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Ecology and morphology as drivers of passerine song differentiation across an island archipelago

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We conducted an integrative analysis to explore possible explanations for the song differentiation in bird populations from the mainland and the Tres Marías Archipelago (TMA) on the central-western Pacific coast of Mexico. We first analysed the effect of isolation on cultural transmission by comparing repertoire size and genetic fixation in four phylogenetically distantly related passerines for which previous studies have documented significant genetic divergence between island and mainland populations. Then, we examined the contributions of potential factors involved in song differentiation (syllable duration and frequencies, frequency range), including morphology (bill size and body weight), genetic divergence and environmental factors (precipitation and temperature). Syllable sharing between TMA and mainland populations was higher for Northern Cardinals *Cardinalis cardinalis* and Golden Vireos *Vireo hypochryseus* but lower for Rufous-backed Thrushes *Turdus rufopalliatus*. Songs of TMA populations showed longer syllable duration in Rufous-backed Thrush and Golden Vireo, and shorter syllable duration in Northern Cardinal than those on the mainland. No significant differences were found between island and mainland populations of Streak-backed Oriole *Icterus pustulatus*. Bill volume differed significantly between TMA and mainland populations of Northern Cardinals and Rufous-backed Thrushes but not Streak-backed Orioles and/or Golden Vireos. Correlations between body size and song frequencies were low but significant for most variables. We conclude that bird song differentiation on these islands is driven by isolation, environmental factors and morphology.

Keywords: acoustic divergence, colonization, founder effect, isolation, Pleistocene.

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Understanding the evolution of species' distributions requires a comprehensive understanding of not only the evolutionary and ecological factors influencing them, such as ecological and geographical barriers, but also cultural transmission (e.g. song learning) between populations (Frugone *et al.* 2019, Luzuriaga-Aveiga & Weir 2019). All these factors may promote population differentiation in genetic, morphological, ecological and behavioural characters. This pattern is commonly observed in species with insular populations (Price 2008, Pavlova *et al.* 2012, Mason *et al.* 2017, Friedman *et al.* 2019, Jezierski *et al.* 2023).

Oceanic islands are of high relevance in studies of evolution and biogeography due to their volcanic origin, isolation, lower biodiversity and differences in environmental conditions (Whittaker & Fernández-Palacios 2007, Whittaker *et al.* 2010). These qualities render oceanic islands favourable for studying processes of colonization (founder effect), divergence between populations in multiple traits and speciation (Borregaard *et al.* 2017). Many hypotheses have been proposed to explain trait differences observed between island and mainland populations. For example, syndromes such as gigantism and dwarfism are common in island populations (Foster 1964, Lomolino 2005), as are morphological differences associated with food availability, coloration (mainly in red, yellow and orange tones; Toews *et al.* 2017, Reudink *et al.* 2021) or specialization towards certain resources associated with competition among conspecifics (Grant & Grant 2006). In addition, trade-offs between bird coloration and song complexity (Tietze & Hahn 2024), character release due to reduced interspecific competition (Morinay *et al.* 2013, Robert *et al.* 2021), or different natural or sexual selection pressures on islands (weaker or stronger depending on the population densities, resource availability and social selection; Price 2008) may be also reflected in song complexity (Catchpole & Slater 2008, Price 2008, Robert *et al.* 2022). Due to the key role of songs in mate attraction and assortative mating, acoustic divergence may eventually lead to speciation (Irwin *et al.* 2001, Price 2008, Wilkins *et al.* 2013, Uy *et al.* 2018). If bird songs are genetically constrained (McCracken & Sheldon 1997, Sober *et al.* 2008), song divergence can be strongly correlated with genetic distance (Päckert *et al.* 2003; but see Tobias *et al.* 2010). Alternatively, bird songs could be constrained by environmental factors independently of genetic differentiation.

Studying the evolution of bird songs in island populations is not easy because multiple factors are involved. Song divergence in island populations may result not only from island syndromes (Foster 1964, Van Valen 1973, Olson *et al.* 2009, Benítez-López *et al.* 2021, Jezierski *et al.* 2023) but also from founder effects (Mayr 1942, see below), genetic or cultural drift (Wilkins *et al.* 2013, Graham *et al.* 2018), changes in natural selection pressures (e.g. ecological conditions or acoustic environment; Ryan & Brenowitz 1985, Sagar *et al.* 2024), and shifts in social or sexual selection pressures (e.g. changes in song traits related to efficient male–male communication or mate attraction; Catchpole & Slater 2008, Wilkins *et al.* 2013). In addition, morphological differences in island populations may promote changes in spectro-temporal traits of bird song (Wallschläger 1980, Ryan & Brenowitz 1985, Clegg & Owens 2002, Mason & Burns 2015). Thus, for example, differences in bill size may be related to frequency and temporal song characters, while bill shape is associated with song pace and trill rates; bill dimensions, however, can vary independently of body size (García & Tubaro 2018, Demery *et al.* 2021). In addition, despite bill shape and size being closely related to climate and diet (Nowicki *et al.* 1992, Podos & Nowicki 2004, Friedman *et al.* 2019), relationships between bill size, body size and song structure are not conclusive (Sagar *et al.* 2024, Zampa *et al.* 2025). Evidence suggests that variation in bird song structure may be also explained by ecological factors, for example acting indirectly on bill size and shape due to adaptations for foraging, rather than directly on sound features for better transmission (Nowicki *et al.* 1992, Podos & Nowicki 2004, Derryberry *et al.* 2018).

The founder effect occurs when a reduced number of individuals from a source population colonize a new area (Baker & Jenkins 1987), resulting in limited behavioural and genetic diversity. Consequently, songs may change due to the loss of acoustic diversity of syllable repertoires (Cavalli-Sforza *et al.* 1982, Catchpole & Slater 2008), all of which may lead to population divergence in a relatively short time (Baker *et al.* 2003). On more isolated islands, limited cultural transmission may result in population and species differentiation (Haesler *et al.* 2007, White 2010; but see Illera *et al.* 2014, Oñate-Casado *et al.* 2026), whereas on islands close

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to the mainland, frequent gene flow and cultural transmission may reduce differentiation between populations, leading to cultural and genetic homogenization (Baker & Jenkins 1987, Lynch & Baker 1994). Comparisons of the structure of acoustic signals between closely related populations may provide insights into the biogeographical events that shaped behavioural traits in current populations and more generally into the link between biogeography and behavioural differentiation (Baker & Jenkins 1987, Potvin & Clegg 2015).

The Tres Marías Islands (TMA) is an archipelago located off the central-western Pacific coast of Mexico, which emerged during the Late Pleistocene (c. 120 kya; McCloy *et al.* 1988). Genetic studies suggest that some land-birds dispersed to the islands c. 120 kya when the mainland and TMA islands were closer (c. 25 km, contrasting with the current c. 95 km) due to a c. 100 m lower sea level (Ortiz-Ramírez *et al.* 2018). After sea level rose, TMA populations underwent differentiation due to higher isolation, probably increasing genetic, morphological and cultural divergence between island and mainland populations, all of which has allowed the recognition of endemic subspecies in many taxa (Grant 1965a, Wilson 1991, Leisler & Winkler 2015, Ortiz-Ramírez *et al.* 2018). This is partially supported by the record of longer bills and larger body sizes in TMA passerines than their mainland counterparts (Grant 1965b, 1968, 1971, Gómez de Silva *et al.* 2020). Thus, the TMA represents an ideal setting to study the effect that genetic, morphological and environmental factors have in the evolution of song divergence of island passerines.

Here, we conducted an integrative analysis to test the effects of isolation, as well as biotic (genetic and morphological) and abiotic factors (temperature and precipitation), on the songs of four TMA passerine species and their mainland counterpart populations. We expect less complex songs (fewer shared syllables and lower repertoire sizes) due to founder effects (e.g. a reduced initial pool of song variants maintained by low cultural flow to island populations). Founder effects are inferred from genetic differentiation and diversity. Bird songs with lower frequencies are expected in island populations due to differences in body size (as reflected in bill volume and body-weight). In addition, we also expect that bird songs from populations inhabiting areas with both higher precipitation and temperature will show lower frequencies due to sound transmission.

We first addressed the probable effect of isolation on cultural transmission by comparing syllable repertoires (repertoire size and syllable sharing among populations) with the genetic fixation index F_{ST} in four distantly related TMA oscine (vocal learning) passerines. This allowed us to contrast whether island populations showing songs with a lower syllable diversity also show low levels of gene flow, which would be consistent with a founder effect and a consequent low cultural transmission. Then, we addressed the role of biotic and abiotic factors involved in song differentiation of the TMA populations by performing a multivariate analysis (mixed-effects models) examining the effect of morphology, genetics and environment.

METHODS

Study area

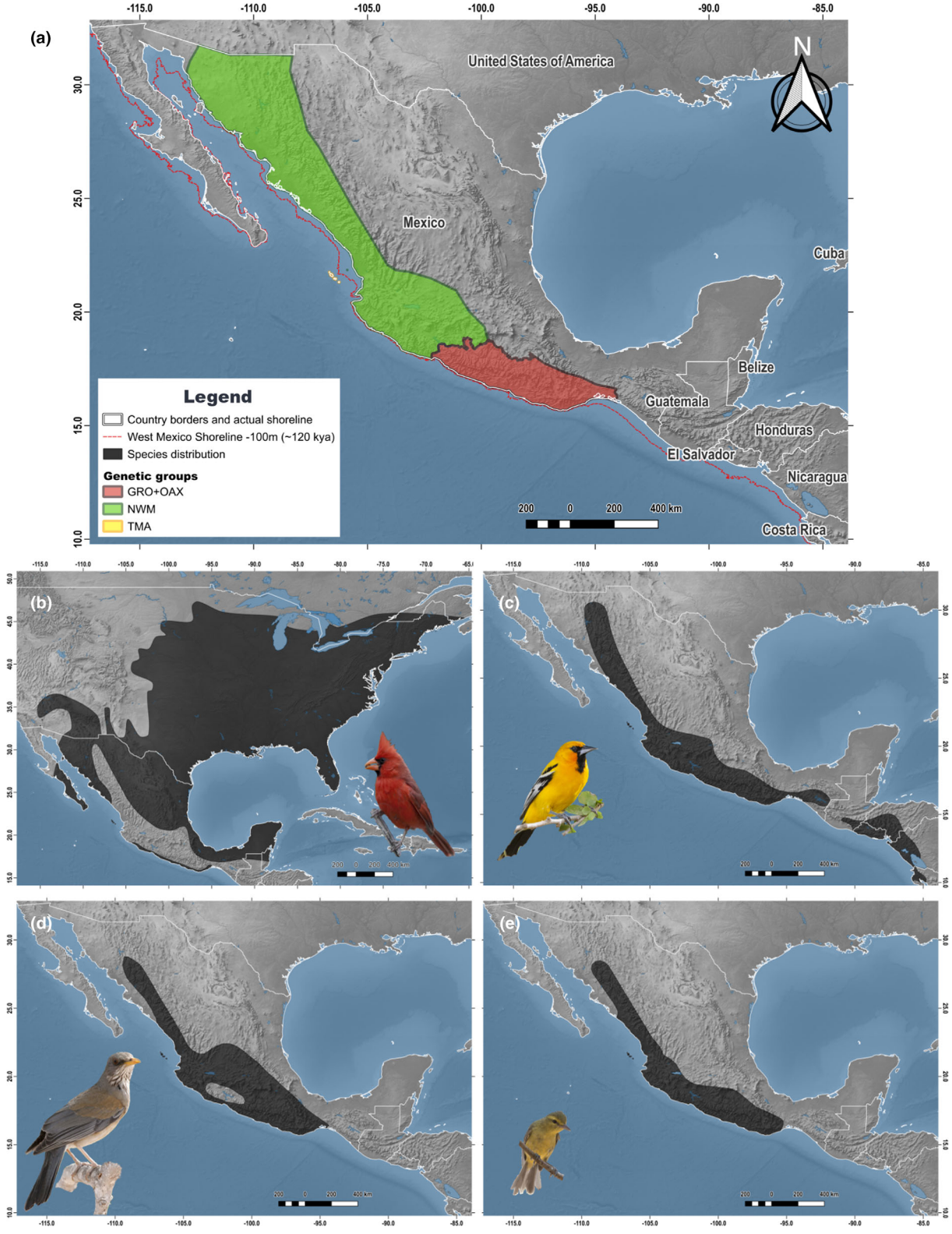
The TMA is composed of four volcanic islands (María Cleofas, María Magdalena, María Madre and San Juanito) located off the central-western coast of Mexico, c. 132 km west of San Blas, Nayarit (Fig. 1). The highest elevation (650 m above sea level) is located on María Madre. The archipelago is mainly covered by seasonally dry tropical deciduous forest and semi-deciduous forest mixed with thorn scrub, mangroves and cacti. The rainy season is from June to December (Grant 1965a, Hahn *et al.* 2012).

Taxon sampling

We analysed four passerine bird species: Northern Cardinal *Cardinalis cardinalis*, Rufous-backed Thrush *Turdus rufopalliatus*, Golden Vireo *Vireo hypochryseus* and Streak-backed Oriole *Icterus pustulatus* (names are simplified from here on as Cardinals, Thrushes, Vireos and Orioles, respectively), distributed across the western coast of Mexico (including the TMA) for which previous studies have documented significant genetic and morphological divergence (bill and body size) between island and mainland populations (e.g. Grant 1965a, Cortés-Rodríguez *et al.* 2008, Smith *et al.* 2011, Smith & Klicka 2013, Arbeláez-Cortés *et al.* 2014, Montaño-Rendón *et al.* 2015, Ortiz-Ramírez *et al.* 2018, Gómez de Silva *et al.* 2020). All study species colonized the TMA

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Figure 1. (a) The Tres Marías Archipelago (21.778°N–21.258°N, 106.698°W–106.198°W) and current geographical distance from the mainland. Genetic groups are represented by colour shades: NWM (green); GRO-OAX (red); and TMA (yellow). Red dashed line depicts the shoreline c. 120 kya (100 m below current sea level). (b) Geographical distribution of Northern Cardinal *Cardinalis cardinalis* (Cardinal). (c) Streak-backed Oriole *Icterus pustulatus* (Oriol). (d) Rufous-backed Thrush *Turdus rufopalliatus* (Thrush). (e) Golden Vireo *Vireo hypochryseus* (Vireo).

around c. 120 kya (Ortiz-Ramírez *et al.* 2018) coinciding with the estimated time of emergence of the archipelago, setting this date as the maximum isolation period for the TMA populations.

We compared TMA and mainland populations because cultural transmission and gene flow may have occurred, and because the current distribution of study species is very similar (Ortiz-Ramírez *et al.* 2018). The present distribution, however, is different in the Cardinal, which is not found on the nearby mainland (Jalisco, Nayarit and south of Sinaloa). Nonetheless, palaeoecological distribution models suggest that its distribution was very similar to the rest of the selected taxa during the Last Glacial Maxima (Smith *et al.* 2011), suggesting modern processes for its current absence but rendering it suitable for comparisons.

Acoustic sampling and song measurements

We obtained song recordings covering the distributional range (mainland western Mexico and the TMA) of the four studied species from digital sound collections (Appendix S1). We supplemented this dataset with our own field recordings during 2006, 2007, 2009, 2012–2014 and 2017. We used the following recording equipment: portable digital recorder (Tascam HD-P2) with a parabolic microphone (Telinga Twinscience Pro 8), and a Marantz PMD 661 MKII recorder with a Sennheiser ME67/K6 shotgun microphone. We recorded the birds we found along trails from 06:00 to 10:30 h and 16:00 to 19:30 h. If it started to rain, we suspended bird song recordings. Environmental conditions were described at the end of each recording. For a satisfactory recording, the recommended maximum distance was 25 m. We positioned recording sites at least 200 m apart from each other along different trails to minimize the likelihood of recording the same individual twice (Ralph *et al.* 1996). However, because the Vireos are highly abundant, we defined a smaller distance between sites for this species (75–150 m). To maximize the number of individuals recorded, whenever multiple individuals sang simultaneously from different directions, we recorded the nearest one first and then attempted to approach the second one; we noted those cases at the end of each recording.

We analysed 160 recordings with a good signal-to-noise ratio (i.e. low background noise and non-overlapping bird songs), as follows: 21 from Cardinals, 40 from Orioles, 31 from Thrushes and 68 from Vireos. Because recordings were obtained from different sources, we standardized them (when necessary) by transforming them from an amplitude resolution of 24 bits to 16 bits and sampling rates of 48–44.1 kHz. For recordings with two channels, we used only the channel with the best signal-to-noise ratio by visual inspection of the spectrograms. Sound spectrograms were generated using Raven Pro 1.4 (http://www.birds.cornell.edu/raven) with 90% overlap in Hann window function and time window of 1024 samples. Because the recordings came from different sources, their duration and quality varied considerably. Therefore, we decided to select at least three to five of the best vocalizations that did not overlap with other sounds and had minimal background noise. We then imported Raven's annotation files into R using *Rraven* (Araya-Salas 2020) for automated selection and measure of song syllables.

We followed Catchpole and Slater (2008) in defining syllables as the song units that occur together in a particular pattern (i.e. a phrase). Under this definition, syllables can be quite simple (one note or element; Catchpole 2000) or more complex (two or more elements conforming a pattern). For syllable selection, we performed automatic detection to get the start and end times of each syllable based on the levels of amplitude previously selected using the *energy_detector* function in the *ohun* package (Araya-Salas *et al.* 2023). After automatic detection, we built an annotation database that was inspected to delete false positives. We also manually corrected

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instances where syllables were clearly shorter or exceeded the syllable length according to a visual inspection of the spectrogram, as implemented in the *seltailor* function in *warbleR* (Araya-Salas & Smith-Vidaurre 2017). We manually selected syllables only for recordings in which no syllables were autodetected, using the function *manualoc*, and added them to the autodetection database. We performed a visual inspection of the variation between island and mainland populations to obtain syllables and song catalogues using the *catalog* function and grouped recordings for each species. We then measured the structure of all annotations using the function *spectro_analysis*, which provides acoustic features related to the distribution of energy in frequency and time and statistical descriptors of dominant frequency contours (Appendix S2). Thus, we grouped syllable variables into three modules: (1) duration, (2) frequency and (3) shape (syllable roundness, flatness or peak shape). All acoustic measurements were quantified using *warbleR* (Araya-Salas & Smith-Vidaurre 2017).

Acoustic range comparison between islands and mainland

Because geographical isolation and avian species composition differences occur between TMA and mainland populations (Hahn *et al.* 2012), we tested whether individuals from TMA populations use different frequency ranges than those from the mainland populations by analysing the dominant frequency range (i.e. subtraction of the minimum dominant frequency from the maximum dominant frequency for each syllable). We then obtained dominant frequency ranges for each population and compared the TMA and mainland using a Kruskal–Wallis test for each species. Additionally, we performed a principal components analysis (PCA) of the frequency variables (minimum and maximum dominant frequencies, dominant frequency range and mean dominant frequency) and syllable duration for TMA and mainland populations with the *princomp* function implemented in the *stats* package in R (R Core Team 2020) and extracted the three principal components (PC1, PC2 and PC3), then plotted them three-dimensionally with the function *scatterplot3d* implemented in the package *scatterplot3d* (Ligges & Maechler 2003).

Vocal repertoire sizes

To account for the repertoire size of each population in each species, we performed an automated classification of syllables using the function *df_DTW* in *warble*R (Araya-Salas & Smith-Vidaurre 2017), which allowed us to estimate the acoustic dissimilarity among the dominant frequency contours of each syllable using dynamic time warping, as implemented in the *dtwDist* function in the *dtw* R package (Giorgino 2009). We then used the *tsne* function in the package *Rtsne* (van der Maaten & Hinton 2008, van der Maaten 2014, Krijthe 2015), which allowed us to visualize clusters of syllables with similar acoustic features; these were classified as syllable types. We then checked whether syllables were correctly classified by visual inspection of the spectrograms, and counted both the number of unique notes and the number of syllables shared between TMA and mainland populations.

Because the enumeration of syllables may not be reliable for species with large repertoires (Botero *et al.* 2008), as in our species set, we first estimated the completeness of our syllable samples using accumulation and rarefaction curves (Chao *et al.* 2014). Then, to compare syllable repertoire sizes between TMA and mainland, we standardized the syllable repertoire samples to achieve >50% coverage (Chao & Jost 2012). This two-step procedure allowed us to perform repertoire size comparisons between TMA and mainland populations of each species.

To mitigate the effect of variation in syllable sampling effort while testing for evidence of founder effects between populations, we first built a presence/absence syllable type matrix for each species and estimated syllable repertoires of TMA and mainland samples using 'iNEXT' (Hsieh *et al.* 2016). After obtaining the sample coverage for island and mainland populations of each species, we identified the lowest sample coverage percentage (>50%) from both populations of each species. We obtained syllable richness corresponding to that sample coverage percentage for the other population. We then compared island and mainland populations of each species by dividing syllable richness of the island population by syllable richness of the mainland population: a value of 1 indicates an equal repertoire size between populations.

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Founder effects in cultural and genetic flow between islands and mainland

We considered high levels of syllable sharing between TMA and the mainland as an indicator of cultural transmission (Jenkins 1978, Rivera-Gutierrez *et al.* 2010, Price & Yuan 2011, Lachlan *et al.* 2013) or recently founded populations. If acoustic divergence is correlated with genetic divergence (e.g. Luo *et al.* 2024), we would expect concordance in the acoustic and genetic difference between populations. We assessed mitochondrial DNA (mtDNA) (*ND2* and *Cytb* genes) genetic differentiation using the F_{ST} fixation index (as previously estimated for these species in Ortiz-Ramírez *et al.* 2018) between TMA and mainland populations. We then contrasted F_{ST} values with the pairwise syllable repertoire-sharing index, which was estimated following Rivera-Gutierrez *et al.* (2010). On the other hand, low levels of genetic differentiation and high levels of syllable sharing could be the result of recent founder effects or high migration rates. To explore founder effects in TMA populations, we considered previously published estimates of genetic diversity (haplotype [h] and nucleotide [π] diversities) for these species (Ortiz-Ramírez *et al.* 2018) and interpreted them according to Grant and Bowen (1998).

To obtain the syllable repertoire-sharing index, we used Dice's similarity index (Dice 1945): $S = 2a/(b + c)$, where 'S' is the similarity between groups, 'a' is the number of syllables shared by the two groups, and 'b' and 'c' are the syllable repertoire sizes of each group. Values = 0 indicate no syllable sharing, while values = 1 indicate complete syllable repertoire sharing.

Morphology and acoustic divergence

To test whether acoustic divergence is driven by body size, bill size or both, we measured 205 adult male specimens of the four selected species, housed in scientific collections in Mexico and the product of fieldwork by several research groups (see Acknowledgements), compiling a sample that included 33 Cardinals, 85 Orioles, 42 Thrushes and 45 Vireos. We obtained three bill measurements (length, depth and width) and lengths of wing chord, tail and tarsus using a Mitutoyo Absolute Digimatic digital calliper (accuracy of 0.1 mm). Bill depth and width were taken at the anterior border of the nostrils; bill length was obtained from the exposed culmen. Wing chord was measured from the carpal joint to the tip of the longest primary feather without flattening the wing. Tarsus length was measured from the tibiotarsus joint to the midpoint of the distal edge of the lowest undivided scute. To minimize variation in bill size due to age and sex, we considered only adult (as determined by specimen skull ossification) male individuals. Body weight was recorded directly from specimen tags.

To test whether bill size differences explain bird song frequency variation between populations, we first removed the influence of body size from bill size measures using a PCA of body measurements (wing chord, tail and tarsus length) according to Huber and Podos (2006). The first principal component (PC1) accounted for 53% of the variation in Cardinals, 68% in Orioles, 60% in Thrushes and 71% in Vireos; most of the PCA loadings corresponded to weight, tarsus length or tail length (Table S1). Then, to obtain bill residuals (per measurement) for each species, we constructed linear regressions between each bill measurement (depth, width and length) and the PC1 of each species. Using these residuals, we estimated bill volume for each population (TMA and mainland) as follows:

$$\text{Vol} = \frac{\pi \times \frac{1}{2}\text{bill depth} \times \frac{1}{2}\text{bill width} \times \text{bill length}}{3}$$

To test whether bird song frequencies (i.e. dominant frequency range, dominant frequency mean, maximum dominant frequency and minimum dominant frequency) are better explained by body size (using body weight as a proxy; Bowman 1979, Palacios & Tubaro 2000, Podos 2001) than by bill volume, we ran Spearman correlations using the mean weight of each group with the frequency of each variable for all of the study species. The significance of these correlations was assessed via the p-values of the Spearman's rho estimates, using the *cor* function as implemented in the stats for R (R Core Team 2020), following recommendations in Akoglu (2018).

Biotic and abiotic factors in bird song divergences

We used precipitation and temperature variables to investigate whether acoustic divergence could be attributed to environmental differences between

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the TMA and mainland (Tobias *et al.* 2010). We extracted values of these environmental variables from each song record of our selected species using the sampling point co-ordinates and the WorldClim climatic environmental layers (Fick & Hijmans 2017), with a resolution of 0.5 min using the function *gedData* of the *raster* R package (Hijmans 2018). We used the mean temperature of the wettest quarter of the year (BIO 8 layer) because it is presumably concordant with higher acoustic activity in birds during the breeding season (Catchpole & Slater 2008, Vokurková *et al.* 2018). We also used the precipitation of the warmest quarter of the year (BIO 18 layer) because in North America acoustic activity occurs mainly during the breeding season (Chiver *et al.* 2015, Vokurková *et al.* 2018). To test whether significant environmental differences between geographical areas were related to acoustic differences, we performed a Kruskal–Wallis test of environmental conditions between the TMA and mainland (MacFarland & Yates 2016). These tests were conducted in R using the *kruskal.test* as implemented in the *stats* package (R Core Team 2020).

We used mixed-effect models to analyse whether acoustic divergence between the TMA and mainland is better explained by genetics, or by the independent and interacting effects of morphological and environmental differences. We used mixed-effect models because sample sizes were unequal between the TMA and mainland, and because our data have a nested structure, with multiple bird song syllables measured from multiple bird songs from each individual, and from several individuals from different populations from different species (Zuur *et al.* 2009, Seoane 2014).

We built models for each of the acoustic variables that may be related to ecological and morphological features: dominant frequency range, dominant frequency mean, maximum dominant frequency, minimum dominant frequency and syllable duration (Podos 2001, Huber & Podos 2006, Tobias *et al.* 2010), and as explanatory variables: bill volume, body size, temperature, precipitation (and their interactions: bill volume × body size, and temperature × precipitation), genetic group and species; syllable types were nested in the songs of each individual (Table S2).

To explore collinearity among explanatory variables, we followed Zuur *et al.* (2010), plotting linear pairwise combinations using the *pairs* function in R and performing a visual inspection to select only those variables without evident collinearity. We then used variance inflation factor (VIF): variables above a threshold of 3 were dropped and we recalculated VIF values until all values were below our selected threshold, which indicated that collinearity is not a major issue (Zuur *et al.* 2009, 2010).

For model selection and validation, we obtained the best fixed effects (bill volume, weight, precipitation, temperature, genetic group and species) using the top-down strategy (Zuur *et al.* 2009), in which we started with the most complex model (as many explanatory variables as possible in the fixed component) and then added different possibilities of random effects (individual and syllable type) to the model. When we obtained different combinations of random effects for the model (Table S2), we selected the best combination via the corrected Akaike information criterion (AIC_c) (Zuur *et al.* 2007, Gałecki & Burzykowski 2013). Once we identified the best fixed effects, we searched for the best model by dropping the non-significant explanatory variable with the highest *p*-value until all explanatory variables were statistically significant. Then, model assumptions were validated by plotting the fitted values against the residuals of the model to detect violations of homogeneity. If there was not a clear tendency in the spread of residuals, we accepted the model; alternatively, we transformed the response variables by applying a $\log_{10}(y+2)$ transformation (Zuur *et al.* 2009).

RESULTS

Acoustic sampling and measurements

From a total of 160 recordings, we selected and measured 2229 syllables as follows: 567 from 21 individual Cardinals, 297 from 40 individual Orioles, 591 from 31 individual Thrushes and 774 from 68 individual Vireos. We found longer syllable duration in TMA populations for Thrushes and Vireos, and shorter syllable duration in TMA populations of Cardinals. No significant differences were found for island or mainland populations of Orioles. The dominant frequency range of syllables was narrower in TMA for Cardinals and Vireos, but in Orioles and Thrushes, we found no significant differences. Syllable mean dominant frequency was lower in TMA populations for Cardinals and Vireos, higher for TMA populations of Thrushes and we did not find significant

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differences in Orioles. The minimum dominant frequency of syllables was lower in mainland populations of Thrushes and Vireos, while the maximum dominant frequencies of syllables were lower in the TMA populations of all species (Table 1).

Acoustic frequency range estimates

The acoustic ranges for Cardinals and Vireos were smaller in the TMA (2.97 ± 1.82 and 0.63 ± 0.31 kHz) than in the mainland (4.28 ± 1.49 and 0.92 ± 0.36 kHz; Kruskal–Wallis test = 61.554, $P < 0.001$; and Kruskal–Wallis test = 188.95, $P < 0.001$). We observed no significant differences between TMA and the mainland for Orioles and Thrushes (Kruskal–Wallis test = 0.033, $P = 0.855$; Kruskal–Wallis test = 1.356, $P = 0.244$). The 3D scatterplots using the principal components showed that acoustic characteristics of TMA populations are not highly differentiated from their mainland counterparts (Supplementary animations available at the GitHub repository: https://github.com/Marco-Ortiz-R/Ecology-and-morphology-as-drivers-of-passerine-song-differentiation-across-an-island-archipelago).

Repertoire sizes

Standardized syllable sample coverage for each species was at 88% for Cardinals, 61% for Thrushes and 89% for Vireos. We were unable to compare repertoire size in Orioles because the TMA population sample coverage was lower than the threshold value of 50% (Fig. S1, Table 2). Once we standardized syllable richness to the lowest sample coverage (Table 2), repertoire size comparisons between TMA and the mainland showed an equal repertoire size for the Cardinals (quotient = 1). The repertoire size in mainland Thrushes is three times longer than TMA Thrushes (quotient = 0.33), while the opposite was observed for Vireos, which had a repertoire size 1.3 times shorter on the mainland (quotient = 1.30).

Cultural transmission, gene flow and founder effects

Syllable sharing between TMA and the mainland was high for Cardinals and Vireos but low for Thrushes (Table 3). According to the F_{ST} values, there is a strong genetic differentiation between TMA and mainland populations, suggesting low gene flow between them (>0.25, Table 3; Ortiz-Ramírez *et al.* 2018). This is also supported by genetic diversity values, which suggest founder effects in all species (Table 3).

Morphology and bird song frequencies

We found significantly larger bill volumes in the TMA populations in comparison with mainland populations for Cardinals and Thrushes, but not for Orioles or Vireos (Table 3). Correlations between body size and bird song frequencies were low but significant for most of the measured variables. For Cardinals, mean dominant frequency, maximum dominant frequency and dominant frequency range of syllables decreased at both larger body sizes and larger bill volumes, while the minimum dominant frequency showed the opposite pattern. In Orioles, the maximum dominant frequency and dominant frequency range increased with increasing body size, while in Thrushes, the mean and maximum dominant frequency decreased with larger body sizes and increased with a larger bill volume. For Vireos, the mean and maximum dominant frequency, as well as the dominant frequency range, increased with larger body sizes but decreased with larger bill volumes (Table 4).

Mixed-effects models

The explanatory variables without collinearity were bill volume, body weight, syllable duration, temperature and precipitation. Our best model considering these variables and their interactions (Table 5) showed that dominant frequency range and maximum dominant frequency were explained by body size variation; larger birds in island populations emitted lower frequencies and narrower frequency ranges. Additionally, although each species has a different dominant frequency range and dominant frequency maximum, TMA populations had narrower dominant frequency ranges and lower dominant frequency maxima (Fig. 2). The model for mean dominant frequency suggests that variation is explained by bill volume and body size; individuals with larger bills and body sizes emitted vocalizations with lower frequencies (Fig. 3).

We found that the mean temperature of the wettest quarter of the year showed less variation in the

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Table 1. Acoustic differences in syllable duration and frequencies between island and the mainland for the four species.

	Species															
	Cardinalis cardinalis				*Icterus pustulatus*				*Turdus rufopalliatus*				*Vireo hypochryseus*			
Variable	Mean (I/M)	sd (I/M)	*H*	*P*-value	Mean (I/M)	sd (I/M)	*H*	*P*-value	Mean (I/M)	sd (I/M)	*H*	*P*-value	Mean (I/M)	sd (I/M)	*H*	*P*-value
Duration (s)	0.192 0.222	0.101 0.066	14.043	<**0.001**	0.098 0.104	0.030 0.018	0.580	0.446	0.471 0.395	0.210 0.152	14.370	**0.001**	0.262 0.171	0.143 0.040	90.995	<**0.001**
Dominant frequency range (kHz)	2.971 4.286	1.817 1.497	61.554	<**0.001**	1.307 1.207	0.837 0.521	0.033	0.855	1.839 1.683	1.441 1.398	1.356	0.244	0.660 0.931	0.381 0.329	188.95	<**0.001**
Mean dominant frequency (kHz)	2.845 3.387	0.659 0.795	52.615	<**0.001**	3.418 3.422	0.340 0.465	0.041	0.839	2.346 2.051	0.525 0.429	25.647	<**0.001**	2.995 3.144	0.293 0.246	87.713	<**0.001**
Minimum dominant frequency (kHz)	1.600 1.620	0.562 0.520	0.056	0.812	2.595 2.808	0.834 0.477	0.107	0.744	1.696 1.448	0.585 0.339	11.076	<**0.001**	2.618 2.595	0.236 0.329	7.390	**0.006**
Maximum dominant frequency (kHz)	4.571 5.906	1.736 1.388	67.386	<**0.001**	3.902 4.015	0.138 0.493	5.783	**0.016**	3.536 3.131	1.346 1.381	11.478	<**0.001**	3.279 3.526	0.421 0.313	170.94	<**0.001**

Mean values and standard deviations are upper for island populations (I) and lower for mainland populations (M); Kruskal–Wallis statistic (H); degrees of freedom = 1. Significant values are shown in bold, $\alpha = 0.05$.

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Table 2. Repertoire sizes (sample coverage estimates and adjusted population repertoire are in parentheses; individual mean syllable repertoires at each population are in square brackets) in islands and mainland for each species.

Species	Area		Species syllable diversity	Adjusted syllable diversity
	TMA	Mainland		
Cardinalis cardinalis	12 (97%) [4.33]	10 (88%) [2.77]	12	10 (TMA)
Icterus pustulatus	4 (40%) [2]	8 (100%) [2.34]	8	NA
Turdus rufopalliatus	19 (61%) [10.66]	99 (88%) [12.27]	104	58 (mainland)
Vireo hypochryseus	9 (89%) [2.37]	10 (98%) [2.24]	11	7 (mainland)

TMA, Tres Marías Archipelago. For *Vireo hypochryseus*, mainland includes all continental populations except for Sinaloa.

Table 3. Syllable sharing (standardized samples); haplotype diversity (h), nucleotide diversity (π), fixation index (F_{ST}) previously published (Ortiz-Ramírez *et al.* 2018); and bill volume comparisons between TMA and mainland populations for the study species, Kruskal–Wallis statistic (H), degrees of freedom (df) and P-values.

Species	Dice similarity index	Population	h/π	F_{ST}	H	df	P-value
Cardinalis cardinalis	0.80	TMA	0.73/0.002	0.77	13.816	1	**<0.001**
		Mainland	0.87/0.015				
Icterus pustulatus	NA	TMA	0.70/0.001	0.34	3.019	1	0.082
		Mainland	0.85/0.004				
Turdus rufopalliatus	0.18	TMA	0.53/0.001	0.84	7.498	1	**0.006**
		Mainland	0.69/0.002				
Vireo hypochryseus	0.75	TMA	0.00/0.000	0.84	1.610	1	0.205
		Mainland	0.89/0.005				

$\alpha = 0.05$, significant values are shown in bold. Note that syllable sharing in *Icterus pustulatus* was not calculated due to sample coverage below the threshold limit.

Table 4. Correlation (r_s, Spearman's rho) between morphological measurements and bird song frequencies for each species.

		Species							
		Cardinalis cardinalis		*Icterus pustulatus*		*Turdus rufopalliatus*		*Vireo hypochryseus*	
Acoustic variables	Morphometric variables	r_s	P-value	r_s	P-value	r_s	P-value	r_s	P-value
Dominant frequency mean	Body size	−0.221	**<0.001**	−0.064	0.267	−0.159	**<0.001**	0.297	**<0.001**
	Bill size adjusted by body size	**−0.284**	**<0.001**	0.070	0.225	0.149	**<0.001**	−0.335	**<0.001**
Dominant frequency minimum	Body size	0.102	**0.015**	0.042	0.471	−0.043	0.295	−0.082	**0.021**
	Bill size adjusted by body size	−0.005	0.911	0.057	0.325	0.159	**<0.001**	0.105	**0.003**
Dominant frequency maximum	Body size	−0.131	**<0.001**	0.217	**<0.001**	−0.125	**0.002**	0.439	**<0.001**
	Bill size adjusted by body size	−0.340	**<0.001**	0.056	0.332	0.080	**0.049**	−0.413	**<0.001**
Dominant frequency range	Body size	−0.104	**0.013**	0.249	**<0.001**	−0.077	0.059	0.448	**<0.001**
	Bill size adjusted by body size	−0.327	**<0.001**	0.215	**<0.001**	−0.006	0.869	−0.465	**<0.001**
Duration	Body size	−0.228	**<0.001**	0.117	**0.043**	−0.200	**<0.001**	0.235	**<0.001**
	Bill size adjusted by body size	0.101	0.795	0.062	0.284	0.030	0.462	−0.490	**<0.001**

Significant values are in bold. $\alpha = 0.05$.

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Table 5. Mixed-effects models that best explain the acoustic variation (according to AIC_c values) in five acoustic features considering morphological variation (bill and body sizes), genetic divergence, environmental conditions (temperature and precipitation) and geographical isolation of the study species.

Variables	Model	Fixed effect	Random effect	Grouping	Difference in AIC_c	df
Dominant frequency range	M8a[a]	Body size + genetic group + species	Individuals	Syllable type	0.0	9
Dominant frequency minimum	M7b[a]	Temperature + precipitation + temperature : precipitation + species	Individuals	Syllable type	0.0	10
Dominant frequency mean	M6c[a]	Bill + body size + temperature : precipitation + precipitation + species	Individuals	Syllable type	0.0	11
Dominant frequency maximum	M8d[a]	Body size + genetic group + species	Individuals	Syllable type	0.0	9
Duration	M7e[a]	Bill + body size + precipitation + species	Individuals	Syllable type	0.0	10

Bill refers to bill volume; genetic group refers to TMA or mainland populations; df, degrees of freedom; ':', a simplified notation to indicate the interaction between two variables. [a]The best AIC_c model for each variable (see all models in Table S2).

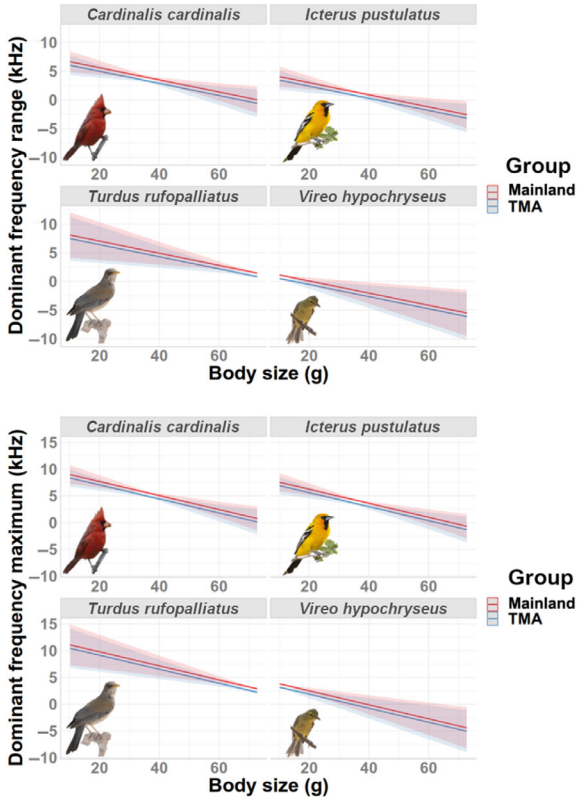


Figure 2. Predicted values for dominant frequency range and dominant frequency maximum for TMA and mainland populations.

TMA than in the mainland, while during the rainy season, precipitation is lower and less variable in the TMA in comparison with the mainland (Table 6). These environmental differences between island and mainland populations affected song frequencies. Larger individuals produce lower frequencies, while their song frequency minimum in areas with low precipitation is not affected as in the TMA. However, in more humid areas, bird songs increased in minimum frequency as temperature increased. Our best model also suggested that the dominant frequency mean is affected by the interaction between precipitation and temperature; in areas with lower temperatures, bird song frequency increased as precipitation increased, while in areas with higher temperatures, bird song frequency decreased as precipitation increased (Fig. 3).

The model for syllable minimum dominant frequency revealed a clear pattern: individuals with larger body size and bill volume emitted syllables with lower minimum dominant frequencies (Fig. 3). It also showed that in less humid areas there was almost no effect of temperature, but in areas of higher precipitation, syllable minimum dominant frequency increased with increasing temperature (Fig. 3).

For syllable duration, we found that island individuals, which usually have larger body sizes and bill volumes, produced shorter syllables. In addition, syllables were longer in more humid areas, as on the mainland (Fig. 4).

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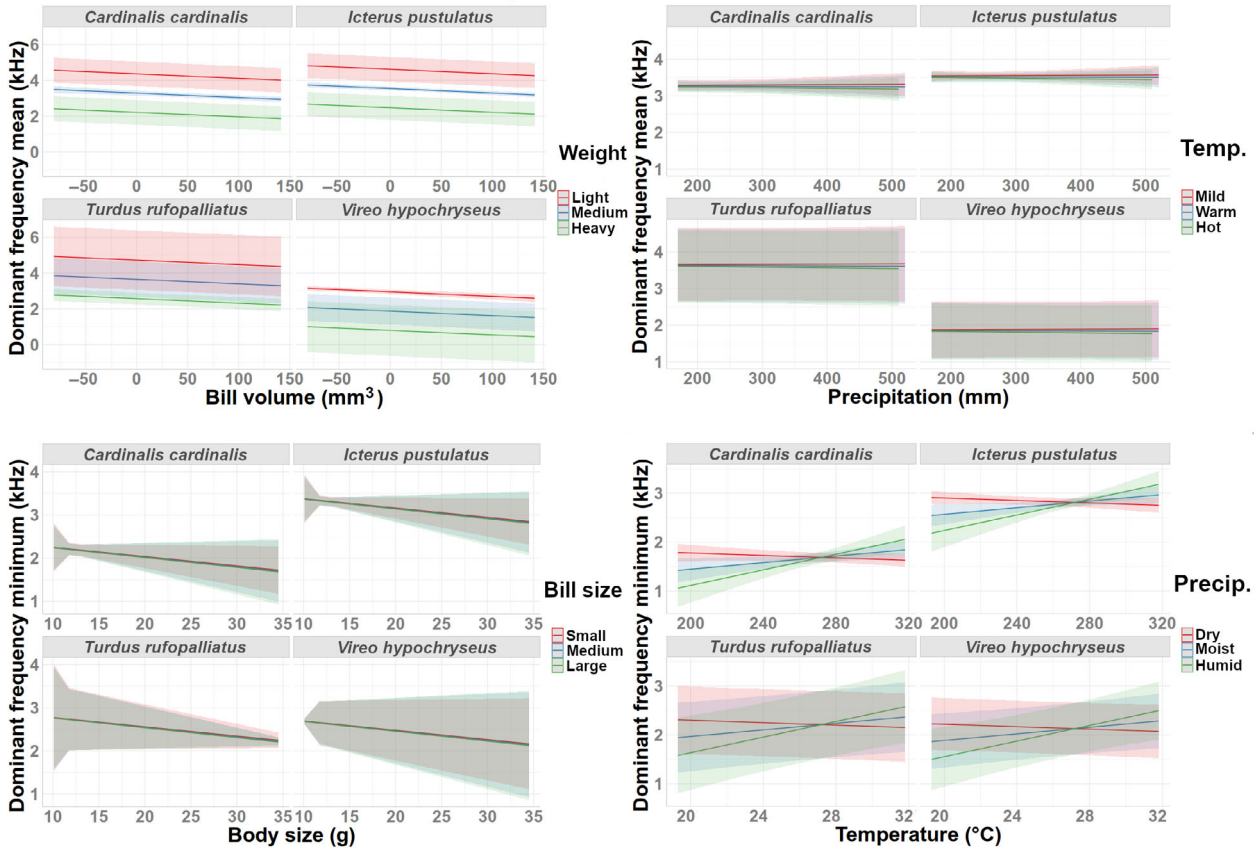


Figure 3. Predicted values for mean dominant frequency according to the best model are best explained by bill volume and body weight as a proxy of body size (light: 13.79 g; medium: 36.63 g; and heavy: 59.46 g; upper left), and precipitation and temperature (mild: 23°C; warm: 25°C; and hot: 27°C; upper right).

DISCUSSION

Overall, we found significant differences in genetic, morphological and acoustic characters between TMA and mainland populations. Our results suggest that differences in bird song frequencies are related to morphology and also that environmental differences between areas are related to bird song frequencies probably due to sound transmission. Although we find no overall support for a general pattern (see also Morinay *et al.* 2013, Robert *et al.* 2022), we provide evidence that morphological and environmental differences, along with geographical isolation, are influencing bird song evolution in TMA populations.

Song differentiation in insular bird populations

We expected our study species to show an expanded acoustic frequency range in the TMA due to a wider acoustic space given a lower species richness (which may indicate less competition for acoustic space as observed in other islands; Robert *et al.* 2019), larger body sizes, larger bill volumes and differences in vegetation structure. Although we observed larger bill volumes in island populations of Cardinals and Thrushes, the acoustic frequency ranges of island populations of Orioles and Thrushes were not different from their mainland counterparts, while the acoustic frequency ranges of Cardinals and Vireos were smaller than on the mainland. This lack of any apparent general pattern was also observed in the Macaronesian islands (Illera *et al.* 2014). However, our results could be explained by syllable repertoire sharing, resulting in similar frequency ranges. Specifically, the island populations of Cardinals and Vireos had the highest syllable sharing rates compared to their mainland counterparts. Our analysis suggests

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Table 6. Environmental differences in temperature and precipitation obtained from WorldClim environmental climatic layers (Fick & Hijmans 2017, bio8 and bio18) between TMA and mainland populations.

		Comparisons
Variables		TMA versus mainland
Temperature	Mean temperature of the wettest quarter of the year	25.8 ± 0.3°C in TMA versus 25.8 ± 4.2°C in mainland ($\chi^2 = 55.66$, df = 1, ***P* < 0.001**)
Precipitation	Precipitation of the warmest quarter of the year	586 ± 45 mm in TMA versus 880 ± 479 mm in mainland ($\chi^2 = 12.06$, df = 1, ***P* < 0.001**)

Significant *P*-values are in bold ($\alpha = 0.05$).

that both body size and environment modify syllable frequencies, which is concordant with the 'insular rule' (Sebastianelli *et al.* 2022a, 2022b), which suggests that birds that sing near the coast increase their dominant frequency to improve communication by avoiding ocean noise. For Orioles and Thrushes, the absence of acoustic frequency range differences seems to be related to ecological factors rather than repertoire sharing. In our study species, the Thrush showed lower repertoire sharing between TMA and the mainland. However, we found that the frequency range was similar to that on the mainland. This suggests that even when populations use different syllables for communication and show significant differences in bill volume, the best sound transmission frequencies apparently constrain bird songs in TMA. These findings are consistent with other studies analysing the effects of morphology and habitat on acoustic divergence (Derryberry *et al.* 2018). However, we do not discard acoustic competition with other bird species, or even with insects, as has been previously reported in Amazonian birds (Torres *et al.* 2019). Further studies are needed on this issue.

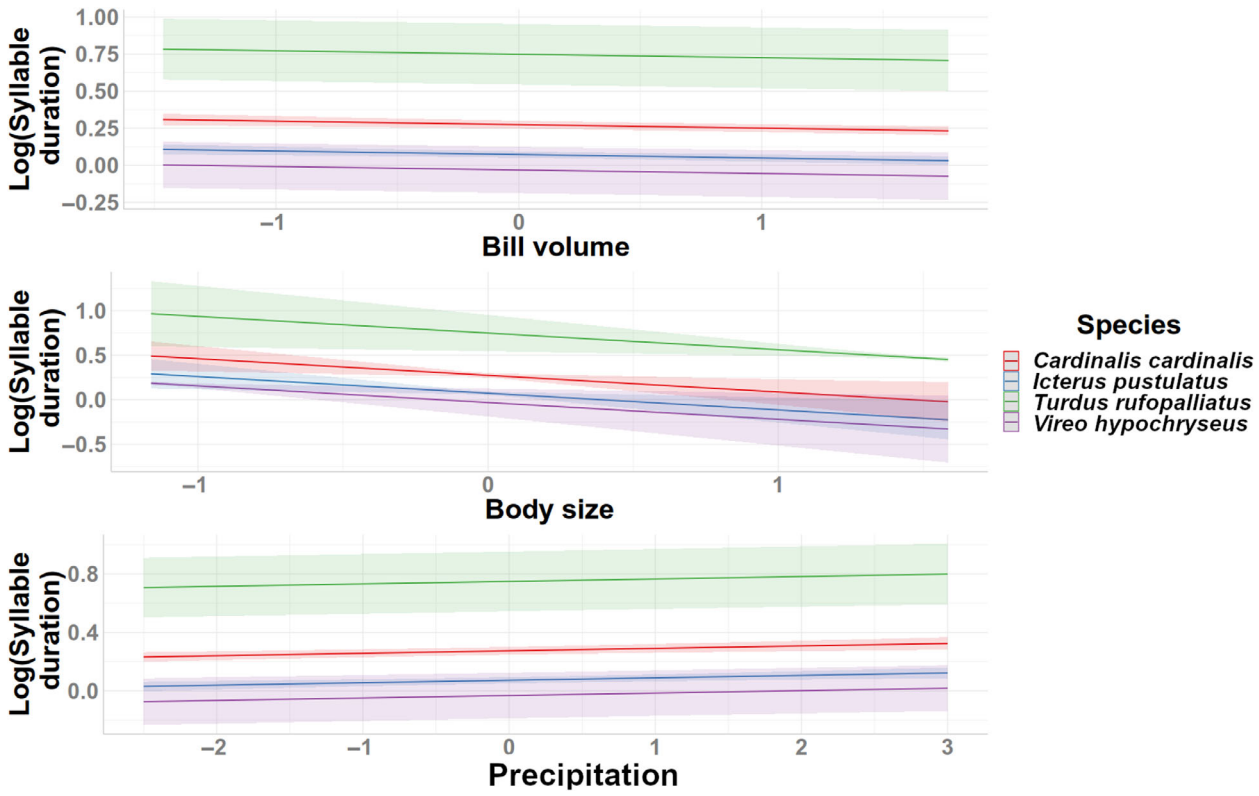


Figure 4. Bird song syllable length is negatively related to bill volume (upper) and shows a stronger negative relationship with body size (middle). Syllable length is positively related to precipitation (bottom).

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Cultural flow and bird song divergence

Our results for repertoire sizes of TMA and mainland populations showed variations among the study species, hindering the development of generalized explanations. Nevertheless, geographical isolation has probably reduced gene flow. This is supported by the very high genetic differentiation F_{ST} values, which suggest that TMA populations have very low to null gene flow with mainland populations. However, as in other island taxa, variation in song traits, presumably related to cultural transmission, was not fully explained by geographical isolation (see Baker *et al.* 2001, Baker *et al.* 2006, Sosa-López & Mennill 2014a, 2014b). Different studies on island birds have reported acoustic divergence in a relatively short time (Päckert *et al.* 2003, Päckert 2006, Baker 2012, Aleixandre *et al.* 2013). This may be the case for the Thrushes, which have both the lowest syllable-sharing index and highest F_{ST} values between TMA and the mainland, suggesting high genetic and cultural differentiation among island and mainland populations due to geographical isolation (Montaño-Rendón *et al.* 2015, Ortiz-Ramírez *et al.* 2018). For the other three study species, if acoustic divergence has increased in the c. 120 kyr since TMA colonization, then greater song differences and lower syllable sharing between the TMA and the mainland would be expected. However, genetic differences in these three species do not correspond in magnitude to the syllable repertoire-sharing index observed (Ortiz-Ramírez *et al.* 2018). Thus, genetic differentiation is apparently not accompanied by acoustic differentiation, but rather the latter may be influenced by environmental factors, which may represent a selective pressure for sound transmission (see below), as supported by studies in Galapagos finches (Grant & Grant 1995, Podos & Nowicki 2004). According to Wilson (1991), the lowlands of Sinaloa (north-western Mexico) are the most environmentally similar area to the TMA. Previous genetic studies on the study species have suggested that TMA populations are more closely related to north-western Mexico populations, which have been found to be the most probable source for the colonization of the TMA (Ortiz-Ramírez *et al.* 2018).

After dispersal, a reduced syllable diversity is expected due to the limited number of colonizers, which may represent a subsample of the vocal repertoire of the source population. However, we did not find evidence for any founder effect on syllable repertoires. Instead, the high number of shared syllables between the TMA and the mainland seems to be unrelated to genetic differentiation between the islands and the mainland. This suggests that syllable sharing could be the result either of colonizers having almost identical syllable repertoires as those of the source populations or that other evolutionary pressures (e.g. sexual selection and/or ecological selection) are maintaining syllable diversity in the TMA (Baker 2006, Graham *et al.* 2018, Robinson & Creanza 2019; but see Illera *et al.* 2014, Oñate-Casado *et al.* 2026). A further possibility is that the fixation of new syllables in island populations in a short time may be the result of low population sizes (Futuyma & Kirkpatrick 2017), or probably due to sexual selection favouring local syllable types within each population (Williams *et al.* 2013).

Morphological variation and bird song differentiation

We found a general pattern in bird song frequencies related to body size and/or bill volume (Demery *et al.* 2021, Sebastianelli *et al.* 2022a, 2022b, Sagar *et al.* 2024). Both the syllable dominant frequency range and the maximum dominant frequency were strongly related to body size and bill volume. All our study species emitted songs with higher frequencies, with larger individuals producing lower frequencies, as expected (Ryan & Brenowitz 1985). This same relationship has been found in antbirds (Seddon 2005) and some species of Thraupidae (Mason & Burns 2015, but see Porzio *et al.* 2018). We found that island populations showed lower frequencies for these acoustic variables and larger body sizes. This relationship may be driven by body size but not by bill volume, as in Asian barbets (González-Voyer *et al.* 2013, but see Luther & Danner 2016). However, a similar pattern was observed for the mean dominant frequency, which is related to both body size and bill size (Podos 2001, Huber & Podos 2006, Sagar *et al.* 2024).

Ecology and acoustic divergence

Our study species showed increased syllable duration in areas with higher precipitation, especially

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in north-western Mexico, the Mexican monsoon region (Gochis *et al.* 2006). North-western Mexico generally has denser vegetation due to the combination of high precipitation and high temperature (Rzedowski 2006, Yan *et al.* 2017), which may represent an obstacle to sound transmission and may modify bird song frequency and syllable rates (Morton 1975, Slabbekoorn & Smith 2002a, 2002b; but see Mikula *et al.* 2021, Freitas *et al.* 2025). Our results for all study species suggest that environmental conditions are significant drivers that ultimately affect syllable duration and frequency. It is interesting that the main factor affecting syllable duration is precipitation rather than bill features, as observed in other birds (Derryberry *et al.* 2012, García & Tubaro 2018, Demery *et al.* 2021). Our findings therefore suggest that bird song evolution is also driven by ecological factors (Derryberry *et al.* 2018, Andrade-González *et al.* 2023). This is further supported in other studies, which concluded that bird song divergence is correlated with sound transmission properties of the habitat rather than with genetic divergence or the effects of body mass or bill size (Tobias *et al.* 2010).

Integrative analyses of bird song differentiation in islands

Our selected models (Table 5) showed a clear pattern in which body size is more relevant than bill volume for syllable duration, although both are statistically significant. Previous studies in tanagers have found that birds with larger bodies tend to produce slower-paced songs (Mason & Burns 2015). Similar results were observed in fringillids, where larger species also tended to have longer note duration (García *et al.* 2014). However, other studies found no relationships between syllable duration and body weight nor bill volume, as in Cardinalidae (García & Tubaro 2018). In North American vireos, highly similar syllable durations are produced regardless of body size (Borror 1972). In *Icterus* orioles, a strong convergence in song regardless of body size has been reported (Price *et al.* 2007). In addition to morphology, our models also suggest that precipitation is a significant factor, which is concordant with previous studies that considered precipitation and other ecological factors to explain bird song variation (Tobias *et al.* 2010, Mason & Burns 2015, Derryberry *et al.* 2018, Reudink *et al.* 2021).

Our analyses support the importance of the environment to explain acoustic variation. Although temperature does not have much influence on syllable frequency in areas with less precipitation, the more humid the area, the stronger the influence of temperature over frequency. We conclude that both biotic and abiotic factors are important for bird song differentiation on the TMA in the four studied species. We found that morphology and the environment are apparently driving bird song evolution independently of genetic divergence, which is congruent with other studies in lowland forest passerine birds (Gogoleva 2020). These factors and geographical isolation have all differentially contributed to acoustic differences between island and mainland populations. However, further analyses should address factors that were not measured in this study such as sexual selection, the presence or absence of other species in maintaining the similarity of song syllables between the two areas, the possibility of acoustic competition with other taxa and the use of singing birds for obtaining morphological measurements for accurate comparisons.

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provided key and useful comments on previous versions of the manuscript and help in data analysis.

AUTHOR CONTRIBUTIONS

Marco F. Ortiz-Ramírez: Conceptualization; methodology; data curation; investigation; validation; formal analysis; writing – original draft; writing – review and editing. **Eduardo Iñigo-Elías:** Data curation; investigation; supervision; writing – original draft; writing – review and editing. **Marcelo Araya-Salas:** Methodology; software; data curation; investigation; writing – original draft; writing – review and editing. **Fernanda Bribiesca-Contreras:** Methodology; data curation; writing – original draft; writing – review and editing. **Luis. A. Sánchez-González:** Conceptualization; methodology; data curation; investigation; formal analysis; writing – original draft; writing – review and editing. **Juan Francisco Ornelas:** Conceptualization; methodology; investigation; formal analysis; writing – original draft; writing – review and editing. **Adolfo G. Navarro-Sigüenza:** Conceptualization; methodology; data curation; investigation; supervision; writing – original draft; writing – review and editing; funding acquisition; resources; formal analysis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

Supplementary animations that support the findings of this study are available at the GitHub repository: https://github.com/Marco-Ortiz-R/Ecology-and-morphology-as-drivers-of-passerine-song-differentiation-across-an-island-archipelago.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Accumulation curves for syllable diversity completeness estimates (to the left) and sample coverage (to the right) for mainland (top) and TMA (bottom) populations of (A) *Cardinalis cardinalis*, (B) *Icterus pustulatus*, (C) *Turdus rufopalliatus* and (D) *Vireo hypochryseus*. Black dots depict the observed value, whereas sample coverage of 1 corresponds to 100% of the estimated syllable diversity. Number of sampling units is the number of individuals recorded.

Table S1. Principal component loadings of morphological variables for each species of this study.

Table S2. Mixed-effects models considered to explain the acoustic variation in five acoustic features related to morphological variation, genetic divergence and geographical isolation among the four species.

Appendix S1. Sound library sources, localities and geographical co-ordinates for each recording of the study species.

Appendix S2. Description and units of the acoustic parameters measured with *warble*R. Variables are grouped in three modules: (1) duration, (2) frequency and (3) shape of notes (roundness, flatness or peak shape).

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