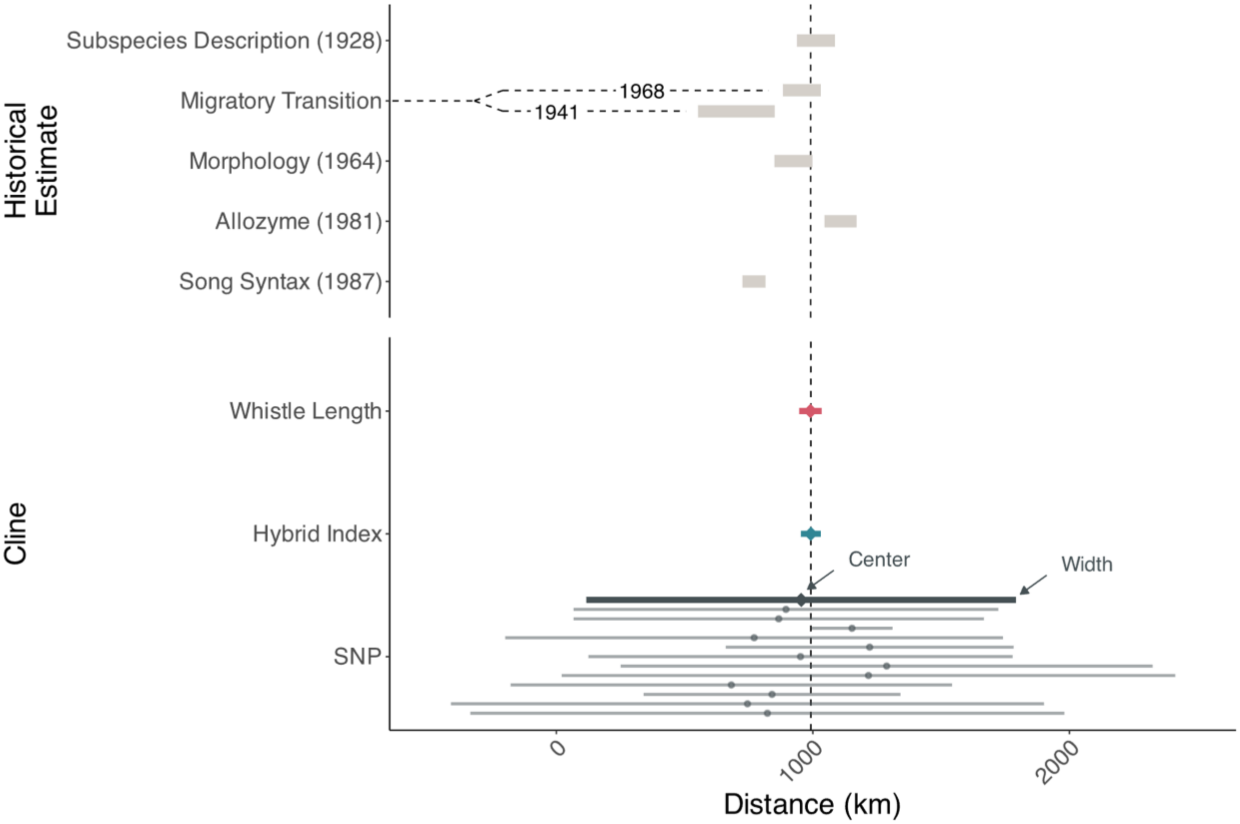


Figure 3.5. Parameters of molecular and trait clines. Dots are the clinal centers, and the horizontal lines are cline widths. All SNP clines are shown, and the bolded line shows the average of the SNP clines. Light gray bars show historical estimates of the contact zone. The subspecies delineation is by Grinnell (1928), the morphology-based estimate is from Banks (1964), the migratory transitions are from Mewaldt et al. (1968) and Blanchard (1941), the allozyme-based estimate is from Corbin (1981), and the song syntax transition is from Baker (1987). Distances are relative to San Juan Island, and the vertical line intersects the center of the hybrid index cline.

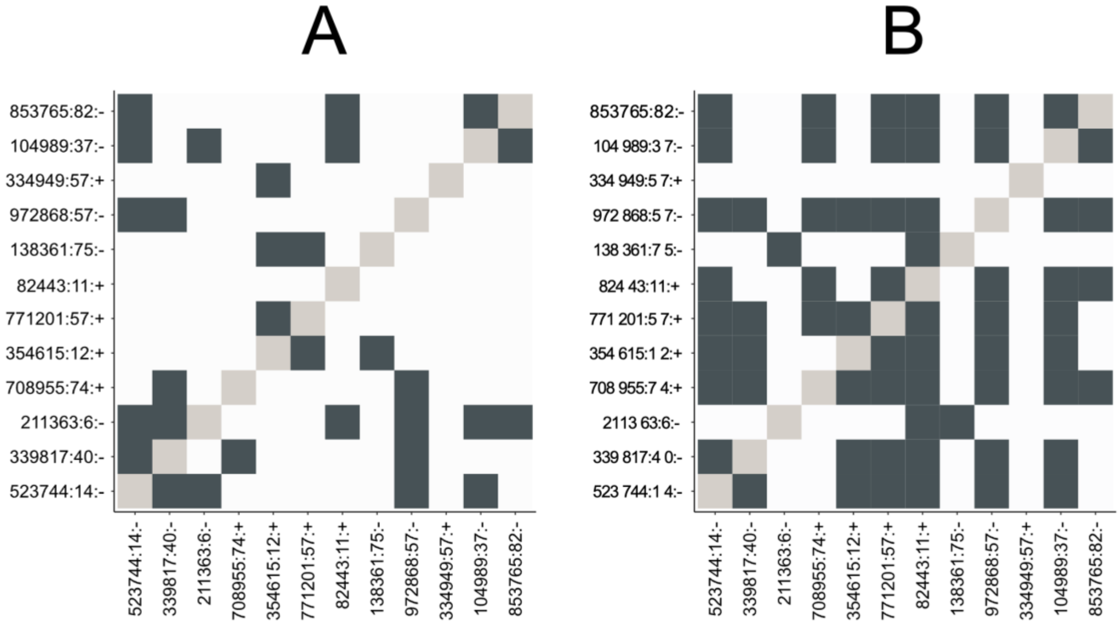


Figure 3.6. The (A) coincidence and (B) concordance of SNP clines. The dark blue cells represent comparisons between SNP clines that are either (A) coincident or (B) concordant, and the diagonal gray cells are null, as they represent comparisons to self.

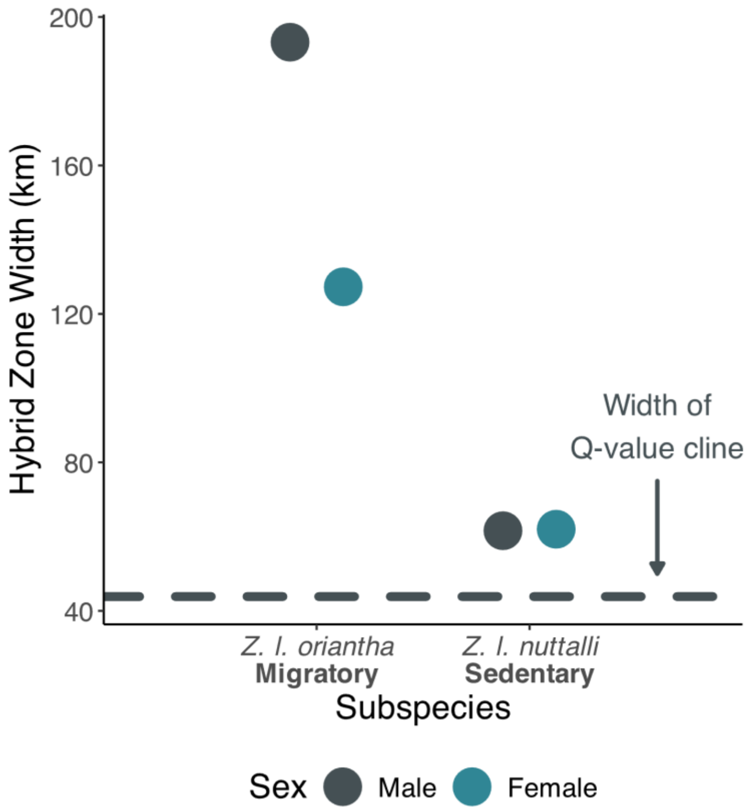


Figure 3.7. Estimates of genetic neutral diffusion. Points show the predicted width of the hybrid zone under neutral diffusion, based on the standard deviation of natal dispersal distance found in each study and 2000 generations since secondary contact. The horizontal dashed line shows the width of the hybrid index cline from this study.

estimates for male and female natal dispersal in *Z. l. oriantha* (Morton, 1992), the contact zone would be 127.3-193.2 km wide under neutral diffusion. The contact zone would be narrower using the estimate of *Z. l. nuttalli* natal dispersal (Baker & Mewaldt, 1978), at 61.60-62.01 km wide. We compared the AICc of the hybrid index cline model (i.e., unconstrained model) to models constrained to the predicted hybrid zone widths, and the constrained models had significantly higher AICc values than the unconstrained model (**Table 3.1**). In other words, the secondary contact zone significantly narrower than a hybrid zone under neutral diffusion.

Isolation by distance

For the genetic, morphological, and song traits, we tested for isolation by distance using Mantel tests. Genetic data showed no significant isolation by distance (R = 0.1137, p = 0.143), indicating that population structure is not solely the outcome of spatially limited gene flow. However, all morphological traits displayed significant isolation by distance, including mass, tarsus length, and bill dimensions. The only significant PC was morphological PC1, which was negatively loaded with mass and bill measurements and positively loaded with tarsus length (**Table 3.2**, **Figure A-9**). *Z. l. nuttalli* larger in terms of mass and bill measurements but had shorter tarsi. Of the 12 song traits tested, only minimum frequency and song length were significant for isolation by distance (**Table 3.2**). Song PC1 was almost entirely and negatively loaded with trill rate, and it was not significant for isolation by distance.

Discussion

Our results demonstrate that divergence in introductory whistle length, likely a key trait in species recognition, is strongly associated with reduced gene flow between *Z. l. nuttalli* and *Z. l. pugetensis*. The hybrid index cline is steep and significantly narrower than expected under neutral diffusion, suggesting the presence of a reproductive barrier. Notably, the clinal variation in introductory whistle length is both coincident and concordant with the hybrid index cline. Thus, divergence in introductory whistle length could be contributing to reproductive isolation at the hybrid zone. The stability of whistle

Table 3.1. Predicted contact zone width based on neutral diffusion. Estimates are based on natal dispersal estimates from *Z. l. oriantha* (Morton, 1992) and *Z. l. nuttalli* (Baker & Mewaldt, 1978). The predicted contact zone widths were calculated using the model proposed by Barton and Gale (Barton & Gale, 1993).

Subspecies	**Sex**	**Dispersal SD**	**Predicted Width**	**ΔAIC**
Z. l. oriantha	Female	1.134	127.3	13.39
Z. l. oriantha	Male	1.721	193.2	26.74
Z. l. nuttalli	Female	0.5524	62.01	3.095
Z. l. nuttalli	Male	0.5488	61.60	3.042

Table 3.2. Mantel test results for song and morphological traits. Traits with statistically significant tests are italicized.

Category	Trait	R-statistic	p-value
Song	Maximum Frequency	0.1047	0.113
Song	***Minimum Frequency***	***0.1975***	***0.02***
Song	***Song Length***	***0.2691***	***0.01***
Song	Bandwidth	-0.112	0.93
Song	Peak Frequency	0.02441	0.565
Song	Introductory Whistle Dominant Frequency	-0.07435	0.836
Song	Complex Note Bandwidth	-0.04682	0.656
Song	Average Complex Note Length	-0.08264	0.838
Song	Trill Bandwidth	-0.06845	0.716
Song	Trill Length	-0.1431	0.984
Song	Average Trill Note Length	-0.05226	0.649
Song	Trill Rate	-0.1402	0.938
Song	Song PC1	-0.1777	0.99
Morphological	***Body Mass***	***0.5105***	***0.001***
Morphological	***Tarsus Length***	***0.7795***	***0.001***
Morphological	***Bill Length***	***0.7795***	***0.001***
Morphological	***Bill depth***	***0.2486***	***0.026***
Morphological	***Bill width***	***0.1799***	***0.014***
Morphological	***Morphological PC1***	***0.7091***	***0.001***
Morphological	Morphological PC2	-0.1238	0.856

length across both historical (ca. 1970) and contemporary (2004-2023) datasets indicates that this pattern is not a result of sampling artifacts. While we observed morphological differences between subspecies, these traits followed a pattern of isolation-by-distance, without a clinal distribution within the hybrid zone. Although migratory behavior was not analyzed in this study, we consider its potential role in maintaining subspecies boundaries; differences in migratory strategies are known to contribute to reproductive isolation in other systems (Ruegg et al., 2012). Taken together, our results suggest that the divergence in introductory whistle length, potentially in combination with migratory differences, serves as a reproductive barrier between these subspecies.

We found support for a tension zone, or a secondary contact zone in which dispersal by parental genotypes is counteracted by selection against hybrids. Tension zones are narrower than hybrid zones under neutral diffusion (i.e., without barriers to gene flow), given the same number of generations since secondary contact. In the absence of reproductive barriers, gene flow gradually widens the hybrid zone and erodes genetic differentiation between populations. But reproductive barriers in tension zones maintain relatively narrow clines by limiting gene flow and introgression (Barton & Gale, 1993; Barton & Hewitt, 1985). Additionally, tension zones tend to move toward areas with low dispersal from parental populations (e.g., less suitable habitat), as they will shift to minimize the length of the contact zone (Barton & Hewitt, 1985). We recovered a hybrid index cline that is narrower than predicted under neutral diffusion, which strongly suggests that gene flow between subspecies is being impeded. Furthermore, the secondary contact zone sits on an area with less suitable habitat—redwood forest, as opposed to the typical habitat of coast scrub. Our hybrid index cline is largely consistent with the historical descriptions of the position of the hybrid zone, starting from the delineation of *Z. l. pugetensis* in 1928 (Banks, 1964; Grinnell, 1928; Mewaldt et al., 1968), suggesting that the secondary contact zone has remained at a location with low parental dispersal. Together, the narrow hybrid index cline and its stable position in a likely area of low dispersal suggest that this is a tension zone.

We posit that the introductory whistle length is a pre-zygotic reproductive barrier that is maintaining the tension zone. Traits that reduce gene flow at a tension zone (e.g., traits used in species recognition) often exhibit steep clines that are coincident and concordant with genetic clines (Baldassarre et al., 2014; Scordato et al., 2017; Walsh et al., 2016). Consistent with this prediction, we found that the cline in introductory whistle length is both coincident and concordant with the hybrid index cline, supporting its role as a reproductive barrier. Furthermore, the introductory whistle functions as a species recognition cue, both inter- and intra-specifically. Golden-crowned and white-crowned sparrows are sister species, and in both species, their introductory whistles alone are enough for species recognition during song learning (Hudson & Shizuka, 2017; Soha & Marler, 2000a). White-crowned sparrow males use introductory whistles to distinguish between subspecies, as well. Males preferentially learn songs from their own subspecies (Nelson, 2000). In addition to song learning, there is also some evidence that introductory whistles could promote assortative mating, although this has not been tested directly. White-crowned sparrows males are more aggressive toward songs from their own subspecies (Lipshutz et al., 2017), and female preference parallels male aggression (Derryberry, 2007), such that females may prefer songs from their own subspecies. Experimental work is needed to test whether females of these two subspecies prefer songs from their own subspecies and whether preference is associated with introductory whistle length. If whistle length is associated with assortative mating, then the alignment of the whistle length and hybrid index clines suggest that assortative mating is effectively reducing gene flow between the subspecies.

We further posit that there is stronger evidence for song maintaining the hybrid zone, compared to morphology. Morphological divergence between subspecies has potential to act as a reproductive barrier, either through pre-zygotic (e.g., hybrid morphologies are ineffective for mate attraction) or post-zygotic (e.g., hybrid morphologies have reduced survival) mechanisms. The subspecies differed in all the measured morphological traits, but none of the traits were clinal. Instead, morphological variation was explained solely through isolation by distance. With no evidence for selection against hybrid morphology, it is likely that song divergence, rather than morphological divergence, plays the dominant role in maintaining reproductive isolation.

In addition to song divergence, the migratory divide between *Z. l. pugetensis* (short-distance migrant) and *Z. l. nuttalli* (sedentary) may contribute to the maintenance of the tension zone. Birds from the hybrid zone exhibit physiological changes preceding spring migration: prenuptial molts, migratory restlessness (Zugunruhe), and delayed gonadal development relative to *Z. l. nuttalli* (Mewaldt et al., 1968). However, populations in the secondary contact zone tend to be sedentary, with some *Z. l. pugetensis* individuals near the secondary contact zone also showing sedentary behavior (Blanchard, 1942). While delayed gonadal development may be adaptive in colder climates, the energy costs of prenuptial molts and migratory restlessness could be maladaptive in sedentary populations. Thus, selection against hybrid migratory traits might reinforce subspecies boundaries. Since sexual selection often works alongside natural selection to maintain population boundaries, it is possible that both song divergence and migratory behavior act in concert to maintain the subspecies boundary in this hybrid zone.

The alignment of the hybrid index and whistle length clines could suggest a hybrid sink, where low population density due to post-zygotic selection against hybrids reduces gene flow, but our findings do not support this hypothesis. In hybrid sinks, frequent hybridization between parental genotypes leads to a population sink driven by low hybrid fitness, resulting in a hybrid zone in which most individuals have parental genotypes and multiple genetic loci exhibit steep, coincident clines (Barton, 1980; Barton & Hewitt, 1985). However, almost all individuals sampled in the hybrid zone were admixed, and none of the clinal loci coincided with the hybrid index cline. Instead, the locus clines were much wider than the genetic cline and frequently discordant and non-coincident with each other. The general inconsistency of SNP clines indicates a lack of strong, consistent selection across loci. These findings, along with the high proportion of admixed individuals, suggest that a hybrid sink is not acting as a significant barrier to gene flow in this system.

Although culturally transmitted signals are often proposed as drivers of population divergence in birds (Edwards et al., 2005; Uy et al., 2018), the relative contribution of genetic inheritance versus cultural transmission in such signals varies. Therefore, genetic encoding and learning both need to be considered when assessing the role of signaling traits in reducing gene flow. Given the complex ontogeny of learned songs, some song traits may be more genetically inherited than culturally transmitted. For instance, temporal features (e.g., note length) tend to be more innate than spectral features (e.g., syllable morphology) (Araki et al., 2016; Love et al., 2019). Furthermore, introductory whistles are universal across white-crowned sparrow songs and part of the species' song neural template, suggesting this difference in introductory whistle length reflects an innate divergence between subspecies (Soha & Marler, 2000a; Whaling et al., 1997). Consequently, our findings suggest that innate song traits may be more important than learned ones in maintaining reproductive isolation between subspecies.

For avian species, song divergence frequently serves as a reproductive barrier, even among closely related species without post-zygotic incompatibilities (Edwards et al., 2005). However, a key challenge remains in understanding when and how intraspecific song variation facilitates the speciation process. Our study provides evidence of a tension zone between *Z. l. nuttalli* and *Z. l. pugetensis*, potentially maintained by divergence in the introductory whistle length—an innate song trait. The clinal alignment of whistle length with the hybrid index, coupled with the behavioral evidence of subspecies-specific song discrimination (Lipshutz et al., 2017), strongly supports the role of the introductory whistle as a reproductive barrier. While cultural transmission is a prominent point of study for white-crowned sparrows, our findings highlight that it is the divergence of an innate trait, rather than learned traits, that is most likely to maintain reproductive isolation in this system. These results underscore the importance of innate song traits in sustaining subspecies boundaries and contribute to our broader understanding of the mechanisms maintaining tension zones.

CONCLUSION

Song divergence between species is an effective reproductive isolator in birds, even between otherwise genetically compatible species (Price & Bouvier, 2002). Further evidence suggests that learned songs help maintain species boundaries (Balakrishnan & Sorenson, 2006; Grant & Grant, 1996). Similar song discrimination occur within species, but evidence for reproductive barriers can be present (Irwin et al., 2008; MacDougall-Shackleton & MacDougall-Shackleton, 2001), absent (Leader et al., 2008; Nelson et al., 2017; Poesel et al., 2017; Wright et al., 2005), or asymmetrical (Brooks & Wimberger, 2023; Hamao, 2016). Moreover, some evidence demonstrates that intraspecific cultural divergence in songs may similarly reduce gene flow between populations (Keighley et al., 2019; Ribot et al., 2012). However, the extent to which culturally transmitted differences—as opposed to genetically heritable differences—in song act as reproductive isolators remains uncertain. For my dissertation, I investigated the relationship between song and genetic differentiation across multiple levels of intraspecific divergence in the white-crowned sparrow (*Zonotrichia leucophrys*), a model species for song learning and cultural evolution.

In Chapter 1, I conducted an empirical study to assess whether geographic associations exist between song divergence and population structure, including a playback experiment to test a behavioral mechanism by which song divergence can maintain population structure. Specifically, I tested whether acoustic dissimilarity between song dialects influences behavioral song discrimination, which could drive population divergence. Previous studies investigated associations between song and genetic population structure but treated dialects as categorical distinctions (Poesel et al., 2017; Soha et al., 2004; Tomback & Baker, 1984). Instead, I focused on the continuous of song on the landscape and found that males discriminate more strongly between dialects that are highly acoustically dissimilar. I therefore concluded that behavioral discrimination is driven by continuous variation in songs, not categorical distinctions between local and foreign dialects. The genetic boundary between populations appears to be spatially associated with the strongest song boundary (i.e., the greatest acoustic dissimilarity across the boundary), suggesting that discrimination between highly divergent songs is maintaining the genetic differentiation between populations.

In Chapter 2, I developed a new R package, BoundaryStats, to implement boundary statistics that have been well described in the literature (Fortin et al., 1996; Jacquez, 1995; Jacquez et al., 2000; Wagner & Fortin, 2005). To my knowledge, BoundaryStats is the first free, open source, and cross-platform implementation available to researchers. I applied boundary analysis using this package to investigate whether song and genetic boundaries from Chapter 1 are spatially correlated. In running boundary statistics, I found that the genetic boundary is significantly associated and overlapping with the song boundary at Monterey Bay, thus corroborating the results from Chapter 1. Between my first two chapters, I found evidence that song divergence is an effective barrier to gene flow between *Z. l. nuttalli* populations.

In Chapter 3, I investigated whether individual song traits are reproductive barriers between two subspecies: *Z. l. nuttalli* and *Z. l. pugetensis*. I conducted a clinal analysis to assess whether the hybrid zone congruent with a tension zone, in which selection against hybridization reduces gene flow between taxa in secondary contact. I also ran further clinal analyses to test whether song or morphological traits are maintaining the tension zone. These analyses revealed that, among the song and morphological traits included in this study, introductory whistle length was the only likely reproductive barrier between subspecies. Evidence suggests that the length of the introductory whistle is highly genetically heritable, rather than learned (Araki et al., 2016; Love et al., 2019; Nelson, 2000; Soha & Marler, 2000; Whaling et al., 1997). Therefore, it appears that a genetic difference in song, rather than a cultural difference, is acting as a reproductive barrier at the subspecies level.

My dissertation research suggests that song divergence can play a role throughout the speciation process. Within the subspecies *Z. l. nuttalli*, I found that overall acoustic dissimilarity between songs is likely reducing gene flow between populations. Between subspecies, I found that difference in introductory whistle length is a reproductive barrier. These conclusions advance our understanding of the contexts in which song plays a role in the speciation process. At multiple levels of genetic differentiation in white-crowned sparrows, song divergence reduces gene flow between genetic groupings, though I did not find support for individual song dialects as reproductive barriers. And despite the interest in cultural differences as potential reproductive barriers, only a genetically inherited song difference appears to be maintaining the subspecies boundary between *Z. l. nuttalli* and *Z. l. pugetensis*. Nevertheless, cultural divergence as a barrier to gene flow is a compelling hypothesis, and I did not explicitly test its role within subspecies or in other avian taxa. Future investigations into the role of culture during population divergence or convergence may clarify its potential role during the speciation process.

REFERENCES

Adimalla, N., Chen, J., & Qian, H. (2020). Spatial characteristics of heavy metal contamination and potential human health risk assessment of urban soils: A case study from an urban region of South India. *Ecotoxicology and Environmental Safety*, *194*(110406). https://doi.org/10.1016/j.ecoenv.2020.110406

Andersson, M. (1994). *Sexual Selection*. Princeton University Press.

Anselin, L. (1995). Local Indicators of Spatial Association—LISA. *Geographical Analysis*, *27*(2), 93–115. https://doi.org/10.1111/j.1538-4632.1995.tb00338.x

Araki, M., Bandi, M. M., & Yazaki-Sugiyama, Y. (2016). Mind the gap: Neural coding of species identity in birdsong prosody. *Science*, *354*(6317), 1282–1287. https://doi.org/10.1126/science.aah6799

Araya-Salas, M., & Smith-Vidaurre, G. (2017). warbleR: An R package to streamline analysis of animal acoustic signals. *Methods in Ecology and Evolution*, *8*(2), 184–191. https://doi.org/10.1111/2041-210X.12624

Baker, M. C. (1975). Song Dialects and Genetic Differences in White-Crowned Sparrows (*Zonotrichia leucophrys*). *Evolution*, *29*(2), 226–241. https://doi.org/10.2307/2407210

Baker, M. C. (1983). The behavioral response of female Nuttall’s White-crowned Sparrows to male song of natal and alien dialects. *Behavioral Ecology and Sociobiology*, *12*, 309–315. https://doi.org/10.1007/BF00302898

Baker, M. C. (1987). Intergradation of Song between Two Subspecies of White-Crowned Sparrows on the West Coast of North America. *Ornis Scandinavica*, *18*(4), 265. https://doi.org/10.2307/3676894

Baker, M. C., Cunningham, M. A., & Thompson, A. D. (1984). Cultural and Genetic Differentiation of Two Subspecies of White-Crowned Sparrow. *The Condor*, *86*(4), 359–367. https://doi.org/10.2307/1366808

Baker, M. C., & Mewaldt, L. R. (1978). Song Dialects as Barriers to Dispersal in White-Crowned Sparrows, *Zonotrichia leucophrys nuttalli*. *Evolution*, *32*(4), 712–722. https://doi.org/10.2307/2407487

Baker, M. C., & Thompson, D. B. (1985). Song Dialects of White-Crowned Sparrows: Historical Processes Inferred from Patterns of Geographic Variation. *The Condor, 87*(1), 127–141. https://doi.org/10.2307/1367144

Balakrishnan, C. N., & Sorenson, M. D. (2006). Song discrimination suggests premating isolation among sympatric indigobird species and host races. *Behavioral Ecology*, *17*(3), 473–478. https://doi.org/10.1093/beheco/arj052

Baldassarre, D. T., White, T. A., Karubian, J., & Webster, M. S. (2014). Genomic and morphological analysis of a semipermeable avian hybrid zone suggests asymmetrical introgrression of a sexual signal. *Evolution, 68*(9), 2644–2657. https://doi.org/10.1111/evo.12457

Banks, R. C. (1964). Geographic variation in the white-crowned sparrow *Zonotrichia leucophrys*. *University of Califonia Press Publications in Zoology*, *70*(1), 1–123.

Baptista, L. F. (1977). Geographic Variation in Song and Dialects of the Puget Sound White-Crowned Sparrow. *The Condor, 79*(3), 356. https://doi.org/10.2307/1368014

Barton, N. H. (1980). The hybrid sink effect. *Heredity*, *44*(2), 277–278. https://doi.org/10.1038/hdy.1980.23

Barton, N. H., & Gale, K. S. (1993). Genetic Analysis of Hybrid Zones. In R. G. Harrison (Ed.), *Hybrid Zones and the Evolutionary Process* (pp. 13–45). Oxford University PressNew York, NY. https://doi.org/10.1093/oso/9780195069174.003.0002

Barton, N. H., & Hewitt, G. M. (1985). Analysis of Hybrid Zones. *Annual Review of Ecology and Systematics*, *16*, 113–148. https://doi.org/10.1146/annurev.es.16.110185.000553

Beeman, K. (1998). *Signal* (Version 5) [Computer software]. Cambridge Electronic Design.

Benites, P., Campagna, L., & Tubaro, P. L. (2015). Song-based species discrimination in a rapid Neotropical radiation of grassland seedeaters. *Journal of Avian Biology*, *46*(1), 55–62. https://doi.org/10.1111/jav.00447

Blain, S. A., Justen, H. C., Easton, W., & Delmore, K. E. (2024). Reduced hybrid survival in a migratory divide between songbirds. *Ecology Letters*, *27*(4), e14420. https://doi.org/10.1111/ele.14420

Blanchard, B. D. (1941). *The white-crowned sparrows (*Zonotrichia leucophrys*) of the Pacific seaboard: Environment and annual cycle*. *46*(1), 178.

Blanchard, B. D. (1942). Migration in Pacific Coast White-Crowned Sparrows. *The Auk*, *59*(1), 47–63. https://doi.org/10.2307/4079162

Boul, K. E., Chris Funk, W., Darst, C. R., Cannatella, D. C., & Ryan, M. J. (2007). Sexual selection drives speciation in an Amazonian frog. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1608), 399–406. https://doi.org/10.1098/rspb.2006.3736

Bowman, D. M. J. S., Ondei, S., Lucieer, A., Foyster, S., & Prior, L. D. (2023). Forest-sedgeland boundaries are historically stable and resilient to wildfire at Blakes Opening in the Tasmanian Wilderness World Heritage Area, Australia. *Landscape Ecology*, *38*, 205–222. https://doi.org/10.1007/s10980-022-01558-x

Brambilla, M., Janni, O., Guidali, F., & Sorace, A. (2008). Song perception among incipient species as a mechanism for reproductive isolation. *Journal of Evolutionary Biology*, *21*(3), 651–657. https://doi.org/10.1111/j.1420-9101.2008.01523.x

Briggs, J. C., & Bowen, B. W. (2012). A realignment of marine biogeographic provinces with particular reference to fish distributions. *Journal of Biogeography*, *39*(1), 12–30. https://doi.org/10.1111/j.1365-2699.2011.02613.x

Brooks, W. E., & Wimberger, P. H. (2023). Asymmetric song recognition does not influence gene flow in an emergent songbird hybrid zone. *Ornithology*, *140*(1), 1–11. https://doi.org/10.1093/ornithology/ukac055

Calsbeek, R., Thompson, J. N., & Richardson, J. E. (2003). Patterns of molecular evolution and diversification in a biodiversity hotspot: The California Floristic Province. *Molecular Ecology*, *12*(4), 1021–1029. https://doi.org/10.1046/j.1365-294X.2003.01794.x

Campbell, P., Arévalo, L., Martin, H., Chen, C., Sun, S., Rowe, A. H., Webster, M. S., Searle, J. B., & Pasch, B. (2019). Vocal divergence is concordant with genomic evidence for strong reproductive isolation in grasshopper mice (*Onychomys*). *Ecology and Evolution*, *9*(22), 12886–12896. https://doi.org/10.1002/ece3.5770

Caro, L. M., Caycedo-Rosales, P. C., Bowie, R. C. K., Slabbekoorn, H., & Cadena, C. D. (2013). Ecological speciation along an elevational gradient in a tropical passerine bird? *Journal of Evolutionary Biology*, *26*(2), 357–374. https://doi.org/10.1111/jeb.12055

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: An analysis tool set for population genomics. *Molecular Ecology*, *22*(11), 3124–3140. https://doi.org/10.1111/mec.12354

Caye, K., Deist, T. M., Martins, H., Michel, O., & François, O. (2016). TESS3: Fast inference of spatial population structure and genome scans for selection. *Molecular Ecology Resources*, *16*(2), 540–548. https://doi.org/10.1111/1755-0998.12471

Chang, C. C., Chow, C. C., Tellier, L. C., Vattikuti, S., Purcell, S. M., & Lee, J. J. (2015). Second-generation PLINK: rising to the challenge of larger and richer datasets. *GigaScience*, *4*, 7. https://doi.org/10.1186/s13742-015-0047-8

Coombes, K. R., & Wang, M. (2022). *PCDimension: Finding the Number of Significant Principal Components* (Version 1.1.13) [Computer software]. https://CRAN.R-project.org/package=PCDimension

Corbin, K. W. (1981). Genic heterozygosity in the white-crowned sparrow: A potential index to boundaries between subspecies. *The Auk*, *98*(4), 669–680.

Creanza, N., Kolodny, O., & Feldman, M. W. (2017). Cultural evolutionary theory: How culture evolves and why it matters. *Proceedings of the National Academy of Sciences*, *114*(30), 7782–7789. https://doi.org/10.1073/pnas.1620732114

Cunningham, M. A., Baker, M. C., & Boardman, T. J. (1987). Microgeographic Song Variation in the Nuttall’s White-Crowned Sparrow. *The Condor*, *89*(2), 261–275. https://doi.org/10.2307/1368479

Danchin, E., Nöbel, S., Pocheville, A., Dagaeff, A.-C., Demay, L., Alphand, M., Ranty-Roby, S., Van Renssen, L., Monier, M., Gazagne, E., Allain, M., & Isabel, G. (2018). Cultural flies: Conformist social learning in fruitflies predicts long-lasting mate-choice traditions. *Science*, *362*(6418), 1025–1030. https://doi.org/10.1126/science.aat1590

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., Sherry, S. T., McVean, G., Durbin, R., & 1000 Genomes Project Analysis Group. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156–2158. https://doi.org/10.1093/bioinformatics/btr330

Danner, J. E., Danner, R. M., Bonier, F., Martin, P. R., Small, T. W., & Moore, I. T. (2011). Female, but Not Male, Tropical Sparrows Respond More Strongly to the Local Song

Dialect: Implications for Population Divergence. *The American Naturalist*, *178*(1), 53–63. https://doi.org/10.1086/660283

Delmore, K. E., & Irwin, D. E. (2014). Hybrid songbirds employ intermediate routes in a migratory divide. *Ecology Letters*, *17*(10), 1211–1218. https://doi.org/10.1111/ele.12326

Demko, A. D., Sosa-López, J. R., & Mennill, D. J. (2019). Subspecies discrimination on the basis of acoustic signals: A playback experiment in a Neotropical songbird. *Animal Behaviour*, *157*, 77–85. https://doi.org/10.1016/j.anbehav.2019.08.021

Derryberry, E. P. (2007). Evolution of bird song affects signal efficacy: An experimental test using historical and current signals. *Evolution*, *61*(8), 1938–1945. https://doi.org/10.1111/j.1558-5646.2007.00154.x

Derryberry, E. P. (2009). Ecology Shapes Birdsong Evolution: Variation in Morphology and Habitat Explains Variation in White-Crowned Sparrow Song. *The American Naturalist*, *174*(1). https://doi.org/10.1086/599298

Derryberry, E. P., Derryberry, G. E., Maley, J. M., & Brumfield, R. T. (2014). HZAR: Hybrid zone analysis using an R software package. *Molecular Ecology Resources*, *14*(3), 652–663. https://doi.org/10.1111/1755-0998.12209

Edwards, S. V., Kingan, S. B., Calkins, J. D., Balakrishnan, C. N., Jennings, W. B., Swanson, W. J., & Sorenson, M. D. (2005). Speciation in birds: Genes, geography, and sexual selection. *Proceedings of the National Academy of Sciences*, *102*(s1), 6550–6557. https://doi.org/10.1073/pnas.0501846102

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., & Mitchell, S. E. (2011). A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. *PLoS ONE*, *6*(5), e19379. https://doi.org/10.1371/journal.pone.0019379

Feldman, C. R., & Spicer, G. S. (2006). Comparative phylogeography of woodland reptiles in California: Repeated patterns of cladogenesis and population expansion. *Molecular Ecology*, *15*(8), 2201–2222. https://doi.org/10.1111/j.1365-294X.2006.02930.x

Fortin, M.-J., Drapeau, P., Jacquez, G. M., & Fortin, M.-J. (1996). Quantification of the Spatial Co-Occurrences of Ecological Boundaries. *Oikos*, *77*(1), 51–60. https://doi.org/10.2307/3545584

Freeman, B. G., & Montgomery, G. A. (2017). Using song playback experiments to measure species recognition between geographically isolated populations: A comparison with acoustic trait analyses. *The Auk*, *134*(4), 857–870. https://doi.org/10.1642/AUK-17-63.1

Fumagalli, M. (2013). Assessing the Effect of Sequencing Depth and Sample Size in Population Genetics Inferences. *PLoS ONE*, *8*(11), e79667. https://doi.org/10.1371/journal.pone.0079667

Gill, F. B., & Murray, B. G. (1972). Discrimination behavior and hybridization of the blue-winged and golden-winged warblers. *Evolution*, *26*(2), 282–293.

González, C., & Ornelas, J. F. (2014). Acoustic Divergence with Gene Flow in a Lekking Hummingbird with Complex Songs. *PLoS ONE*, *9*(10), e109241. https://doi.org/10.1371/journal.pone.0109241

Goudet, J. (2005). HIERFSTAT, a package for R to compute and test hierarchical *F*-statistics. *Molecular Ecology Notes*, *5*(1), 184–186. https://doi.org/10.1111/j.1471-8286.2004.00828.x

Goudey, C. B., & Smith, D. W. (1994). *USDA Forest Service, California Ecological Sections* [Dataset].

Grant, B. R., & Grant, P. R. (1996). Cultural Inheritance of Song and Its Role in the Evolution of Darwin’s Finches. *Evolution*, *50*(6), 2471–2487.

Grant, P. R., & Grant, B. R. (1997). Genetics and the origin of bird species. *Proceedings of the National Academy of Sciences*, *94*(15), 7768–7775. https://doi.org/10.1073/pnas.94.15.7768

Grant, P. R., & Grant, B. R. (2018). Role of sexual imprinting in assortative mating and premating isolation in Darwin’s finches. *Proceedings of the National Academy of Sciences*, *115*(46), E10879–E10887. https://doi.org/10.1073/pnas.1813662115

Grinnell, J. (1928). Notes on the Systematics of West American Birds. III. *The Condor*, *30*(3), 185–189. https://doi.org/10.2307/1363276

Hafner, D. J., & Petersen, K. E. (1985). Song Dialects and Gene Flow in the White-Crowned Sparrow, *Zonotrichia leucophrys nuttalli*. *Evolution*, *39*(3), 687–694.

Hamao, S. (2016). Asymmetric response to song dialects among bird populations: The effect of sympatric related species. *Animal Behaviour*, *119*, 143–150. https://doi.org/10.1016/j.anbehav.2016.06.009

Harbison, H., Nelson, D. A., & Hahn, T. P. (1999). Long-Term Persistence of Song Dialects in the Mountain White-Crowned Sparrow. *The Condor*, *101*(1), 133–148. https://doi.org/10.2307/1370454

Hebets, E. A., Bern, M., McGinley, R. H., Roberts, A., Kershenbaum, A., Starrett, J., & Bond, J. E. (2021). Sister species diverge in modality-specific courtship signal form and function. *Ecology and Evolution*, *11*(2), 852–871. https://doi.org/10.1002/ece3.7089

Heinemann, D. (1981). Song Dialects, Migration, and Population Structure of Puget Sound White-Crowned Sparrows. *The Auk*, *98*(3), 512–521.

Hewitt, G. M. (1988). Hybrid zones-natural laboratories for evolutionary studies. *Trends in Ecology & Evolution*, *3*(7), 158–167. https://doi.org/10.1016/0169-5347(88)90033-X

Higashi, M., Takimoto, G., & Yamamura, N. (1999). Sympatric speciation by sexual selection. *Nature*, *402*, 523–526. https://doi.org/10.1038/990087

Hijmans, R. J. (2022). *geosphere: Spherical Trigonometry* (Version 1.5-18) [Computer software]. https://CRAN.R-project.org/package=geosphere

Hijmans, R. J. (2023). *terra: Spatial Data Analysis* (Version 1.7-65) [R]. https://CRAN.R-project.org/package=terra

Hong, B., Bonczak, B. J., Gupta, A., Thorpe, L. E., & Kontokosta, C. E. (2021). Exposure density and neighborhood disparities in COVID-19 infection risk. *Proceedings of the National Academy of Sciences*, *118*(13), e2021258118. https://doi.org/10.1073/pnas.2021258118

Hudson, E. J., & Price, T. D. (2014). Pervasive Reinforcement and the Role of Sexual Selection in Biological Speciation. *Journal of Heredity*, *105*(S1), 821–833. https://doi.org/10.1093/jhered/esu041

Hudson, E. J., & Shizuka, D. (2017). Introductory whistle is sufficient for early song recognition by golden-crowned sparrow nestlings. *Animal Behaviour*, *133*, 83–88. https://doi.org/10.1016/j.anbehav.2017.09.018

Hulme-Beaman, A., Rudzinski, A., Cooper, J. E. J., Lachlan, R. F., Dobney, K., & Thomas, M. G. (2020). GEOORIGINS: A new method and R package for trait mapping and geographic provenancing of specimens without categorical constraints. *Methods in Ecology and Evolution*, *11*(10), 1247–1257. https://doi.org/10.1111/2041-210X.13444

Irwin, D. E., Thimgan, M. P., & Irwin, J. H. (2008). Call divergence is correlated with geographic and genetic distance in greenish warblers (*Phylloscopus trochiloides*): A strong role for stochasticity in signal evolution? *Journal of Evolutionary Biology*, *21*(2), 435–448. https://doi.org/10.1111/j.1420-9101.2007.01499.x

Jacquez, G. M. (1995). The map comparison problem: Tests for the overlap of geographic boundaries. *Statistics in Medicine*, *14*(21–22), 2343–2361. https://doi.org/10.1002/sim.4780142107

Jacquez, G. M. (2010). Geographic boundary analysis in spatial and spatio-temporal epidemiology: Perspective and prospects. *Spatial and Spatio-Temporal Epidemiology*, *1*(4), 207–218. https://doi.org/10.1016/j.sste.2010.09.003

Jacquez, G. M., Maruca, S., & Fortin, M.-J. (2000). From fields to objects: A review of geographic boundary analysis. *Journal of Geographical Systems*, *2*, 221–241. https://doi.org/10.1007/PL00011456

Janes, J. K., Miller, J. M., Dupuis, J. R., Malenfant, R. M., Gorrell, J. C., Cullingham, C. I., & Andrew, R. L. (2017). The K = 2 conundrum. *Molecular Ecology*, *26*(14), 3594–3602. https://doi.org/10.1111/mec.14187

Janicke, T., Marie-Orleach, L., Aubier, T. G., Perrier, C., & Morrow, E. H. (2019). Assortative Mating in Animals and Its Role for Speciation. *The American Naturalist*, *194*(6), 865–875. https://doi.org/10.1086/705825

Jombart, T. (2008). *adegenet*: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, *24*(11), 1403–1405. https://doi.org/10.1093/bioinformatics/btn129

Jonker, R. M., Kraus, R. H. S., Zhang, Q., Van Hooft, P., Larsson, K., Van Der Jeugd, H. P., Kurvers, R. H. J. M., Van Wieren, S. E., Loonen, M. J. J. E., Crooijmans, R. P. M. A., Ydenberg, R. C., Groenen, M. A. M., & Prins, H. H. T. (2013). Genetic consequences of breaking migratory traditions in barnacle geese *Branta leucopsis*. *Molecular Ecology*, *22*(23), 5835–5847. https://doi.org/10.1111/mec.12548

Keighley, M. V., Heinsohn, R., Langmore, N. E., Murphy, S. A., & Peñalba, J. V. (2019). Genomic population structure aligns with vocal dialects in Palm Cockatoos (*Probosciger aterrimus*); evidence for refugial late-Quaternary distribution? *Emu - Austral Ornithology*, *119*(1), 24–37. https://doi.org/10.1080/01584197.2018.1483731

Kenyon, H. L., Alcaide, M., Toews, D. P. L., & Irwin, D. E. (2017). Cultural isolation is greater than genetic isolation across an avian hybrid zone. *Journal of Evolutionary Biology*, *30*(1), 81–95. https://doi.org/10.1111/jeb.12989

Kenyon, H. L., Toews, D. P. L., & Irwin, D. E. (2011). Can Song Discriminate between MacGillivray's and Mourning Warblers in a Narrow Hybrid Zone? *The Condor*, *113*(3), 655–663. https://doi.org/10.1525/cond.2011.100182

Komsta, L., & Novomestky, F. (2022). *moments: Moments, Cumulants, Skewness, Kurtosis and Related Tests* [Computer software]. CRAN.

Kopp, M., Servedio, M. R., Mendelson, T. C., Safran, R. J., Rodríguez, R. L., Hauber, M. E., Scordato, E. C., Symes, L. B., Balakrishnan, C. N., Zonana, D. M., & Van Doorn, G. S. (2018). Mechanisms of Assortative Mating in Speciation with Gene Flow: Connecting Theory and Empirical Research. *The American Naturalist*, *191*(1), 1–20. https://doi.org/10.1086/694889

Kuchta, S. R., Parks, D. S., Mueller, R. L., & Wake, D. B. (2009). Closing the ring: Historical biogeography of the salamander ring species *Ensatina eschscholtzii*. *Journal of Biogeography*, *36*(5), 982–995. https://doi.org/10.1111/j.1365-2699.2008.02052.x

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). **lmerTest** Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, *82*(13), 1–26. https://doi.org/10.18637/jss.v082.i13

Leader, N., Geffen, E., Mokady, O., & Yom-Tov, Y. (2008). Song dialects do not restrict gene flow in an urban population of the orange-tufted sunbird, Nectarinia osea. *Behavioral Ecology and Sociobiology*, *62*(8), 1299–1305. https://doi.org/10.1007/s00265-008-0558-8

Lipshutz, S. E., Overcast, I. A., Hickerson, M. J., Brumfield, R. T., & Derryberry, E. P. (2017). Behavioural response to song and genetic divergence in two subspecies of white-crowned sparrows (*Zonotrichia leucophrys*). *Molecular Ecology*, *26*(11), 3011–3027. https://doi.org/10.1111/mec.14002

Love, J., & Goller, F. (2021). Processes underlying complex patterns of song trait evolution in a *Setophaga* hybrid zone. *Ecology and Evolution*, *11*(12), 7264–7277. https://doi.org/10.1002/ece3.7559

Love, J., Hoepfner, A., & Goller, F. (2019). Song Feature Specific Analysis of Isolate Song Reveals Interspecific Variation in Learned Components. *Developmental Neurobiology*, *79*(4), 350–369. https://doi.org/10.1002/dneu.22682

Luo, A. R., Lipshutz, S., Phillips, J., Brumfield, R. T., & Derryberry, E. P. (2024). Song and genetic divergence within a subspecies of white-crowned sparrow (*Zonotrichia leucophrys nuttalli*). *PLoS ONE*, *19*(5), e0304348. https://doi.org/10.1371/journal.pone.0304348

Luther, D. A., Phillips, J., & Derryberry, E. P. (2016). Not so sexy in the city: Urban birds adjust songs to noise but compromise vocal performance. *Behavioral Ecology*, *27*(1), 332–340. https://doi.org/10.1093/beheco/arv162

Maan, M. E., & Seehausen, O. (2011). Ecology, sexual selection and speciation. *Ecology Letters*, *14*(6), 591–602. https://doi.org/10.1111/j.1461-0248.2011.01606.x

MacDougall-Shackleton, E. A., & MacDougall-Shackleton, S. A. (2001). Cultural and genetic evolution in mountain white-crowned sparrows: Song dialects are associated with population structure. *Evolution*, *55*(12), 2568–2575. https://doi.org/10.1111/j.0014-3820.2001.tb00769.x

Manni, F., Guérard, E., & Heyer, E. (2004). Geographic patterns of (genetic, morphological, linguistic) variation: How barriers can be detected with Monmonier's algorithm. *Human Biology*, *76*(2), 173–190.

Marler, P., & Tamura, M. (1962). Song "Dialects" in Three Populations of White-Crowned Sparrows. *The Condor*, *64*(5), 368–377. https://doi.org/10.2307/1365545

Mason, N. A., Burns, K. J., Tobias, J. A., Claramunt, S., Seddon, N., & Derryberry, E. P. (2017). Song evolution, speciation, and vocal learning in passerine birds. *Evolution*, *71*(3), 786–796. https://doi.org/10.1111/evo.13159

Matocq, M. D. (2002). Phylogeographical structure and regional history of the dusky-footed woodrat, *Neotoma fuscipes*. *Molecular Ecology*, *11*(2), 229–242. https://doi.org/10.1046/j.0962-1083.2001.01430.x

McGregor, P. K. (2000). Playback experiments: Design and analysis. *Acta Ethologica*, *3*, 3–8. https://doi.org/10.1007/s102110000023

Mejías, M. A., Roncal, J., & Wilson, D. R. (2021). Territorial responses of male Bermuda White-eyed Vireos (*Vireo griseus* subsp. *Bermudianus*) reflect phylogenetic similarity of intruders and acoustic similarity of their songs. *Journal of Field Ornithology*, *92*(4), 431–449. https://doi.org/10.1111/jofo.12384

Mendelson, T. C., & Safran, R. J. (2021). Speciation by sexual selection: 20 years of progress. *Trends in Ecology & Evolution*, *36*(12), 1153–1163. https://doi.org/10.1016/j.tree.2021.09.004

Mewaldt, L. R., Kibby, S. S., & Morton, M. L. (1968). Comparative Biology of Pacific Coastal White-Crowned Sparrows. *The Condor*, *70*(1), 14–30. https://doi.org/10.2307/1366505

Mijangos, J. L., Gruber, B., Berry, O., Pacioni, C., & Georges, A. (2022). *DARTR* v2: An accessible genetic analysis platform for conservation, ecology and agriculture. *Methods in Ecology and Evolution*, *13*(10), 2150–2158. https://doi.org/10.1111/2041-210X.13918

Morton, M. L. (1992). Effects of Sex and Birth Date on Premigration Biology, Migration Schedules, Return Rates and Natal Dispersal in the Mountain White-Crowned Sparrow. *The Condor*, *94*(1), 117–133. https://doi.org/10.2307/1368802

Myers, E. A., Rodríguez-Robles, J. A., DeNardo, D. F., Staub, R. E., Stropoli, A., Ruane, S., & Burbrink, F. T. (2013). Multilocus phylogeographic assessment of the California Mountain Kinsnake (*Lampropeltis ampropeltis zonata*) suggests alternative patterns of diversification for the California Floristic Province. *Molecular Ecology*, *22*(21), 5418–5429. https://doi.org/10.1111/mec.12478

Nelson, D. A. (1998). Geographic Variation in Song of Gambel's White-Crowned Sparrow. *Behaviour*, *135*(3), 321–342. https://doi.org/10.1163/156853998793066258

Nelson, D. A. (2000). A preference for own-subspecies' song guides vocal learning in a song bird. *Proceedings of the National Academy of Sciences*, *97*(24), 13348–13353. https://doi.org/10.1073/pnas.240457797

Nelson, D. A., Hallberg, K. I., & Soha, J. A. (2004). Cultural Evolution of Puget Sound White-Crowned Sparrow Song Dialects. *Ethology*, *110*(11), 879–908. https://doi.org/10.1111/j.1439-0310.2004.01025.x

Nelson, D. A., Khanna, H., & Marler, P. (2001). Learning by instruction or selection: Implications for patterns of geographic variation in bird song. *Behaviour*, *138*(9), 1137–1160. https://doi.org/10.1163/156853901753287172

Nelson, D. A., Marler, P., & Palleroni, A. (1995). A comparative approach to vocal learning: Intraspecific variation in the learning process. *Animal Behaviour*, *50*, 83–97. https://doi.org/10.1006/anbe.1995.0223

Nelson, D. A., Nickley, B. M., Poesel, A., Gibbs, H. L., & Olesik, J. W. (2017). Inter-dialect dispersal is common in the Puget Sound white-crowned sparrow. *Behaviour*, *154*(7–8), 809–834. https://doi.org/10.1163/1568539X-00003445

Nelson, D. A., & Poesel, A. (2009). Does learning produce song conformity or novelty in white-crowned sparrows, *Zonotrichia leucophrys*? *Animal Behaviour*, *78*(2), 433–440. https://doi.org/10.1016/j.anbehav.2009.04.028

Nelson, D. A., & Poesel, A. (2014). Tutor choice and imitation accuracy during song learning in a wild population of the Puget Sound white-crowned sparrow. *Behavioral Ecology and Sociobiology*, *68*(11), 1741–1752. https://doi.org/10.1007/s00265-014-1782-z

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Gustavo, C., Chirico, M., De Caceres, M., Durand, S., … Weedon, J. (2022). *vegan: Community Ecology Package* (Version 2.6-4) [Computer software]. https://CRAN.R-project.org/package=vegan

Ostertagová, E., Ostertag, O., & Kováč, J. (2014). Methodology and Application of the Kruskal-Wallis Test. *Applied Mechanics and Materials*, *611*, 115–120. https://doi.org/10.4028/www.scientific.net/AMM.611.115

Ottenburghs, J., Kraus, R. H. S., Van Hooft, P., Van Wieren, S. E., Ydenberg, R. C., & Prins, H. H. T. (2017). Avian introgression in the genomic era. *Avian Research*, *8*(1), 30. https://doi.org/10.1186/s40657-017-0088-z

Panhuis, T. M., Butlin, R., Zuk, M., & Tregenza, T. (2001). Sexual selection and speciation. *Trends in Ecology & Evolution*, *16*(7), 364–271.

Payne, R. J. H., & Krakauer, D. C. (1997). Sexual selection, space, and speciation. *Evolution*, *51*(1), 1–9. https://doi.org/10.1111/j.1558-5646.1997.tb02382.x

Pebesma, E. J., & Wesseling, C. G. (1998). Gstat: A program for geostatistical modelling, prediction and simulation. *Computers & Geosciences*, *24*(1), 17–31. https://doi.org/10.1016/S0098-3004(97)00082-4

Petrinovich, L., & Patterson, T. L. (1981). The Responses of White-crowned Sparrows to Songs of Different Dialects and Subspecies. *Zeitschrift Für Tierpsychologie*, *57*(1), 1–14. https://doi.org/10.1111/j.1439-0310.1981.tb01309.x

Petrinovich, L., & Patterson, T. L. (1982). The White-Crowned Sparrow: Stability, Recruitment, and Population Structure in the Nuttall Subspecies (1975-1980). *The Auk*, *99*(1), 1–14. https://doi.org/10.2307/4086016

Phillips, J. N., & Derryberry, E. P. (2017a). Equivalent effects of bandwidth and trill rate: Support for a performance constraint as a competitive signal. *Animal Behaviour*, *132*, 209–215. https://doi.org/10.1016/j.anbehav.2017.08.012

Phillips, J. N., & Derryberry, E. P. (2017b). Vocal performance is a salient signal for male–male competition in White-crowned Sparrows. *The Auk*, *134*(3), 564–574. https://doi.org/10.1642/AUK-17-2.1

Phillips, J. N., Rochefort, C., Lipshutz, S., Derryberry, G. E., Luther, D., & Derryberry, E. P. (2020). Increased attenuation and reverberation are associated with lower maximum frequencies and narrow bandwidth of bird songs in cities. *Journal of Ornithology*, *161*(2), 593–608. https://doi.org/10.1007/s10336-020-01751-2

Podos, J. (1997). A performance constraint on the evolution of trilled vocalizations in a songbird family (Passeriformes: Emberizidae). *Evolution*, *51*(2), 537–551. https://doi.org/10.1111/j.1558-5646.1997.tb02441.x

Podos, J. (2007). Discrimination of geographical song variants by Darwin’s finches. *Animal Behaviour*, *73*(5), 833–844. https://doi.org/10.1016/j.anbehav.2006.11.001

Poesel, A., Fries, A. C., Miller, L., Gibbs, H. L., Soha, J. A., & Nelson, D. A. (2017). High levels of gene flow among song dialect populations of the Puget Sound white-crowned sparrow. *Ethology*, *123*(9), 581–592. https://doi.org/10.1111/eth.12632

Polakowska, A. E., Fortin, M.-J., & Couturier, A. (2012). Quantifying the spatial relationship between bird species’ distributions and landscape feature boundaries in southern Ontario, Canada. *Landscape Ecology*, *27*(10), 1481–1493. https://doi.org/10.1007/s10980-012-9804-6

Porter, C. K., & Benkman, C. W. (2019). Character displacement of a learned behaviour and its implications for ecological speciation. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1908), 20190761. https://doi.org/10.1098/rspb.2019.0761

Price, T. D., & Bouvier, M. M. (2002). The evolution of F_1 postzygotic incompatibilities in birds. *Evolution*, *56*(10), 2083–2089. https://doi.org/10.1111/j.0014-3820.2002.tb00133.x

Purcell, S., & Chang, C. (2015). *PLINK* (Version 2.0) [Computer software]. www.cog-genomics.org/plink/2.0/

Qvarnström, A., Haavie, J., Sæther, S. A., Eriksson, D., & Pärt, T. (2006). Song similarity predicts hybridization in flycatchers. *Journal of Evolutionary Biology*, *19*(4), 1202–1209. https://doi.org/10.1111/j.1420-9101.2006.01140.x

Raj, A., Stephens, M., & Pritchard, J. K. (2014). fastSTRUCTURE: Variational Inference of Population Structure in Large SNP Data Sets. *Genetics*, *197*(2), 573–589. https://doi.org/10.1534/genetics.114.164350

Reilly, S. B., Corl, A., & Wake, D. B. (2015). An integrative approach to phylogeography: Investigating the effects of ancient seaways, climate, and historical geology on multi-locus phylogeographic boundaries of the Arboreal Salamander (*Aneides lugubris*). *BMC Evolutionary Biology*, *15*(1), 241. https://doi.org/10.1186/s12862-015-0524-9

Ribot, R. F. H., Buchanan, K. L., Endler, J. A., Joseph, L., Bennett, A. T. D., & Berg, M. L. (2012). Learned Vocal Variation Is Associated with Abrupt Cryptic Genetic Change in a Parrot Species Complex. *PLoS ONE*, *7*(12), e50484. https://doi.org/10.1371/journal.pone.0050484

Ritchie, M. G. (2007). Sexual Selection and Speciation. *Annual Review of Ecology, Evolution, and Systematics*, *38*(1), 79–102. https://doi.org/10.1146/annurev.ecolsys.38.091206.095733

Ruegg, K., Anderson, E. C., & Slabbekoorn, H. (2012). Differences in timing of migration and response to sexual signalling drive asymmetric hybridization across a migratory divide. *Journal of Evolutionary Biology*, *25*(9), 1741–1750. https://doi.org/10.1111/j.1420-9101.2012.02554.x

Safner, T., Miller, M. P., McRae, B. H., Fortin, M.-J., & Manel, S. (2011). Comparison of Bayesian Clustering and Edge Detection Methods for Inferring Boundaries in Landscape Genetics. *International Journal of Molecular Sciences*, *12*(2), 865–889. https://doi.org/10.3390/ijms12020865

Saura, S., & Martınez-Millan, J. (2000). Landscape patterns simulation with a modified random clusters method. *Landscape Ecology*, *15*, 661–678.

Scordato, E. S. C., Wilkins, M. R., Semenov, G., Rubtsov, A. S., Kane, N. C., & Safran, R. J. (2017). Genomic variation across two barn swallow hybrid zones reveals traits associated with divergence in sympatry and allopatry. *Molecular Ecology*, *26*(20), 5676–5691. https://doi.org/10.1111/mec.14276

Searcy, W. A., & Beecher, M. D. (2009). Song as an aggressive signal in songbirds. *Animal Behaviour*, *78*(6), 1281–1292. https://doi.org/10.1016/j.anbehav.2009.08.011

Searcy, W. A., Nowicki, S., Hughes, M., & Peters, S. (2002). Geographic Song Discrimination in Relation to Dispersal Distances in Song Sparrows. *The American Naturalist*, *159*(3).

Searfoss, A. M., Pino, J. C., & Creanza, N. (2020). Chipper: Open-source software for semi-automated segmentation and analysis of birdsong and other natural sounds. *Methods in Ecology and Evolution*, *11*(4), 524–531. https://doi.org/10.1111/2041-210X.13368

Servedio, M. R., & Boughman, J. W. (2017). The Role of Sexual Selection in Local Adaptation and Speciation. *Annual Review of Ecology, Evolution, and Systematics*, *48*(1), 85–109. https://doi.org/10.1146/annurev-ecolsys-110316-022905

Shaw, K. L., & Lesnick, S. C. (2009). Genomic linkage of male song and female acoustic preference QTL underlying a rapid species radiation. *Proceedings of the National Academy of Sciences*, *106*(24), 9737–9742. https://doi.org/10.1073/pnas.0900229106

Sibly, R. M., Pagel, M., Curnow, R. N., & Edwards, J. (2019). How phenotypic matching based on neutral mating cues enables speciation in locally adapted populations. *Ecology and Evolution*, *9*(23), 13506–13514. https://doi.org/10.1002/ece3.5806

Sluijs, I. V. D., Dijkstra, P. D., Lindeyer, C. M., Visser, B., Smith, A. M., Groothuis, T. G. G., Alphen, J. J. M. V., & Seehausen, O. (2013). A test of genetic association among male nuptial coloration, female mating preference, and male aggression bias within a polymorphic population of cichlid fish. *Current Zoology*, *59*(2), 221–229. https://doi.org/10.1093/czoolo/59.2.221

Soha, J. A., & Marler, P. (2000a). A species-specific acoustic cue for selective song learning in the white-crowned sparrow. *Animal Behaviour*, *60*(3), 297–306. https://doi.org/10.1006/anbe.2000.1499

Soha, J. A., & Marler, P. (2000b). A species-specific acoustic cue for selective song learning in the white-crowned sparrow. *Animal Behaviour*, *60*(3), 297–306. https://doi.org/10.1006/anbe.2000.1499

St-Louis, V., Fortin, M.-J., & Desrochers, A. (2004). Spatial association between forest heterogeneity and breeding territory boundaries of two forest songbirds. *Landscape Ecology*, *19*(6), 591–601. https://doi.org/10.1023/B:LAND.0000042849.63040.a9

Strydom, T., & Poisot, T. (2023). SpatialBoundaries.jl: Edge detection using spatial wombling. *Ecography*, *2023*(5), e06609. https://doi.org/10.1111/ecog.06609

Tarroso, P., Pereira, R. J., Martínez-Freiría, F., Godinho, R., & Brito, J. C. (2014). Hybridization at an ecotone: Ecological and genetic barriers between three Iberian vipers. *Molecular Ecology*, *23*(5), 1108–1123. https://doi.org/10.1111/mec.12671

Toews, D. P. L. (2017). From song dialects to speciation in white-crowned sparrows. *Molecular Ecology*, *26*(11), 2842–2844. https://doi.org/10.1111/mec.14104

Tomback, D. F., & Baker, M. C. (1984). Assortative mating by white-crowned sparrows at song dialect boundaries. *Animal Behaviour*, *32*(2), 465–469. https://doi.org/10.1016/S0003-3472(84)80282-1

Turbek, S. P., Browne, M., Di Giacomo, A. S., Kopuchian, C., Hochachka, W. M., Estalles, C., Lijtmaer, D. A., Tubaro, P. L., Silveira, L. F., Lovette, I. J., Safran, R. J., Taylor, S. A., & Campagna, L. (2021). Rapid speciation via the evolution of pre-mating isolation in the Iberá Seedeater. *Science*, *371*(6536), eabc0256. https://doi.org/10.1126/science.abc0256

Uy, J. A. C., Irwin, D. E., & Webster, M. S. (2018). Behavioral Isolation and Incipient Speciation in Birds. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 1–24. https://doi.org/10.1146/annurev-ecolsys-110617-062646

Van Ham, M., Manley, D., Bailey, N., Simpson, L., & Maclennan, D. (Eds.). (2012). *Neighbourhood Effects Research: New Perspectives*. Springer Netherlands. https://doi.org/10.1007/978-94-007-2309-2

Verzijden, M. N., Ten Cate, C., Servedio, M. R., Kozak, G. M., Boughman, J. W., & Svensson, E. I. (2012). The impact of learning on sexual selection and speciation. *Trends in Ecology & Evolution*, *27*(9), 511–519. https://doi.org/10.1016/j.tree.2012.05.007

Wagner, H. H., & Fortin, M.-J. (2005). Spatial analysis of landscapes: Concepts and statistics. *Ecology*, *86*(8), 1975–1987. https://doi.org/10.1890/04-0914

Wagner, H. H., & Fortin, M.-J. (2013). A conceptual framework for the spatial analysis of landscape genetic data. *Conservation Genetics*, *14*(2), 253–261. https://doi.org/10.1007/s10592-012-0391-5

Waller, L. A., Turnbull, B. W., Clark, L. C., & Nasca, P. (1992). Chronic disease surveillance and testing of clustering of disease and exposure: Application to leukemia incidence and TCE-contaminated dumpsites in upstate New York. *Environmetrics*, *3*(3), 281–300. https://doi.org/10.1002/env.3170030303

Walsh, J., Shriver, W. G., Olsen, B. J., & Kovach, A. I. (2016). Differential introgression and the maintenance of species boundaries in an advanced generation avian hybrid zone. *BMC Evolutionary Biology*, *16*(65). https://doi.org/10.1186/s12862-016-0635-y

Wei, X., & Larsen, C. P. S. (2019). Methods to Detect Edge Effected Reductions in Fire Frequency in Simulated Forest Landscapes. *ISPRS International Journal of Geo-Information*, *8*(6), 277. https://doi.org/10.3390/ijgi8060277

Whaling, C. S., Solis, M. M., Doupe, A. J., Soha, J. A., & Marler, P. (1997). Acoustic and neural bases for innate recognition of song. *Proceedings of the National Academy of Sciences*, *94*(23), 12694–12698. https://doi.org/10.1073/pnas.94.23.12694

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis* [R]. https://ggplot2.tidyverse.org

Wiley, C., Ellison, C. K., & Shaw, K. L. (2012). Widespread genetic linkage of mating signals and preferences in the Hawaiian cricket *Laupala*. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1731), 1203–1209. https://doi.org/10.1098/rspb.2011.1740

Wilkins, M. R., Seddon, N., & Safran, R. J. (2013). Evolutionary divergence in acoustic signals: Causes and consequences. *Trends in Ecology & Evolution*, *28*(3), 156–166. https://doi.org/10.1016/j.tree.2012.10.002

Williams, H., Robins, C. W., Norris, D. R., Newman, A. E. M., Freeman-Gallant, C. R., Wheelwright, N. T., & Mennill, D. J. (2019). The buzz segment of Savannah sparrow song is a population marker. *Journal of Ornithology*, *160*, 217–227. https://doi.org/10.1007/s10336-018-1611-7

Wright, T. F., Rodriguez, A. M., & Fleischer, R. C. (2005). Vocal dialects, sex-biased dispersal, and microsatellite population structure in the parrot *Amazona auropalliata*. *Molecular Ecology*, *14*(4), 1197–1205. https://doi.org/10.1111/j.1365-294X.2005.02466.x

Wright, T. F., & Wilkinson, G. S. (2001). Population genetic structure and vocal dialects in an Amazon parrot. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *268*(1467), 609–616. https://doi.org/10.1098/rspb.2000.1403

Yang, Y., Servedio, M. R., & Richards-Zawacki, C. L. (2019). Imprinting sets the stage for speciation. *Nature*, *574*, 99–102. https://doi.org/10.1038/s41586-019-1599-z

Yeh, D. J. (2019). Assortative Mating by an Obliquely Transmitted Local Cultural Trait Promotes Genetic Divergence: A Model. *The American Naturalist*, *193*(1), 81–92. https://doi.org/10.1086/700958

Zink, R. M., Dittmann, D. L., & Rootes, W. L. (1991). Mitochondrial DNA Variation and the Phylogeny of Zonotrichia. *The Auk*, *108*(3), 578–584. https://doi.org/10.2307/4088098

APPENDIX

APPENDIX I: ADDITIONAL FIGURES FOR CHAPTER 1

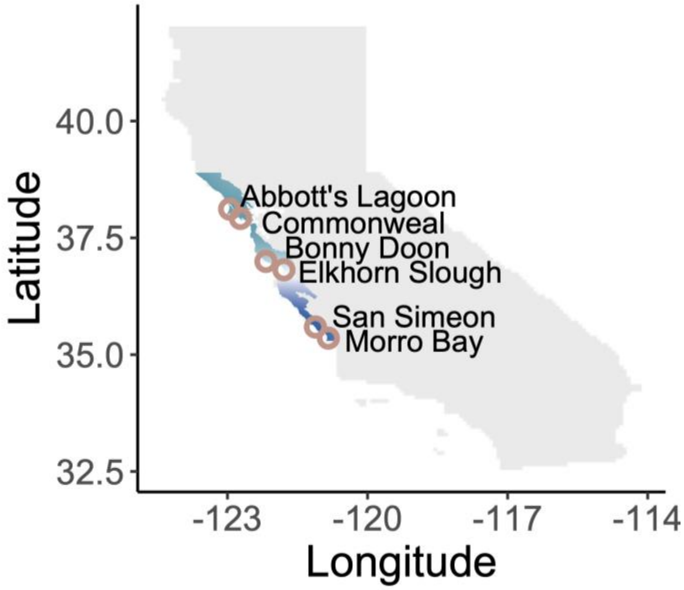


Figure A-1. Song playback experiment sites.

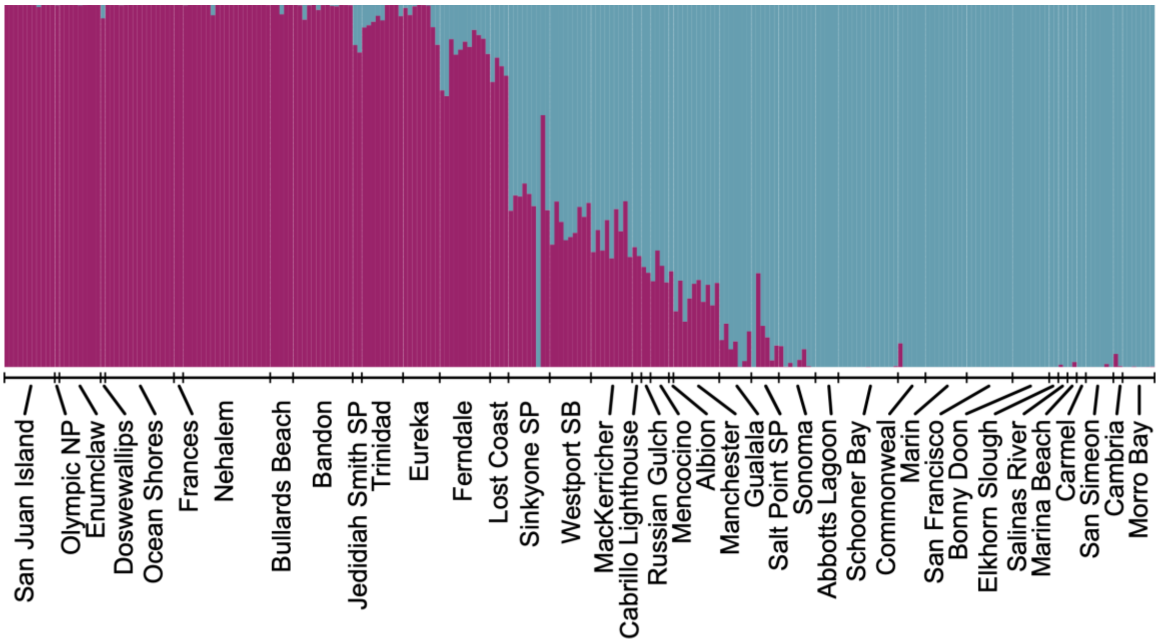


Figure A-2. Averaged k = 2 fastSTRUCTURE results for Z. l. nuttalli and Z. l. pugetensis. Populations are sorted from north to south. Pink indicates Z. l. pugetensis, and blue indicates Z. l. nuttalli.

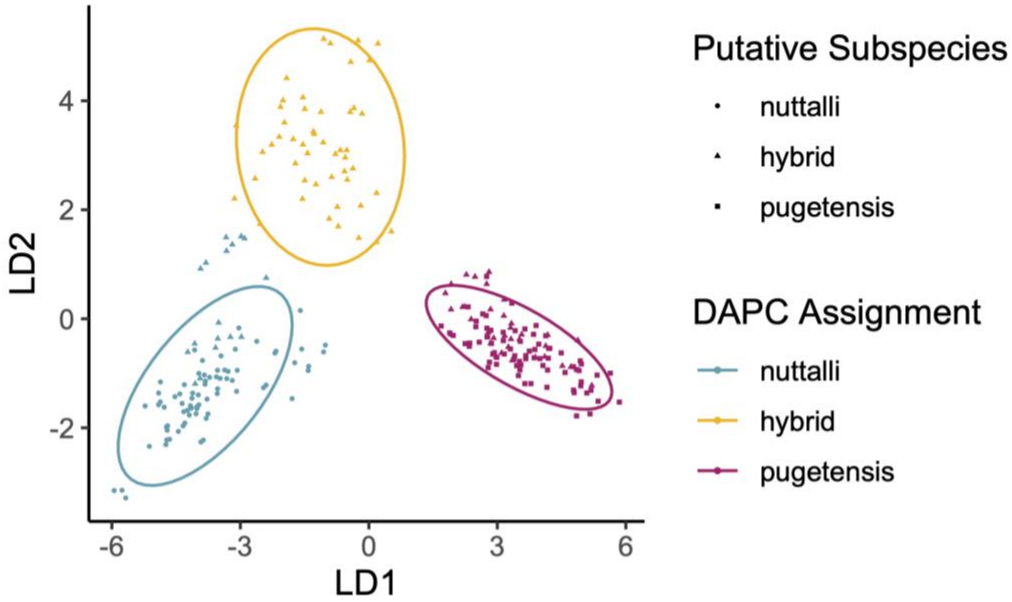


Figure A-3. Scatterplot of first two linear discriminants from DAPC.

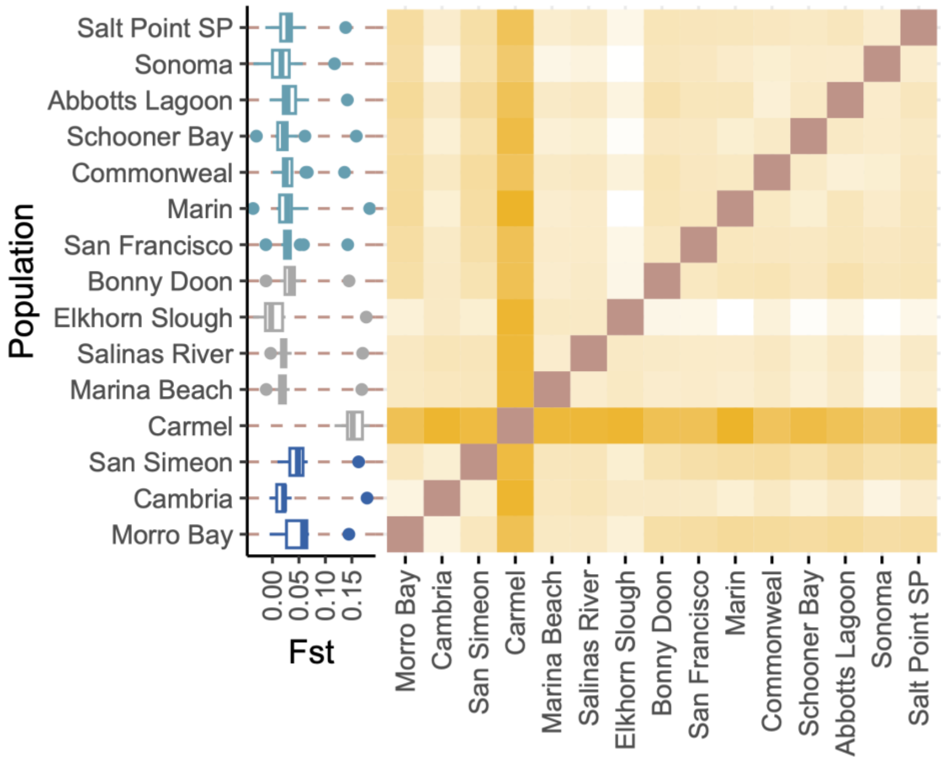


Figure A-4. Heat map and boxplot of population-level pairwise Fst values. Populations are sorted north (top right) to south (bottom left). Pink heat map cells represent null values (comparison to self).

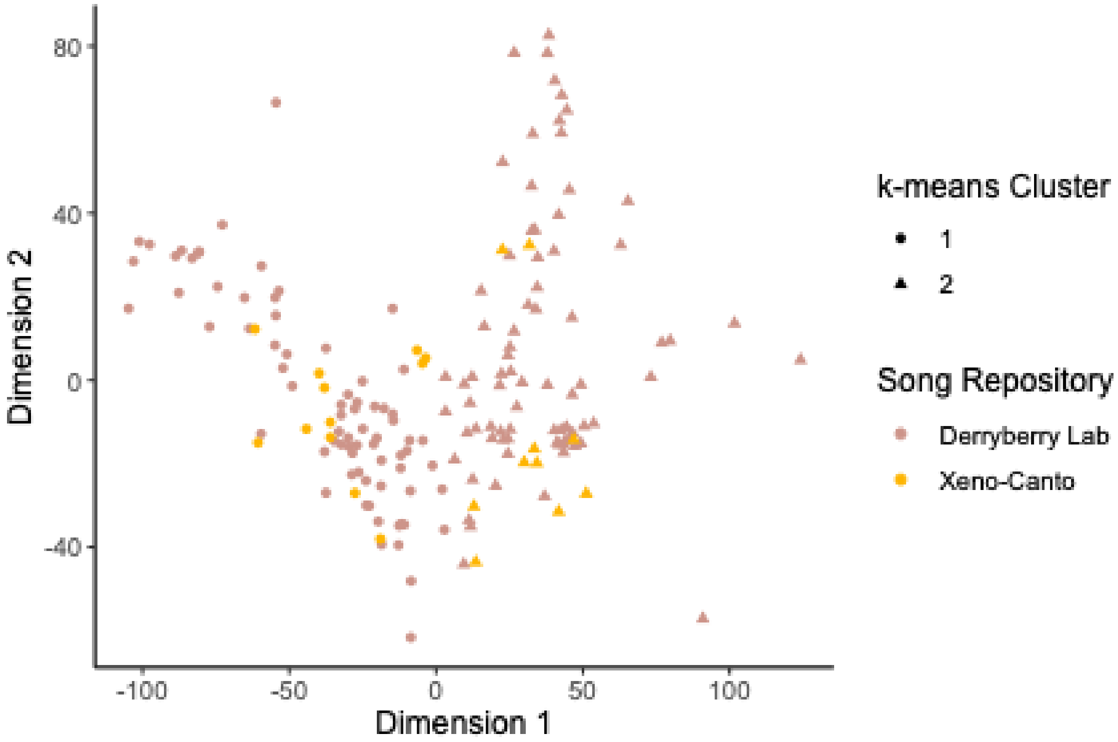
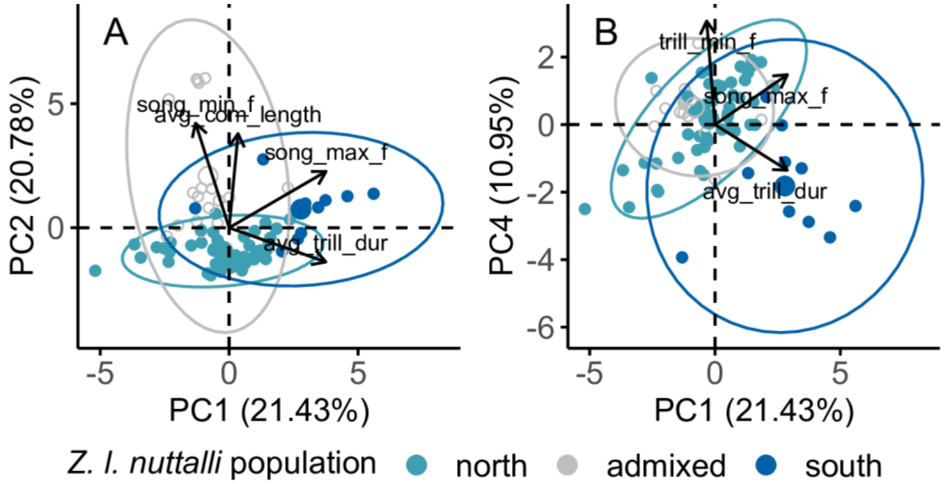


Figure A-5. MDS of songs used to calculate acoustic dissimilarity.



C Song.Trait	PC1	PC2	PC3	PC4
Song Length	0.181	-0.119	-0.163	-0.011
Whistle Length	0.182	-0.026	-0.392	0.149
Avg Complex Note Length	0.036	0.403	-0.255	-0.329
Trill Length	-0.012	0.081	-0.284	-0.064
Trill Note Length	0.392	-0.147	-0.103	-0.257
Trill Rate	-0.309	0.319	0.112	0.050
Overall Bandwidth	0.308	0.200	0.026	0.143
Complex Note Bandwidth	0.176	-0.022	0.565	0.053
Trill Bandwidth	0.365	0.233	-0.201	-0.199
Overall Maximum Frequency	0.393	0.241	0.102	0.282
Overall Minimum Frequency	-0.137	0.448	-0.233	0.160
Complex Note Maximum Frequency	0.312	0.229	0.249	0.314
Complex Note Minimum Frequency	0.067	-0.336	-0.092	0.397
Trill Maximum Frequency	0.327	-0.331	-0.131	-0.163
Trill Minimum Frequency	-0.045	-0.020	-0.335	0.585
Whistle Dominant Frequency	0.200	0.252	0.161	-0.058

Figure A-6. PCA of song trait values. (A) The first and second principal components of song trait variation. (B) The first and fourth principal components of song trait variation. (C) Table with variable loadings for statistically significant PCs.

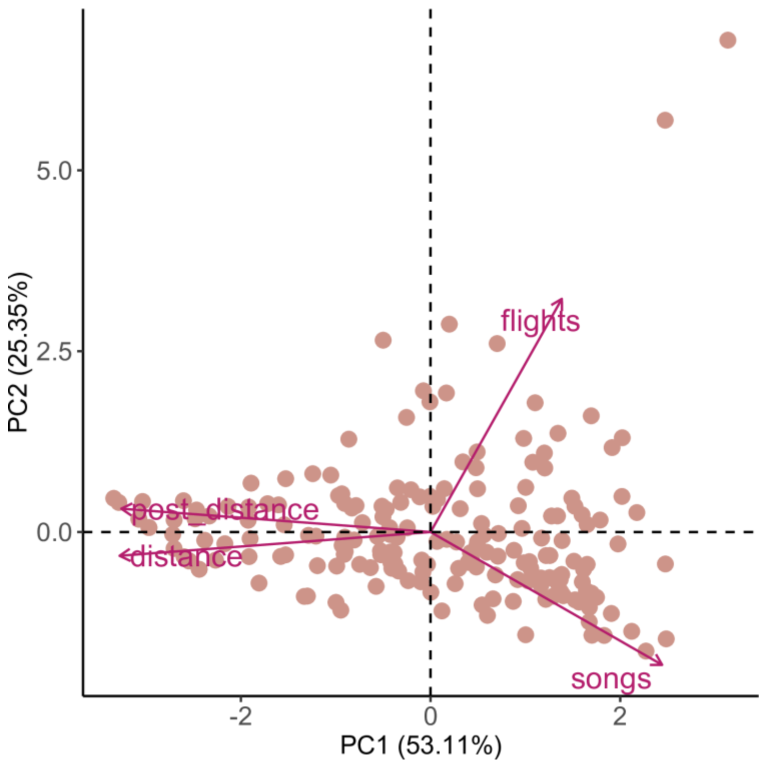


Figure A-7. PC1 and PC2 of playback experiment behavioral variables.

APPENDIX II: ADDIDITONAL FIGURES FOR CHAPTER 2

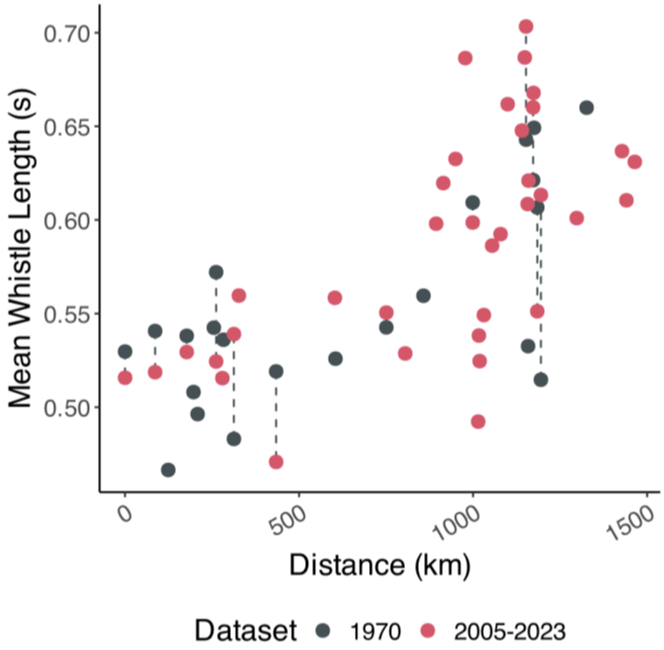


Figure A-8. Comparison of introductory whistle length in historical and contemporary datasets.

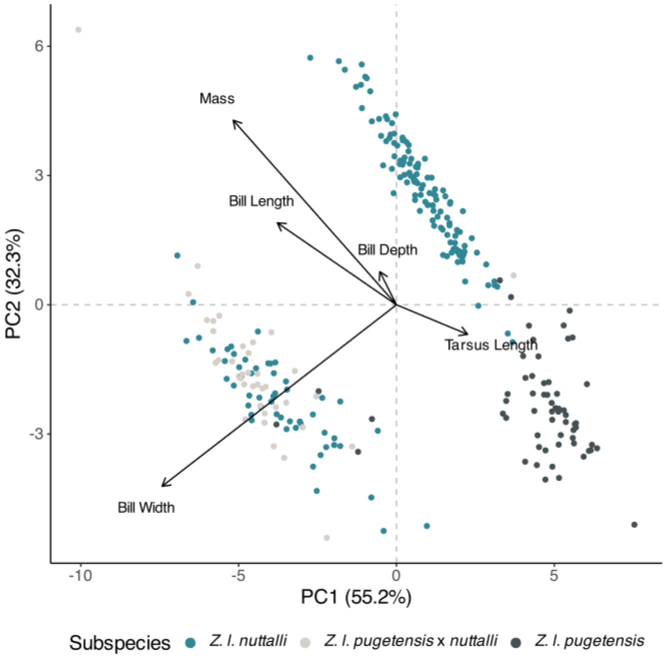


Figure A-9. PCA of morphological traits. Axes are the first two PCs, which are the only two significant PCs, as determined using the Broken Stick method.

VITA

Amy Luo was born and raised Solon, Ohio, in the suburbs of Cleveland. She received her Bachelor of Science in Evolution and Ecology from Ohio State University in 2019. During her undergraduate studies, Amy completed her undergraduate thesis on tropical fungus-growing ants with Dr. Rachelle Adams. Amy began her PhD in 2019 with Dr. Elizabeth Derryberry at the University of Tennessee, Knoxville, where she studied the interactions between cultural and biological evolution in white-crowned sparrows.