

# Behavioural response to songs between genetically diverged allopatric populations of Darwin's small tree finch in the Galápagos

Diane Colombelli-Négrel<sup>1</sup>  | Sonia Kleindorfer<sup>1,2</sup> 

<sup>1</sup>College of Science and Engineering, Flinders University, Adelaide, South Australia, Australia

<sup>2</sup>Konrad Lorenz Research Centre for Behaviour and Cognition, Department of Behavioural and Cognitive Biology, University of Vienna, Vienna, Austria

## Correspondence

Sonia Kleindorfer, College of Science and Engineering, Flinders University, Adelaide, SA 5001, Australia.  
Email: sonia.kleindorfer@flinders.edu.au

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## Abstract

Empirical data that identify contemporary mechanisms of divergence shed light on how species could multiply. In this study, we measured population genetic structure, song syllable diversity and response to simulated intruder song in Darwin's small tree finch (*Camarhynchus parvulus*) on Santa Cruz and Floreana Islands, Galápagos archipelago. Our aim was to test whether the magnitude of contemporary behavioural response in resident birds was consistent with patterns of genetic or cultural differences between populations. We analysed genetic structure and the occurrence of song syllable types, and experimentally measured the response of resident birds to intruder bird song from different geographical origin (i.e., island) or syllable type. We discovered a weak signal of population genetic structure between Santa Cruz and Floreana Islands. Although some song syllables occurred on both islands, others were unique to each island; Santa Cruz Island males used more unique syllables than Floreana Island males. Both Santa Cruz and Floreana resident males discriminated their response towards a simulated intruder song based on the geographical origin of the intruder song, but not on the syllable type sung by the intruder. We conclude that the populations are diverging in genetic and cultural traits and identified a signal of contemporary behavioural response that could maintain divergence upon secondary contact.

## KEYWORDS

allopatry, culture, evolution, gene flow, mating signal divergence, song

## 1 | INTRODUCTION

Biological mechanisms that drive differentiation among populations can be used to target conservation practice and help understand evolutionary processes (Coyne & Orr, 2004; Crandall et al., 2000; see also Friesen et al., 2007). When allopatric populations diverge, the increased variance in traits often precedes reproductive isolation measured upon secondary contact. A shift

in species-level variance across geographically separated populations can have marked consequences for reproductive isolation between the populations, which can later lead to differences in species recognition and to speciation (Coyne & Orr, 2004; Mayr, 1963). Furthermore, diverged populations generally enhance biodiversity (see Pratt, 2010) and can represent significant evolutionary units and distinct targets of conservation (see Crandall et al., 2000). Documenting population differentiation is,

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therefore, a useful activity for biodiversity monitoring in general, to ensure that distinct genetic, morphological or behavioural traits are recognized (see Lesica & Allendorf, 1995; Moritz, 1994) and conserved.

There is growing understanding of the value of conserving both genetic and cultural variety (Brakes et al., 2019). Genetic or cultural information alone do not accurately predict population-level variance (e.g., González & Ornelas, 2014). Genetically depauperate populations may contain individuals that vary in behaviour, and this behavioural variance may itself be a target of selection (e.g., Grant & Grant, 1997a; Knowlton et al., 1992; Wilson et al., 2000). Furthermore, genetic information does not necessarily indicate traits that promote or sustain reproductive isolation. Divergence in behavioural phenotypes, for example, often occurs at a much faster rate than genetic mutation of particular markers (Mallet, 2005; but see Winger & Bates, 2015).

Some animal groups are especially useful to study the interplay between rates of divergence in learned cultural traits and genetic change. Songbirds are the model system to explore changes in learned behaviour, such as song, and its role in the formation and maintenance of barriers to gene flow because song plays a critical role in species recognition as well as in mate choice (Catchpole & Slater, 2008; Price, 2008). One remarkable feature of songs in songbirds is that they can be influenced by both genetic inheritance (heritable traits such as bill morphology may constrain trill rate) (e.g., Derryberry et al., 2018; Huber & Podos, 2006; Nowicki et al., 1992; Podos, 2001) and cultural inheritance (songbirds learn their song syllables or elements from a tutor) (e.g., Evans & Kleindorfer, 2016; Grant & Grant, 1996; Greig et al., 2012; Zann, 1996). Divergence in songs may also occur as a result of acoustic adaptation to the environment (Cardoso & Atwell, 2011; Derryberry, 2009; Slabbekoorn & Smith, 2002a) or variation in female preference (Danner et al., 2011; Gibbs, 1990). Whatever the mechanism driving song divergence, the divergent song can act as a reproductive isolating mechanism (Grant & Grant, 2008a; Haavie et al., 2004; Lachlan & Servedio, 2004) and often precede genetic or phenotypic divergence (Slabbekoorn & Smith, 2002b; Slender et al., 2018).

When divergent allopatric populations come into secondary contact, mating signals and preference for those signals may converge, remain distinct or diverge (De Kort et al., 2002; Dingle et al., 2010; Greig & Webster, 2013; Haavie et al., 2004). Whether this variation in song matters for evolutionary change depends on the discriminatory capacity and behavioural response to the new variants in the intended receivers of the signal. Birds can discriminate conspecific songs between populations (Bradley et al., 2013; Derryberry, 2011; Kleindorfer et al., 2013; Podos, 2007, 2010) and female preference for particular songs can increase genetic differentiation between populations (Baker, 1983; Ellers & Slabbekoorn, 2003; Fleischer & Rothstein, 1988). Therefore, testing behavioural response to song provides a good opportunity to measure how song divergence could result in behavioural barriers to gene flow (see Colbeck et al., 2010; Dingle et al., 2010; Greig et al., 2015; Greig & Webster, 2013; Slender et al., 2018).

The 17 Darwin's finch species on the Galapagos Islands are a textbook example of the different phases involved in allopatric speciation (e.g., Grant & Grant, 2002, 2009; Grant et al., 2000). In addition to morphological change, the song of Darwin's ground finches (*Geospiza spp.*) has also been shown to diverge in these geographically separated populations (Grant & Grant, 1997a, 1997b). Darwin's finch song consists of several repetitions of the same syllable type and the syllables are believed to be culturally transmitted. Young male finches copy their syllable type from a male tutor (usually the father or attending male) (Bowman, 1979, 1983; Grant & Grant, 1996, 2018; Millington & Price, 1985), and thereafter, the syllable of an adult Darwin's finch remains the same throughout its life, which was shown in ground finches (Gibbs, 1990; Grant & Grant, 1996) and, across two years in the same males, in small tree finches (*Camarhynchus parvulus*) (Christensen et al., 2006). The syllable type in Darwin's finches is subject to copying error and other sources of variance, which could affect evolutionary trajectories (Grant & Grant, 1996, 2008b; Grant et al., 2001); when new syllable types are introduced to a population, they provide clues as to the source population of immigrants (see Grant & Grant, 2014; Grant et al., 2001; Lamichhane et al., 2018). To date, no study in the Darwin's finch group has compared syllable repertoire of allopatrically separated populations of the same species in relation to patterns of gene flow, which is the aim of this study.

We measured population genetic structure and song syllable prevalence in two allopatric populations of Darwin's small tree finch on Santa Cruz and Floreana Island, Galápagos archipelago. The aim of the study was to assess the extent of genetic and song syllable differentiation between allopatric populations and to measure the response of resident birds to the song of a simulated intruder that differs in geographical origin and/or song syllable type. We analysed microsatellite loci to infer genetic structure and created a 'syllable library' of the songs to identify the proportion of unique and shared syllable types across islands. To experimentally test if resident birds show a different behavioural response to different intruder song phenotypes, we broadcast intruder song using playback and measured the resident male response. We predicted that (a) resident birds would respond more strongly to playback of local songs (from the same island) than foreign songs (from a different island) and (b) males would respond more strongly to homotypic songs (defined as songs with the same syllable type as their own). This prediction was derived from the increased threat to the territory owner of a conspecific with shared songs (see Beecher & Campbell, 2005; Burt et al., 2001).

## 2 | MATERIALS AND METHODS

### 2.1 | Study sites and species

We collected data at Los Gemelos in the highlands of Santa Cruz Island (described in Kleindorfer et al., 2006) and at the base of Cerro Pajas in the highlands of Floreana Island (described in Kleindorfer,

O'Connor, et al., 2014), in the Galápagos archipelago, Ecuador (Figure 1). The highland *Scalesia* zone on Santa Cruz Island is an ever-green humid forest dominated by *Scalesia pedunculata* (Asteraceae). The highland *Scalesia* zone on Floreana Island is dominated by *S. pedunculata* and *Croton scouleri*. We selected Santa Cruz Island due to its central location in the archipelago and Floreana Island due to the presence of the island endemic medium tree finch (*Camarhynchus pauper*) as medium tree finches compete for resources and hybridize with small tree finches (Kleindorfer & Dudaniec, 2020; Kleindorfer, O'Connor, et al., 2014; Peters et al., 2017). Fieldwork was undertaken during the breeding season (January–March) between 2000 and 2013 (refer Table 1 for sample size per year).

The small tree finch (13 g) is 1 of 17 recognized Darwin's finch species (Passeriformes: Thraupidae) on the Galápagos Islands (Grant & Grant, 2008c; Grant & Grant, 2014; Kleindorfer, Fessl, et al. 2019; Lamichhane et al., 2015; Petren et al., 2005). They are

mostly insectivores but also consume flower parts, fruit, seeds and foliage (Kleindorfer, Fessl, et al. 2019; Loo et al., 2019; Peters & Kleindorfer, 2015; Tebbich et al., 2004). Male age can be estimated by the proportion of black on the crown and chin, which increases with each annual moult: yearling males have no black on their crown or chin (black 0), whereas males with fully black crown and chin (black 5) are at least five years old (minimum longevity in Darwin's tree finches is 12 + years; Langton & Kleindorfer, 2019). The onset of breeding occurs after a period of higher rainfall (Kleindorfer & Dudaniec, 2020). Males build a display nest, mostly in *S. pedunculata* trees, and sing to attract prospective mates (Christensen et al., 2006; Kleindorfer, 2007; Lack, 1947). We located nests by systematic searches in the study area for evidence of singing males, nest building behaviour or pair activity at the nest (see methods in Kleindorfer, 2007; Kleindorfer & Dudaniec, 2009). We recorded the location of each nest using a Garmin GPS 60 (Garmin Ltd).



**FIGURE 1** Map of the main Galapagos Islands. Small tree finch occurs on Isabela, Fernandina, Santiago, Pinzón, Santa Cruz, Santa Fé, San Cristóbal and Floreana Islands. The two study sites (Santa Cruz and Floreana Islands) are in bold

**TABLE 1** Sample size for blood samples (genetic data), song recordings (syllable type) and playback experiments on Floreana and Santa Cruz Islands between 2000 and 2013

	2000	2001	2002	2004	2005	2006	2010	2012	2013	Total
Blood samples										
Floreana Island				41	43	12	36			132
Santa Cruz Island		15	23	28	7					73
Song recordings										
Floreana Island				15	6	10	30			61
Santa Cruz Island	5	14	5	7			30			61
Playback experiments										
Floreana Island							15	20	16	51
Santa Cruz Island							22	18		40

## 2.2 | Genetic divergence

We collected blood samples (0.01 ml) from the jugular vein of 205 adults (132 from Floreana Island and 73 from Santa Cruz Island) and stored on FTA® cards (Smith & Burgoyne, 2004). We extracted all DNA samples from the FTA cards following a variation of method #4 for nucleated erythrocytes (Smith & Burgoyne, 2004; described in Galligan et al., 2012). Each individual was genotyped at 11 autosomal microsatellite loci: Gf01, Gf03, Gf04, Gf05, Gf06, Gf07, Gf09, Gf11, Gf12, Gf13 and Gf15 (Petren, 1998). We performed polymerase chain reaction (PCR) amplification following Galligan et al., (2012). PCR multiplexes were separated and analysed using capillary electrophoresis (ABI 3730 DNA analyser) at the Australian Genome Research Facility Ltd, Adelaide. We sized and scored alleles for each locus using GENEMAPPER® Software version 4.0 (Applied Biosystems) (refer also Petren, 1998; Petren et al., 1999; Petren et al., 2005).

We assessed the presence of null alleles, scoring errors and large-allele dropout using MICRO-CHECKER (Van Oosterhout et al., 2004). Our data were cross-referenced against the hybrid database (Kleindorfer & Dudaniec, 2020; Kleindorfer, O'Connor, et al., 2014; Peters et al., 2017), and we removed 49 hybrids on Floreana Island from our dataset (final sample size 156 adults). We calculated deviations from Hardy-Weinberg equilibrium (HWE; Guo & Thompson, 1992; Weir, 1996) and linkage disequilibrium per locus and putative population using default parameters in GENEPOP web version 4 (Rousset, 2008). We then checked for HWE patterns per locus across years. Significance was evaluated after sequential Bonferroni correction (Rice, 1989).

We estimated the optimal number of genetic clusters present in the populations using the Bayesian approach implemented in STRUCTURE version 2.3.2 (Falush et al., 2003; Pritchard et al., 2000).

STRUCTURE divides individuals into a number of clusters ( $K$ ) based on multilocus genotypic data and assuming HWE and linkage equilibrium. The program uses a Markov chain Monte Carlo (MCMC) procedure to estimate  $P(X|K)$ , the posterior probability that the data fit the hypothesis of  $K$  clusters. The program also calculates the fractional membership of each individual in each cluster ( $Q$ ). We used the admixture model (in which individuals may have mixed ancestry, set  $\alpha = 1$ ), with sampling location as prior information (locprior) and with correlated allele frequencies (200,000 burn-in period, 1,000,000 MCMC, mean frequency = 0.095,  $SD = 0.165$  and  $\lambda = 1$ ). We performed 10 independent runs of each  $K = 1-8$ , and we determined the optimal number of  $K$  using the Evanno method (Evanno et al., 2005) and the locprior model as it can provide more accurate inference of individual ancestry in data sets where the signal of structure is weak (Pritchard et al., 2009). We then performed an analysis of molecular variance (AMOVA) to assess hierarchical genetic structure using GENALEX version 6.5 (Peakall & Smouse, 2012), with significance assessed from 1,000 permutations. We examined the genetic variance accounted for within all individuals, among individuals within islands, and among islands.

We calculated observed ( $H_O$ ) and expected ( $H_E$ ; Nei, 1987) heterozygosity for each locus (overall and by putative population) with GENALEX version 6.5 (Peakall & Smouse, 2012). We assessed genetic diversity by calculating inbreeding coefficient ( $F_{IS}$ ) and pairwise  $F_{ST}$  using GENEPOP (Weir & Cockerham, 1984).

## 2.3 | Song syllable differences

To test for differences in song syllable occurrence between the two populations (i.e., which syllable type is used by different males in their

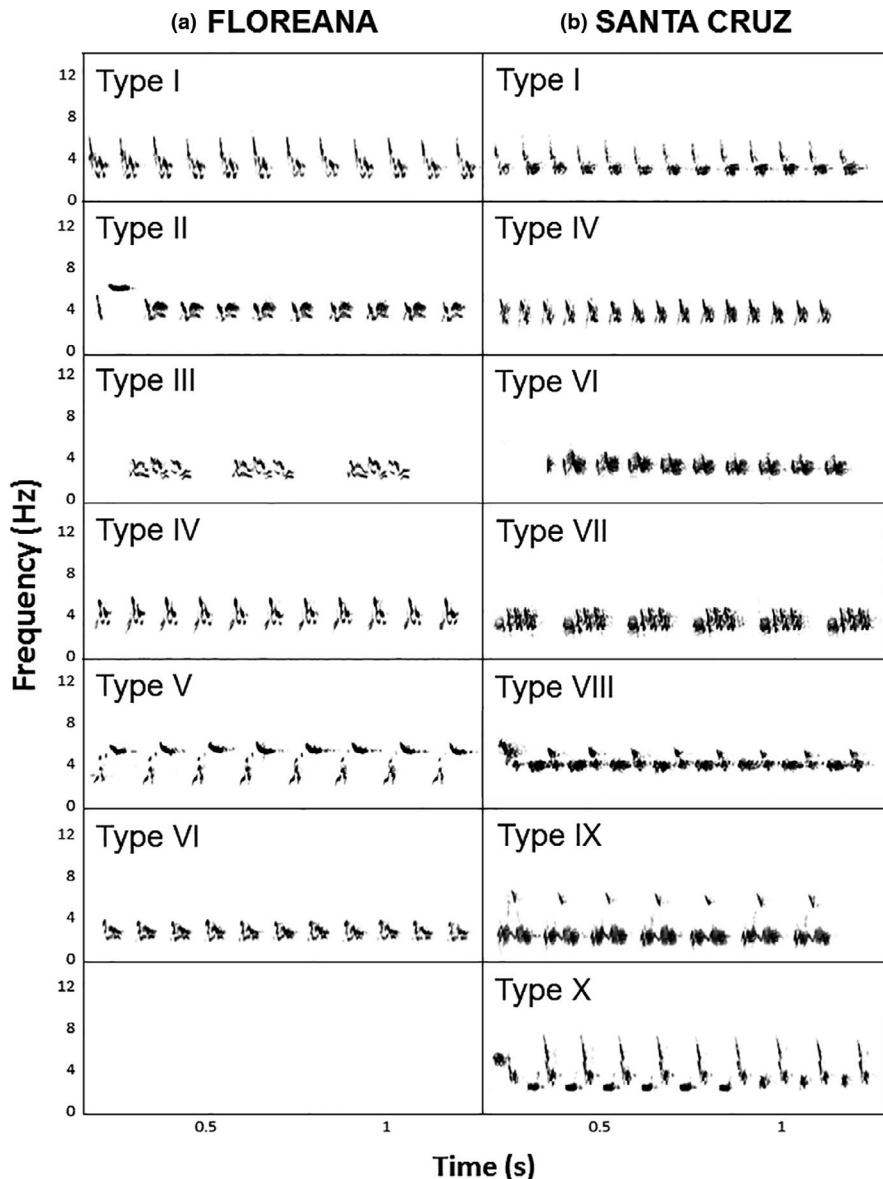
**TABLE 2** Physical characteristics used to classify syllable types from small tree finch males on Santa Cruz Island ( $N = 61$ ) and Floreana Island ( $N = 61$ )

Syllable type	Characteristics
Type I	Each syllable consists of a high-low modulation in frequency that is at least 0.1 s long; average number of elements per syllable is $8 \pm 1$
Type II	Each syllable consists of a low-high-low modulation in frequency that is at least 0.1 s long; first element is followed by a sweep at a higher frequency (approx. 6kHz) that lasts 0.1s; average number of elements per syllable is $10 \pm 1$
Type III	Each syllable consists of three double patterns of low-high-low modulations in frequency; average number of elements per syllable is $3 \pm 1$
Type IV	Each syllable consists of a low-high-low modulation in frequency that is at least 0.1 s; average number of elements per syllable is $11 \pm 1$
Type V	Each syllable consists of a low-high-low modulation in frequency that is at least 0.05s followed by a higher frequency sweep (around 6kHz) of at least 0.05s; average number of elements per syllable is $8 \pm 1$
Type VI	Each syllable consists of one double pattern of low-high-low modulation in frequency; average number of elements per syllable is $10 \pm 1$
Type VII	Each syllable consists of three high-low modulations in frequency preceded by a low frequency trill of 0.05s; average number of elements per syllable is $6 \pm 1$
Type IX	Each syllable consists of a low-high-low modulation in frequency that is at least 0.05s with a higher frequency sweep (around 6kHz) of at least 0.05s; average number of elements per syllable is $7 \pm 1$
Type X	Each syllable consists of a high-low modulation in frequency that is at least 0.1 s long; first element is a sweep at approx. 5kHz that lasts 0.1s; average number of elements per syllable is $10 \pm 1$

songs), we recorded a total of 975 songs from 122 small tree finch males (Santa Cruz Island: 554 songs from 61 males; Floreana Island: 421 songs from 61 males) (refer sample size in Table 1). We used a Sennheiser ME 80 directional microphone (Sennheiser microphones) connected to a Sony WMD6 cassette recorder in 2000–2002, a Sony DCD-100 DAT recorder in 2004–2005 (Sony Corporation) and a Telinga parabolic microphone (Telinga Microphones) connected to a portable Sound Devices 722 digital audio-recorder (Sound Devices LLC) in 2006–2010 to record wav files at 48 kHz sampling rate, 24-bit depth. We transferred all recordings to an APPLE MACPRO for visualization and editing with AMADEUS PRO 1.5 (Hairersoft Inc., Switzerland) and analysis with RAVEN PRO VERSION 1.6 Sound Analysis Software (Cornell Lab of Ornithology Bioacoustics Research Program). We created spectrograms for each recording using the Hann algorithm (DFT = 512 samples; frequency resolution = 124 Hz; time resolution = 11.6 ms; frame overlap = 50%).

Small tree finch songs are made up of one syllable type repeated several times (Christensen et al., 2006; Kleindorfer, Custance,

et al., 2019). From the spectrograms, we identified and categorized syllable types on the basis of structural similarities as previously described in Darwin's finches (refer also Bowman, 1983; Gibbs, 1990; Ratcliffe & Grant, 1985) (Table 2; Figure 2). We defined a syllable as a 'unit of elements' and an element as a 'single trace on the spectrogram' (Catchpole & Slater, 2008). To confirm that the syllables were structurally different, we used spectrogram cross-correlation analysis to examine the similarity between the different syllable types for each island using five examples from different individuals per syllable type (RAVEN PRO version 1.5, Cornell Lab of Ornithology; band-pass filtered from 1,000 to 16,000 Hz). From a previous study, we know that small tree finch males did not change the number of syllables per song across years nor did they change their syllable type (Christensen et al., 2006). We also recorded the number of syllables per song (averaged for each male). Previous study found no significant difference between the song characteristics of small tree finches and hybrid birds (Peters & Kleindorfer, 2017). Hybrids and small tree finches also did not differ in syllable type (D. Colombelli-Négrel & S. Kleindorfer, unpublished data).



**FIGURE 2** Examples of syllable types per song on (a) Floreana Island and (b) Santa Cruz Island. Each song consisted of a repetition of one syllable, with differences in syllable types between males and islands. Syllables II, III and V only occurred on Floreana Island, although syllable types VII–X only occurred on Santa Cruz Island. Refer supplementary material for a description of the physical characteristics used to discriminate between syllable types

## 2.4 | Playback experiments

In 2010, 2012 and 2013, we tested the response of 91 territorial males (40 on Santa Cruz Island, 51 on Floreana Island) to the playback of (a) small tree finch song that differed in geographical origin (same vs. different island) and syllable type (homotypic vs. heterotypic) ( $N = 60$ ), and (b) Galapagos yellow warbler songs (*Dendroica petechia*; control,  $N = 31$ ). For the conspecific song playback, we used: (a) songs from the same island [Santa Cruz Island stimuli on Santa Cruz Island ( $N = 12$ ) and Floreana Island stimuli on Floreana Island ( $N = 18$ )] or (b) songs from a different island [Floreana Island stimuli on Santa Cruz Island ( $N = 13$ ) and Santa Cruz Island stimuli on Floreana Island ( $N = 17$ )]. The conspecific playback songs also differed in syllable type as follows: (c) homotypic songs ( $N = 22$ ; syllable type of the intruder was the same as for the resident bird) and (d) heterotypic songs ( $N = 38$ ; syllable type of the intruder was different from the syllable type of the resident bird). The broadcast syllable was (a) a potentially familiar conspecific syllable from the resident bird's island but different from that of the resident bird, or (b) an unfamiliar unique syllable from the other island. We did not design the study to distinguish the effects of a 'unique island' heterotypic or 'shared island' heterotypic syllable on the resident male's response but accounted for this in our analyses (see below). Of the 91 resident males, 60 were unpaired and 31 were paired. We tested male rather than female response because previous study showed that females rarely respond to playback experiments (Ratcliffe & Grant, 1985). In support for this argument, in our study, females were seen within 10 m from the speaker in only 19% of the experiments and crossed over the speaker at low intensity (one cross) in 4% of the experiments. Therefore, given low statistical power, we did not analyse female response.

We prepared stimuli from previously recorded songs with a good signal-to-noise ratio; a high pass filter removed sounds  $<1.5$  kHz. We normalized the stimuli ( $-15$  db) and saved them as uncompressed 16-bit broadcast wave files (.wav). We prepared a total of 49 playback tracks (25 from Floreana Island and 24 from Santa Cruz Island); each with 3 min of preplayback silence (pre) followed by 3 min of playback (trial). The 3 min of stimulus (trial) consisted of six evenly spaced songs (from the same individual) in the first minute, a minute of silence, and then a repeat of the minute of songs. We then transferred these stimuli onto an Apple iPod.

We did all playback experiments at the onset of the breeding season (February) between 0600 and 1100, the time of peak song activity. Once we located a singing male and its nest (male nest height in our trials was between 4 and 7 m), we placed the playback speaker (Accurian 40-159, Radio Shack Corporation, USA, or Altec Lansing *inMotion* im600; Altec Lansing Corporation) and iPod on the ground under the nest and started the appropriate stimulus track (played at  $-80$  dB at 1 m), which was randomly selected. We never started a trial until the male was observed within a 20 m radius of its nest prior to the experiment. All birds were only tested once. All birds resumed normal activity (were observed to forage) within 10-15 min after the experiment. We never tested neighbours on the same day or with the same stimulus.

Two observers were posted 15-20 m around the focal male's territory, hidden in the vegetation to minimize the impact of human presence on the behaviour of the focal birds. Darwin's finches are largely unaffected by the presence of human observers within 5 m (Goodale & Podos, 2010) and rapidly resume normal parental activities even after physical nest disturbance (Kleindorfer, pers. obs.). We noted: (a) latency(s) to first response; (b) the minimum distance (approach distance, m) of the focal bird from the speaker during the playback stimulus; and (c) the number of flights over the speaker during the playback stimulus. We also recorded the number of (d) songs and (e) buzzes (both used during territory intrusions; Colombelli-Négre & Kleindorfer, unpublished data) during the playback trial.

## 2.5 | Statistical analysis

We analysed all song and playback data with PASW Statistics (PASW version 22.0 for Windows; SPSS Inc.). All means are presented  $\pm SE$ . We present  $\text{Eta}^2$  (proportion of variance attributed to an effect) as measure of effect size (Steyn & Ellis, 2009; Thompson, 2006). Multiple comparisons were corrected with Bonferroni adjustments. To determine if the syllable types were significantly different from one another on each island, we used a Mantel test in XLSTATS version 2020.5.1.1077 (Addinsoft) to compare the matrix of similarity produced by the spectrographic cross-correlation in RAVEN PRO version 1.5 to a second 'hypothesis' matrix with a binary code, where 1 represents within individual comparisons and 0 represents between-individual comparisons in the equivalent position. To analyse song differences between the two islands, we performed a MANOVA on the number of syllables per song and syllable types with islands (Santa Cruz, Floreana) as fixed factor. We then carried out a discriminant function analysis (DFA) with a leave-one-out cross-validation method to quantify the extent to which individuals could be classified to their island of origin on the basis of their songs (syllable type, average number of syllables). We used ANOVA to test for variation in the number of syllables per song and the frequency of the syllable types across years for each island. Playback data did not satisfy conditions of normality. We used Mann-Whitney tests to compare playback response (latency, approach distance, number of flights, songs and buzzes) to treatment song versus control. As small tree finches responded more strongly to small tree finch song (refer results), we excluded response to control stimuli from the analyses and focused on response to conspecific stimuli. We reduced the male response variables using principal components analysis (PCA) with varimax rotation. The PCA analysis provided two components with eigenvalues  $>1$ , which explained 70% of the variance: 42% of the variance was accounted for by PCA Behaviour (factor loading  $-0.81$  short latency to respond,  $-0.84$  close approach distance and 0.75 many flights over the speaker; eigenvalue 2.10), and 28% was accounted for by PCA Vocalization (factor loading 0.80 number of songs and 0.78 number of buzzes; eigenvalue 1.39). High PCA scores indicated a strong response for both PCA Behaviour and PCA Vocalization.

To examine whether the geographical origin or the syllable type of the intruder song influenced male response intensity to playback, we used GLMMs with geographical song origin (same vs. different island), syllable type (homotypic vs. heterotypic), familiarity (whether the syllable type occurred on the island of the tested bird), island (Santa Cruz vs. Floreana) and pairing status (paired vs. unpaired) as fixed factors and male ID as a random factor.

### 3 | RESULTS

#### 3.1 | Genetic divergence

Micro-checker found no evidence of scoring errors, stuttering or large-allele dropout at any of the loci; however, it did infer null alleles (as suggested by general excess of homozygotes for most allele size classes) at Gf11 for Santa Cruz Island and at Gf01, Gf04, Gf06 and Gf09 for Floreana Island. Missing data were 0%–2% across loci. In total, four loci (Gf4, Gf9, Gf11 and Gf15) showed significant departure from HWE after Bonferroni correction, but only one locus (Gf15) departed from HWE in both putative populations. Gf11 only departed from HWE on Santa Cruz, whereas Gf4 and Gf9 only departed from HWE on Floreana; homozygote excess related with the presence of null alleles (as found with Micro-Checker) might explain these deviations. None of the loci showed significant deviations from HWE for more than one of the analysed years. None of the loci were significantly linked. We, therefore, retained a total of 10 loci (Gf01, Gf03, Gf04, Gf05, Gf06, Gf07, Gf09, Gf11, Gf12 and Gf13) for the analysis.

STRUCTURE calculations of the delta K with locprior revealed two genetic clusters  $K = 2$  (Figure 3; see also Figure S1). Clusters were representative of groups of putative populations: the percentage of individuals correctly assigned with their source island with a probability of population membership  $\geq 0.90$  was 100%. The AMOVA showed that about 89% of the variation was at the individual level ( $df = 156$ ; sum of squares = 425.50, estimated variance = 2.73), 9% was among individuals within islands ( $df = 154$ ; sum of squares = 505.60, estimated variance = 0.28) and 2% was among islands ( $df = 1$ ; sum of squares = 11.19, estimated variance = 0.05).

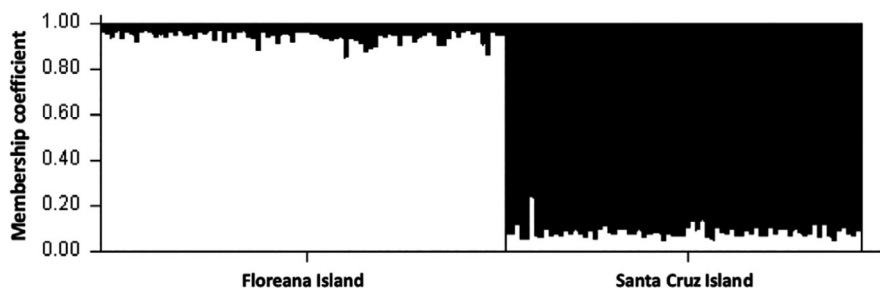
High heterozygosity and low inbreeding ( $F_{IS}$ ) were common among the loci analysed (Table S1). Across all individuals, the number of alleles per locus ranged from 2 to 22 (mean  $9.85 \pm SE 1.12$ ) and expected heterozygosity ranged from 0.06 to 0.88 (mean  $0.66 \pm SE 0.06$ ) (Table S1). Inbreeding ranged from  $-0.16$  to  $0.39$  (mean  $0.09 \pm SE 0.03$ ) (Table S1). Pair-wise  $F_{ST}$  values between islands were weak but significantly different ( $F_{ST} = 0.017$ ,  $p = 0.001$ ).

#### 3.2 | Song syllable differences

We identified a total of 10 syllable types (Figure 2, Table 2). Each song was made up of one syllable type that was repeated 2–16 times (mean  $\pm SE = 8.71 \pm 0.26$  syllable per song), and all recorded songs could be assigned to one syllable type. The different syllable types were significantly different on each island (Mantel test - Santa Cruz:  $r = 0.45$ ,  $p < 0.0001$ ; Floreana:  $r = 0.26$ ,  $p < 0.0001$ ). Only 40% (4/10) of the syllable types were shared between the two islands (i.e., used by males on both islands; Figures 2 and 4). We categorized six syllable types on Floreana Island (types I–VI), with type I being the most common and being sung by 57% of males (Figure 2). We identified nine syllable types on Santa Cruz Island (types I, IV, and VI–X), with type VI and IX being the most common and being sung by 33% and 28% of males, respectively (Figure 2). Syllable types II, III and V only occurred on Floreana Island, whereas syllable types VII, VIII, IX and X only occurred on Santa Cruz Island (Figure 2).

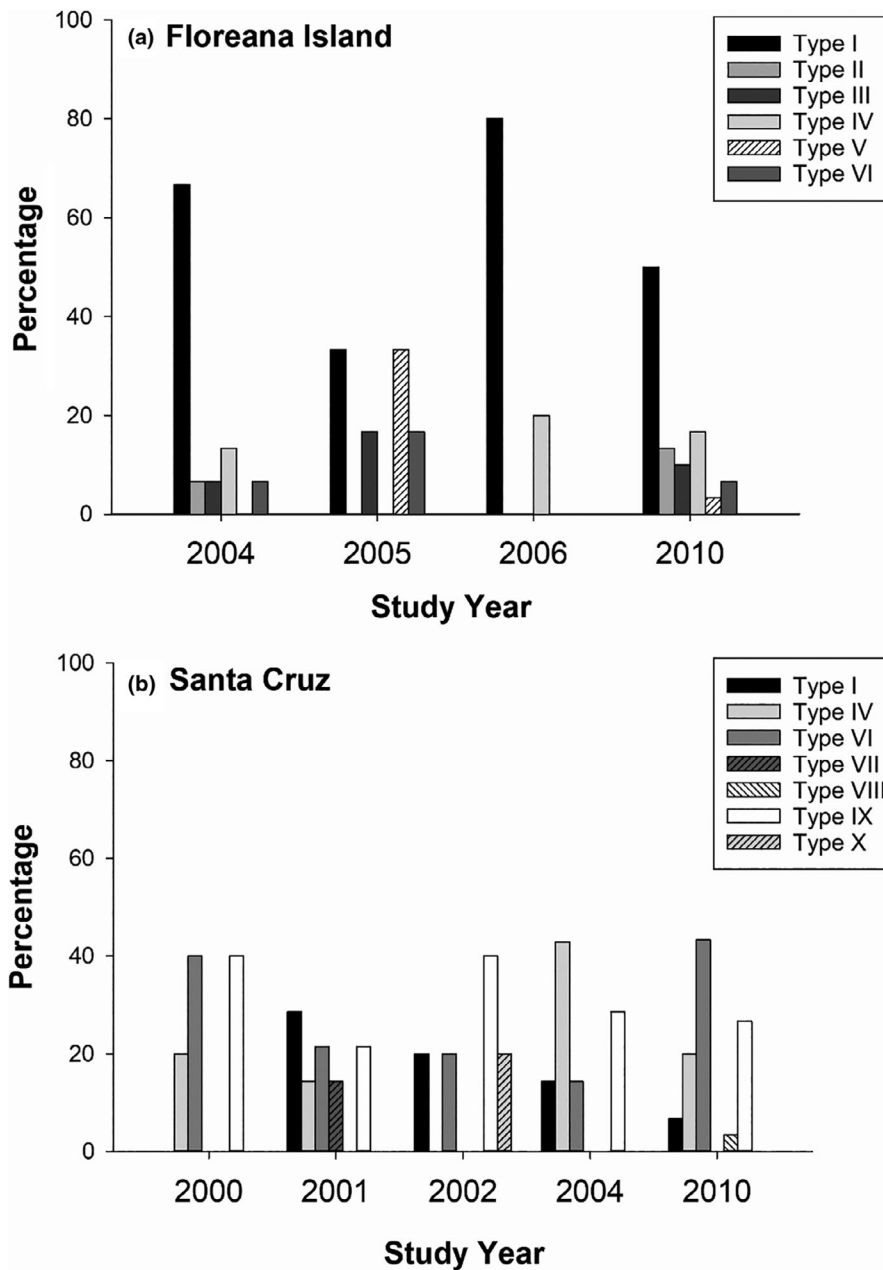
The occurrence of each syllable type differed significantly between islands (MANOVA:  $F_{1,121} = 85.61$ ;  $p < 0.0001$ ;  $\eta^2 = 1.00$ ; Figure 4), but not the number of syllables per song ( $F_{1,121} = 1.49$ ;  $p = .22$ ;  $\eta^2 = 0.23$ ). The DFA analysis revealed significant differences in songs between the two islands (Wilks'  $\lambda = 0.58$ ;  $p < 0.0001$ ). Cross-validated DFA classified 76% of songs to the correct island, which was higher than the percentage of correct classification by chance (50%; Table 3).

On Floreana Island, the occurrence of syllable types differed significantly between years (ANOVA:  $F_{5,23} = 11.01$ ;  $p < 0.0001$ ;  $\eta^2 = 0.75$ ) but not the number of syllables per song ( $F_{3,60} = 0.60$ ;  $p = .62$ ;  $\eta^2 = 0.03$ ). On Santa Cruz Island, the occurrence of syllable types ( $F_{6,34} = 7.43$ ;  $p < 0.0001$ ;  $\eta^2 = 0.61$ ), and the number of



**FIGURE 3** Results of the STRUCTURE analysis with locprior of the 156 small tree finch individuals sampled across two islands (Floreana, Santa Cruz) in the Galapagos archipelago. Each column represents a single individual's estimated membership coefficients in the two clusters ( $K = 2$ ). Black bars show the border between groups

**FIGURE 4** The percentage of syllable types in small tree finch males on (a) Floreana Island ( $N = 61$ ) and (b) Santa Cruz Island ( $N = 61$ ). The data are shown across study year



**TABLE 3** Assignments (%) from the DFA of Darwin's small tree finch songs for two islands (Santa Cruz, Floreana)

	Santa Cruz	Floreana
Santa Cruz	<b>84.7</b>	15.3
Floreana	26.2	<b>73.8</b>

Bold values represent the percentage of songs assigned to the correct island.

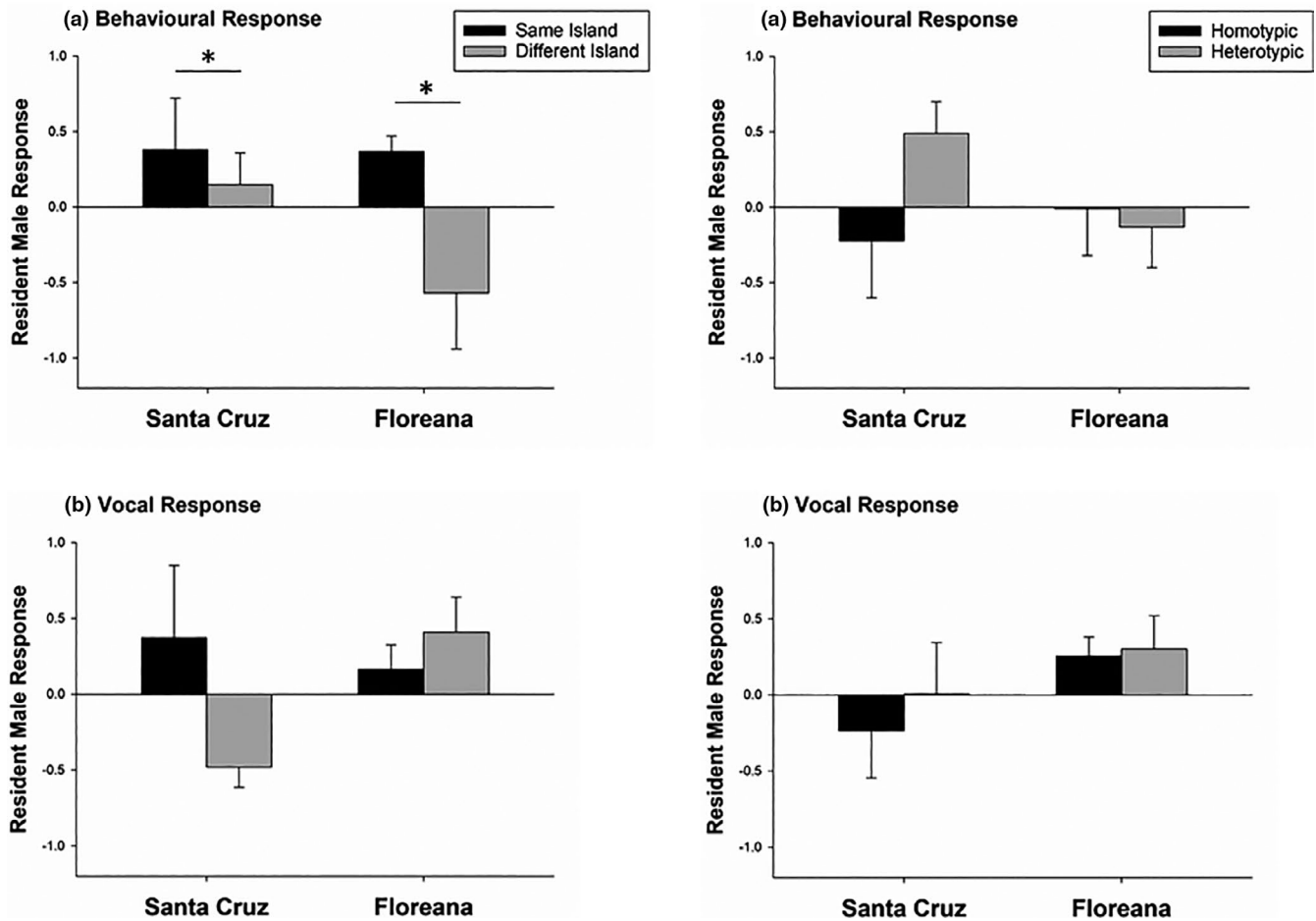
syllables per song ( $F_{4, 60} = 3.20$ ;  $p = 0.02$ ;  $\eta^2 = 0.19$ ) changed between years. For example, syllable types X, VII and VIII were only present in the year 2002, 2007 and 2010, respectively. The details of the occurrence of syllable types over the years are presented in Figure 4.

### 3.3 | Playback response to songs with different geographical origin or syllable type

Overall, individuals responded faster ( $U = 121.5$ ,  $df = 89$ ,  $p < .0001$ ), approached closer ( $U = 501.0$ ,  $p < 0.0001$ ) and with more flights ( $U = 262.0$ ,  $p < 0.0001$ ), songs ( $U = 426.0$ ,  $p < 0.0001$ ) and buzzes ( $U = 492.5$ ,  $p < 0.0001$ ) in response to small tree finch songs than to the control.

Examining response to conspecific song playback only, resident males had a stronger behavioural response, but did not vary their vocal response, to broadcast of intruder song with shared geographical origin (PCA Behaviour:  $F_{1,54} = 4.98$ ;  $p = 0.03$ ; PCA Vocalization:  $F_{1,54} = 1.24$ ;  $p = 0.27$ ; Figure 5). Song syllable type of the intruder song, familiarity (whether the syllable type occurred on the island



**(1) Geographic Origin of the Intruder Song****(2) Syllable Type of the Intruder Song**

**FIGURE 5** The response of 60 resident birds to the playback of intruder song that differed in (1) geographical origin of the intruder song [same island (black) or different island (grey) compared to the resident male] and (2) syllable type [homotypic (black) versus heterotypic (grey)]. The response is shown as derived PCA scores for (a) behavioural response and (b) vocal response. Higher PCA scores indicate a stronger response for both PCA Behaviour (shorter latency to respond, closer approach distance and many flights over the speaker) and PCA Vocalization (higher number of songs and buzzes). The data are for 25 resident males on Santa Cruz Island and 35 resident males on Floreana Island. The asterisk indicates significant results

**TABLE 4** GLMM results for behavioural response (PCA Behaviour) and vocalization response (PCA Vocalization) in resident males exposed to the experimental broadcast of intruder song

Predictor variables	PCA Behaviour		PCA Vocalization	
	$F_{1,54}$	$p$	$F_{1,54}$	$p$
Geographical origin	4.98	0.03	1.24	0.27
Syllable type	0.98	0.33	0.19	0.66
Familiarity	0.06	0.81	0.12	0.73
Island	1.08	0.30	1.17	0.28
Pairing status	0.97	0.33	2.02	0.16

We tested male response against the fixed factors 'geographical song origin' (same versus different island), 'syllable type' (homotypic versus heterotypic), 'familiarity' (whether the syllable type occurred on the island of the tested bird), 'island' (Santa Cruz versus Floreana) and 'pairing status' (paired versus unpaired) with 'Male ID' as random factor.

of the tested bird), pairing status or island did not influence resident male response (all  $p > 0.16$ ; Table 4, Figure 5).

## 4 | DISCUSSION

Populations living on different islands offer unique opportunities to evaluate mechanisms underlying population divergence in allopatry (e.g., Grant & Grant, 2008c; Lerner et al., 2011). This study is the first to investigate differences in population genetic structure and song structure between allopatric populations of Darwin's small tree finches. The populations had weak genetic differentiation between Santa Cruz and Floreana Islands. Song syllables on each island were both unique and shared (i.e., the syllable type used by a singing male could be used on both islands or only on one of the islands), with Santa Cruz Island males using more unique syllable types than Floreana Island males. Overall, males had a stronger response to

intruder song from their own geographical area. Song homotypy per se was not a key factor in eliciting a response on either island. Our results suggest that Darwin's small tree finch populations on Santa Cruz and Floreana Islands have started to diverge genetically and acoustically.

Non-adaptive stochastic effects, such as genetic drift or mutation, have been shown to explain population genetic differences in other species, especially in small populations with low gene flow (Frankham, 1997; Hoek et al., 2010). Here, we found a weak signal of population genetic structure between the two allopatric populations. However, our data showed high heterozygosity (mean  $0.66 \pm SE 0.06$ ) and low inbreeding (mean  $0.09 \pm SE 0.03$ ), which suggests that drift was probably not a major factor in the genetic differentiation observed here (refer also Petren et al., 2005). Our findings are parsimonious with the idea that at least some of the variance we report could be caused by different patterns of intraspecific introgression on each island. In Darwin's finches, hybridization is rather common and may account for 2%–56% of birds in a Darwin's finch population sampled in a given year (Grant & Grant, 1997a, 2016; Grant et al., 2003, 2004; Kleindorfer & Dudaniec, 2020; Kleindorfer, O'Connor, et al., 2014). On Daphne Major Island, researchers documented hybridization between medium ground finch (*Geospiza fortis*) and cactus finch (*Geospiza scandens*), which resulted in morphological and genetic convergence between the two species (Grant et al., 2003, 2004), and on another occasion gave rise to a new species composed of in-bred descendent offspring of the original hybrid pair (Grant & Grant, 2014; Lamichhaney et al., 2018; Lamichhaney et al., 2019). On Floreana Island, researchers have been monitoring a cluster of hybrid finches that are the result of pairings between small and medium tree finches (Kleindorfer & Dudaniec, 2020; Kleindorfer, O'Connor, et al., 2014; Peters et al., 2017). This influx of medium tree finch genes into the small finch population on Floreana Island should lower genetic connectivity with other small tree finch populations elsewhere. Alternatively, high immigration from other islands to Santa Cruz Island (perhaps due to prevailing wind conditions) would result in different genetic structure between the two islands. In support for this idea, there was genetic differentiation on Floreana Island but not Santa Cruz or Isabela Islands for the accidentally introduced parasitic fly *Philornis downsi* (Dudaniec et al., 2008). Genetic sampling across the Galápagos archipelago would be useful to test the idea of gene flow and genetic differentiation in relation to barriers to dispersal (refer also Caplat et al., 2016; Koop et al., 2020).

Because syllable types in most bird species can persist over many years (Baker & Gammon, 2008; Ficken & Popp, 1995), evidence for syllable sharing between populations may be particularly important for examining past and current patterns of divergence or intermixing (Podos & Warren, 2007) as well as the timing of colonization (Lachlan et al., 2013). Here, we found that only 40% of the syllable types were shared between the two islands, with birds on Santa Cruz Island singing more unique syllable types than those living on Floreana Island. This suggests that the syllable pool in the Floreana Island population may be depauperate relative to the Santa Cruz

Island population (see also Baker, 1996). Such difference in syllable types between the two islands could simply be explained by founder effects (Baker, 1996) and cultural drift (Lachlan & Slater, 2003; Lynch & Baker, 1993) if fewer individuals colonized the more remote Floreana Island. In Darwin's finches, song differences between populations have been shown to arise in allopatry through random selection of syllable types in the newly founded population (Grant & Grant, 1995), followed by random extinction of other syllable types and small copying errors in transmission from father to son (Grant & Grant, 1996; Grant & Grant, 1997a, b). Colonization can also increase song diversity if there are multiple cultural source populations. Given that Santa Cruz Island may receive more immigrant birds from more islands than does Floreana Island (as discussed above), the new arrivals to Santa Cruz Island could introduce novel syllable types. Exchange of individuals between populations in other bird species has been shown to homogenize song pools, similar to processes shaping genetic diversity (e.g., Greig & Webster, 2013), which could explain the distribution of population-level syllable types.

Surprisingly, despite divergence in song types between islands, males on both islands did not differentiate between syllable types of the intruder songs. However, these results need to be interpreted with caution due to our small sample sizes for each playback type. Additional playback experiments testing males with two different songs in randomized order may be necessary to fully address the importance of syllable type for song discrimination in Darwin's finches. Yet, similar to previous studies in birds (reviewed in Parker et al., 2018) – including Darwin's finches (Brumm et al., 2010; Grant & Grant, 2002; Kleindorfer et al., 2013; Podos, 2007, 2010; Ratcliffe & Grant, 1985) – we found that territorial males on both islands showed a stronger response to local versus foreign songs, may be using other features of the songs not captured by spectrograms, such as the length of the inter-syllable intervals, their temporal pattern (equal or unequal) or some tonal features of the syllables (their internal structure) (Favaro et al., 2015; Grant & Grant, 2002). A study in African penguins (*Spheniscus demersus*), for example, showed that up to 14 acoustic parameters within a single vocalization could be used for individual recognition (Favaro et al., 2015), and animal vocalizations have been shown to encode more than one type of information (such as sex, age, size or even behavioural state) about the emitter of the signal (e.g., D'Amelio et al., 2017; Hollén & Manser, 2007; Matrosova et al., 2011; Schneiderova & Policht, 2011). Given the higher response of resident males to local songs, males may have perceived local songs as more 'threatening' than songs from another island. Males may be more motivated to repel local conspecific competitors if they are perceived as posing a greater risk for territory take-over, nest usurpation, extra-pair copulations with resident females, or the added risk of high density neighbours for ecto-parasitism (Dudaniec & Kleindorfer, 2006; Fessl et al., 2018; Kleindorfer & Dudaniec, 2016; Kleindorfer et al., 2009, 2014). Divergence in learned acoustic signals can rapidly restrict gene flow by promoting song divergence and thereby enhancing assortative mating (Grant & Grant, 2002; Greig & Webster, 2013; Halfwerk et al., 2016; Lipshutz et al., 2016). The observed stronger

response to local versus foreign songs could, therefore, also be an indication that the two populations have evolved behavioural barriers to reproduction, which remains to be tested (refer also Freeman & Montgomery, 2017). Future research could attempt to test female response to determine whether females show the same pattern of discriminated response to songs from different islands (refer also Grant & Grant, 2002).

Interestingly, in response to playback of intruder songs, Darwin's finches used behavioural response more often than vocal response, perhaps given reduced visibility in dense forest habitat. The findings also support the idea that physical contests may have different costs than vocal challenges (Akçay et al., 2011; Searcy & Beecher, 2009). A study across four *Troglodytes* wren species, for example, showed that all wren species responded to playback of conspecific signals with a high behavioural response but that only the brown-throated wrens (*Troglodytes brunneicollis*) and the Cozumel wrens (*T. beani*) also responded to conspecific signals with a high vocal response (Sosa-López et al., 2016). In Darwin's small tree finches, younger males tend to buzz more often than older males (both while singing to attract mates and to defend their territory) but are also more often attacked by the larger medium tree finch males (pers. obs), suggesting some costs to vocal response towards intruders. Additional research into the costs associated with behavioural versus vocal response (and how these may mediate territory defence) are clearly needed.

In conclusion, we found significant differences in songs and genetics between two allopatric populations of Darwin's small tree finches. We argue that such variation could result in reproductive isolation in the future. A research focus on contemporary behavioural response profiles to diverging mating signals between allopatric populations and cultural signals increase our understanding of the mechanisms that promote biodiversity and inform models that predict whether genetic and cultural change will be maintained upon secondary contact.

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#### CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

#### PEER REVIEW

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#### ORCID

Diane Colombelli-Négrel  <https://orcid.org/0000-0002-9572-1120>

[org/0000-0002-9572-1120](https://orcid.org/0000-0002-9572-1120)

Sonia Kleindorfer  <https://orcid.org/0000-0001-5130-3122>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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