

GENETIC, MORPHOLOGICAL, AND ACOUSTIC EVIDENCE REVEALS LACK OF DIVERSIFICATION IN THE COLONIZATION PROCESS IN AN ISLAND BIRD

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Songbirds with recently (i.e., early Holocene) founded populations are suitable models for studying incipient differentiation in oceanic islands. On such systems each colonization event represents a different evolutionary episode that can be studied by addressing sets of diverging phenotypic and genetic traits. We investigate the process of early differentiation in the spectacled warbler (*Sylvia conspicillata*) in 14 populations separated by sea barriers from three Atlantic archipelagos and from continental regions spanning from tropical to temperate latitudes. Our approach involved the study of sexual acoustic signals, morphology, and genetic data. Mitochondrial DNA did not provide clear population structure. However, microsatellites analyses consistently identified two genetic groups, albeit without correspondence to subspecies classification and little correspondence to geography. Coalescent analyses showed significant evidence for gene flow between the two genetic groups. Discriminant analyses could not correctly assign morphological or acoustic traits to source populations. Therefore, although theory predicting that in isolated populations genetic, morphological, or acoustic traits can lead to radiation, we have strikingly failed to document differentiation on these attributes in a resident passerine throughout three oceanic archipelagos.

KEY WORDS: Avian evolution, gene flow, lack of diversification, Macaronesia, oceanic islands.

To understand the mechanisms operating in the differentiation of populations is one of the most important challenges in evolution. Speciation theory provides the conceptual framework for understanding how species can evolve under restricted and unrestricted gene flow (Turelli et al. 2001), and empirical studies have provided evidence for both circumstances (e.g., Emerson and Oromí 2005; Papadopulos et al. 2011; Warren et al. 2012).

Mechanisms involved in speciation processes have fascinated island evolutionary biologists inasmuch as they provide the opportunity to study selection pressures from incipient till complete speciation events, with the best known examples coming from the bird literature (e.g., Denninson and Baker 1991; Mayr and Diamond 2001; Grant and Grant 2008; Uy et al. 2009; Milá et al. 2010). Recent results investigating phylogenetic relationships and



phenotypic similarities in early differentiation of island birds have also revealed homoplasious evolution (i.e., birds sharing traits because of convergence) among lineages (Warren et al. 2005; Cibois et al. 2007; Kirchman 2009), perhaps because individuals inhabiting small island environments show a high degree of niche conservatism, thus experiencing similar selective pressures and responding in a similar way. Therefore, although it is feasible to document the relative importance of each element operating during the diversification process in those taxa with advanced stages of differentiation, such as those with conspicuous phenotypic variation (e.g., Grant and Grant 2010; Warren et al. 2012), the extent to which slight variation in plumage color and morphological traits are influencing the recognition of conspecifics (after a recent split from the parental population) is an issue not well understood (but see Toewes and Irwin 2008; Podos 2010).

Because oceanic islands are geographically discrete units, and have not been connected to the mainland, they provide tractable study systems to understand evolutionary diversification processes (Emerson 2002; Grant and Grant 2008; Illera et al. 2012). Much effort has been dedicated to infer evolutionary histories among species, subspecies, and populations with advanced stages of differentiation (Illera et al. 2011; Melo et al. 2011; Chaves et al. 2012; Jönsson et al. 2014). However, few studies have evaluated genetic and morphological changes in animals with Holocene colonization and diversification times (Clegg et al. 2002a,b; Illera et al. 2007; Spurgin et al. 2014). Genetic data per se do not provide the reasons explaining the mechanisms operating in population differentiation; however, such information in combination with data on phenotypic variation (e.g., morphological, acoustic, behavioral, etc.) can highlight specific traits that might be involved in reproductive isolation (Sætre and Sæther 2010). To establish whether the origin of reproductive isolation occurs before (prezygotic) or after (postzygotic) zygote formation, and determine their relative contribution on the gene flow prevention is still a challenge in our understanding of the speciation process (Marie Curie SPECIATION network 2012). In wild animal populations, postzygotic isolation mechanisms are not easily traceable because of the inherent complexity of estimating offspring fitness, and only in hybrid zones can the contribution of post over prezygotic mechanisms be addressed with precision (e.g., Shuker et al. 2005; Wiley et al. 2009). On the other hand, song and morphology are considered the most important prezygotic reproductive barriers in birds and their variation can be more readily quantified (Grant and Grant 2008; Price 2008). Nevertheless, despite a plethora of studies investigating how and when song and morphology diverge (e.g., Grant and Grant 1997, 2010; Laiolo and Rolando 2003; Kirschel et al. 2009; Tobias et al. 2010), their specific role and interaction in the diversification process is not well understood yet (Wilkins et al. 2013). Random processes together with sexual

and natural selection through competition, predation, and different environmental pressures may all be involved in the origin of divergence in morphology and song (e.g., Nicholls et al. 2006; Grant and Grant 2010; Medina and Francis 2012; Lapiedra et al. 2013).

Songbirds with recently (i.e., early Holocene) founded populations are suitable models for studying incipient differentiation because each colonization event represents an independent evolutionary episode within which it is feasible to estimate how song, morphology, and genes have diverged on short time scales. The spectacled warbler (*Sylvia conspicillata*) is a sexually dimorphic small passerine (approximately 8.5 g) with a patchy distribution around the Mediterranean basin, but with a broad distribution across the oceanic North Atlantic archipelagos (Macaronesia) of Madeira, the Canary Islands, and Cape Verde (Cramp 1992; Del Hoyo et al. 2006). The species inhabits open and semiarid habitats with scattered trees and bushes from the coast to high altitudes. Despite its broad distribution only two subspecies, described on the basis of slight difference in feather coloration, are recognized: *S. conspicillata conspicillata* occurs around the Mediterranean region and *S. c. orbitalis* is found in Macaronesia. In general, Mediterranean mainland populations are considered medium-distance migrants wintering in the pre-Saharan or Saharan range (Cramp 1992; Tellería et al. 1999; BirdLife International 2012), whereas Macaronesian and Mediterranean island populations are sedentary with some evidence for altitudinal movements within islands (Cramp 1992). The species is locally abundant and widespread across Macaronesian archipelagos, being especially abundant in the Canary Islands (Trujillo 2007) and the Cape Verde islands (Hazevoet 1995; P. Laiolo and J. C. Illera, unpubl. data). Males sing a complex song with a variable number of syllables (4–69) that indicates great capability for innovation (Palmero et al. 2012, 2014). Innovation by founder individuals can lead to sexual signal divergence among populations (Lynch and Baker 1994), and ultimately lead to speciation, a phenomenon that has often been described in old-world warblers (Irwin et al. 2001; Brambilla et al. 2008). In addition, song divergence is typically more rapid than morphological divergence due to both cultural transmission and the relatively rapid divergence in sexually selected traits (e.g., Seddon et al. 2013). Therefore, the spectacled warbler appears an ideal species to investigate colonization patterns and incipient differentiation in morphology, song, and genetic structure among island populations across Macaronesia.

The goal of this study is to examine the colonization process of the spectacled warbler in the Macaronesian islands to quantify population differentiation and understand which traits are more important during the divergence. We use a combination of morphological, acoustic, and genetic (mtDNA and microsatellite) data obtained along a latitudinal gradient of 3580 km across

Macaronesia and the closest continental areas (north Africa and the Iberian Peninsula). We predict some incipient differentiation between insular and continental populations on the basis of the two described subspecies, but it is also plausible to expect to find some significant differentiation among island populations as the sea is expected to constitute an effective barrier to dispersal, at least between the most distant islands (Illera et al. 2012). In addition, the wide latitudinal gradient, spanning from the temperate zone to the tropics, entails dramatic climatic variation that is likely to fuel phenotypic differentiation (Blackburn et al. 1999). Because song in oscine birds is learnt, this trait is expected to accumulate more variation and at a much faster rate through founder effects, copying errors or local adaptations during the first phases of the colonization process than other attributes. Overall, under this scenario of colonization we predict to find a “song radiation” pattern across Macaronesia preceding the morphological and genetic changes.

Material and Methods

SAMPLE COLLECTION

Warblers were sampled across a latitudinal gradient of 3580 km. Sampling included 12 Macaronesian islands across the three archipelagos where the species breeds, plus the two closest continental areas with breeding populations (the Iberian Peninsula and Morocco; Fig. 1). Birds were captured at multiple localities to maximize the variability within each population (except in Morocco where only one locality was sampled due to limited information about its distribution) using mist nets and song playback. For each population, we caught 22–39 individuals (Table 1). Sampling was carried out from January to June 2010 (Madeira, Canary Islands, and the Iberian Peninsula), September 2010 (Cape Verde), and from February to April 2011 (Canary Islands, Morocco, and Porto Santo). Birds were ringed with unique numbered aluminum ring from the relevant Spanish or Portuguese authorities, or with a colored plastic ring in the Moroccan and Cape Verde populations. Age and sex (but see below) was determined based on color and feather molt pattern (Cramp 1992), and eight morphometric traits (see below) were measured. Blood samples (about 40 μ l) were collected by brachial or jugular venipuncture, preserved in 800 μ l of absolute ethanol in a screw-cap microfuge tube, and stored at room temperature. Finally, birds were released in the same place where they were caught.

LABORATORY PROCEDURES

DNA was extracted using Qiagen’s DNeasy tissue kit according to the manufacturer’s protocol. Because some individuals showed a phenotypic pattern compatible with male and female, we sexed all individuals using the molecular procedure specified in Griffiths et al. (1998).

A fragment of 998 base pairs (bp) of the cytochrome *b* gene was amplified for 10 individuals per population, and sequenced using the primers H4A (Harshman 1996), L14841 (Kocher et al. 1989), and H15767 (Edwards et al. 1991) following the conditions described in Illera et al. (2008). In addition, we obtained two additional spectacled warbler cytochrome *b* sequences from birds caught in Israel (JF502289) and Senegal (AJ534539) available from GenBank.

All individuals were genotyped for 15 polymorphic microsatellites (Table S1), arranged into three multiplex reactions obtained with the software Multiplex Manager 1.0 (Holleley and Geerts 2009). Polymerase chain reactions (PCR) were set up in 2 μ l reactions using a method based on Kenta et al. (2008). Negative controls were included to detect potential contamination. PCRs were performed with the following conditions: initial denaturing phase of 95°C for 15 min was followed by 40 cycles of 94°C for 30 sec, 56°C for 90 sec, and 72°C for 60 sec. A final hold of 60°C for 30 min completed the reaction. PCR products were diluted 1 in 700, separated on a MegaBACE 1000 (GE Healthcare, La Laguna) analyzer, and sized using the software MegaBase Fragment Profiler version 1.2 (Amersham Biosciences, Fairfield, CT, 2003. <http://www.amershambiosciences.com>).

MITOCHONDRIAL ANALYSES

Sequences were aligned by eye using BioEdit version 7.0.9 (Hall 1999). The program DnaSP version 5.10.01 (Librado and Rozas 2009) was used to calculate the number of haplotypes, haplotype diversity (h , Nei 1987), the degree of polymorphism (π , Nei 1987), and theta ($\theta = 2N_e\mu$, Tajima 1996) values for each population. Genetic differentiation between populations was examined using analyses of molecular variance (AMOVAs) as implemented in ARLEQUIN version 3.5.1.2 (Excoffier and Lischer 2010). To test the hypothesis that genetic differences are stronger among than within archipelagos, we grouped islands at the level of the archipelago and treated the two continental populations as one group. We performed a similar approach considering the two subspecies described, that is, we treated all archipelagos as one group and the mainland populations as another group. Significance of AMOVA analyses was tested using 10,000 random permutations.

Finally, phylogenetic relationships among mitochondrial sequences were determined through parsimony haplotype network using the program TCS version 1.21 (Clement et al. 2000). We used the default limit of 5% and missing data or gaps were treated as a fifth state.

ANALYSES OF MICROSATELLITE DATA

Genetic diversity including observed (H_O) and expected heterozygosities (H_E), number of alleles, allelic richness, and inbreeding coefficient (F_{IS}) were calculated with the programs ARLEQUIN

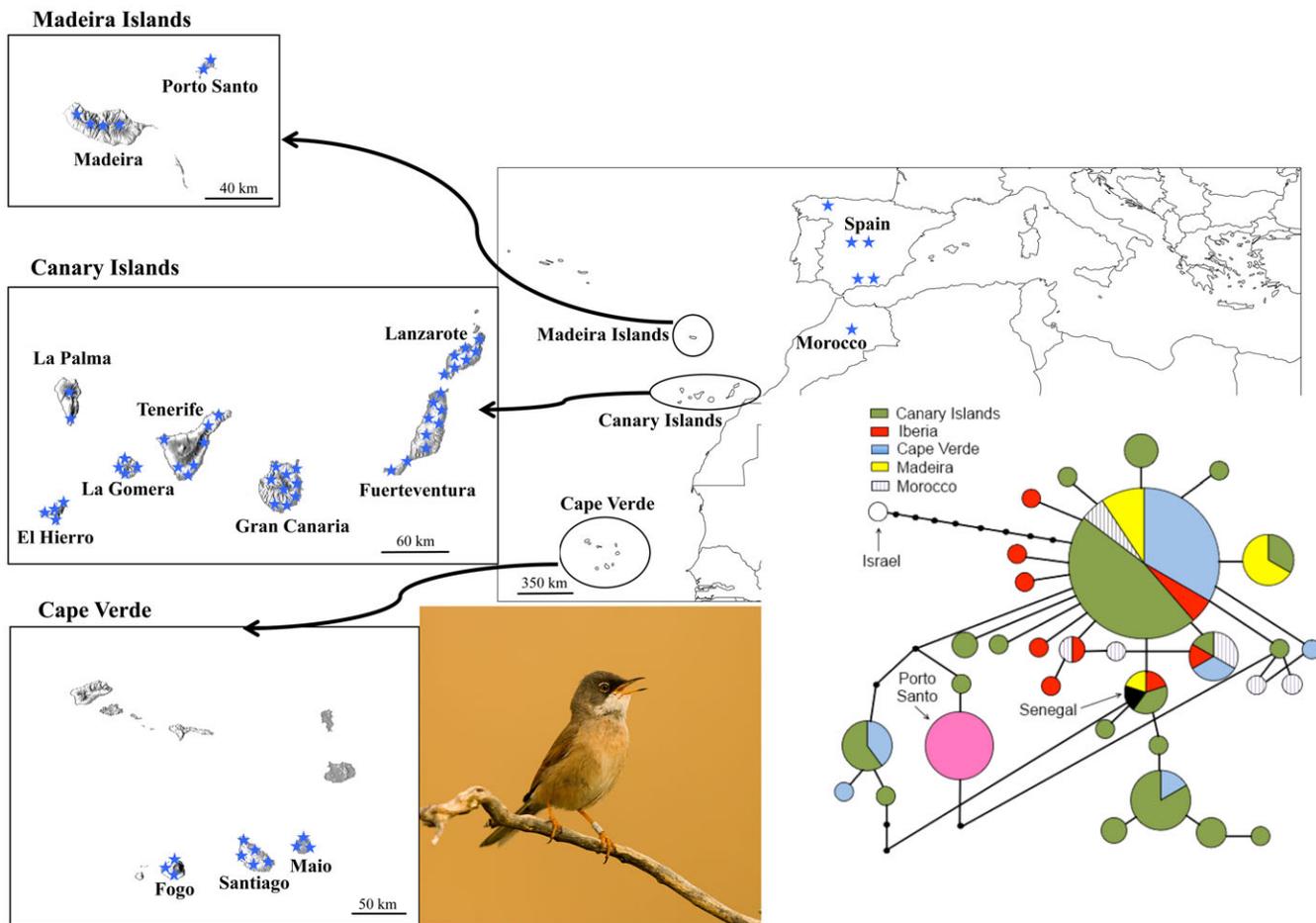


Figure 1. Map showing the archipelagos (with island names) and continental areas where spectacled warblers were sampled. Geographic situation of localities sampled are depicted with blue stars. Minimum spanning network of the spectacled warbler based on the cytochrome *b* mitochondrial gene is also shown. Ellipse sizes are proportional to the number of individuals included.

and FSTAT (Goudet 2002). Genetic differentiation among populations was examined using AMOVAs with the software ARLEQUIN, using the same approach performed with the mitochondrial sequences (see above). We estimated genetic structure among populations with pairwise F_{ST} and Jost's D (D_{EST}) values (Jost 2008) using the programs ARLEQUIN and SMOGD (Crawford 2010). D_{EST} values are based on the effective number of alleles rather than on the expected heterozygosity as F_{ST} (Jost 2008).

We investigated the occurrence of population structure, without using previous information on the origin of each individual, by performing a discriminant analysis of principal components (DAPCs) as described by Jombart et al. (2010). With this approach, genetic data were reduced in a first step to principal components to ensure that variables used in the subsequent discriminant analysis are uncorrelated, and then we used the K -means algorithm to identify the clusters. We evaluated the numbers of clusters (K) between 2 and 14 (number of sampled geographical areas) and the Bayesian information criterion (BIC) was used to

evaluate the relevance of different K values to population structure. A discriminant analysis was then applied to a dataset of the first 30 principal components, to verify the discrimination of the groups of individuals identified by the first step. Using DAPC, we obtained the probabilistic assignment of individuals to each group. In addition, we investigated the occurrence of population structure, without using previous information on the origin of each individual, with the Bayesian algorithm implemented in the software STRUCTURE version 2.3.3 (Pritchard et al. 2000). We used the admixture model (i.e., individuals may have mixed ancestry) and correlated allele frequency model between populations (i.e., expected allele frequencies between populations could be similar). We performed five independent iterations with $K = 1-14$, with a burn-in length of 100,000 and 1,000,000 Markov Chain Monte Carlo (MCMC) repetitions. We used the ad hoc statistic, ΔK , provided by Evanno et al. (2005) to evaluate the relevance of K to population structure using the online software STRUCTURE HARVESTER (Earl and vonHoldt 2012).

Table 1. Populations studied and standard diversity indices obtained from 15 microsatellite loci analyzed.

	<i>N</i>	<i>N_a</i>	Mean <i>N_a</i> (±SD)	AR (±SD)	<i>H_O</i> (±SD)	<i>H_E</i> (±SD)	<i>F_{IS}</i>	Size
Iberian Peninsula								
Spain	32	77	5.50 ± 3.34	4.70 ± 0.75	0.41 ± 0.26	0.53 ± 0.26	0.22	492,175
North Africa								
Morocco	30	88	5.87 ± 3.76	5.36 ± 0.85	0.37 ± 0.24	0.53 ± 0.27	0.30	446,550
Madeira archipelago								
Madeira	23	58	4.46 ± 0.44	3.95 ± 0.60	0.44 ± 0.22	0.55 ± 0.24	0.19	828
Porto Santo	22	53	3.79 ± 1.53	3.57 ± 0.42	0.37 ± 0.24	0.45 ± 0.21	0.18	42.17
Canary Islands								
Fuerteventura	27	69	4.60 ± 2.85	4.38 ± 0.67	0.32 ± 0.25	0.50 ± 0.27	0.36	1659.74
Lanzarote	25	73	4.87 ± 3.25	4.73 ± 0.79	0.36 ± 0.21	0.53 ± 0.24	0.33	845.94
Gran Canaria	31	69	4.60 ± 3.07	4.26 ± 0.66	0.36 ± 0.22	0.52 ± 0.26	0.31	1560.1
Tenerife	32	69	4.6 ± 3.99	4.36 ± 0.92	0.34 ± 0.25	0.50 ± 0.27	0.31	2034.38
La Gomera	32	61	4.36 ± 2.90	3.78 ± 0.68	0.30 ± 0.22	0.45 ± 0.25	0.33	369.76
La Palma	35	56	3.73 ± 2.22	3.44 ± 0.48	0.24 ± 0.22	0.43 ± 0.24	0.45	708.32
El Hierro	23	59	4.14 ± 2.54	3.89 ± 0.65	0.34 ± 0.21	0.49 ± 0.27	0.31	268.71
Cape Verde								
Maio	31	57	4.07 ± 1.07	3.72 ± 0.33	0.39 ± 0.23	0.52 ± 0.19	0.26	270
Santiago	39	64	4.57 ± 2.03	3.87 ± 0.47	0.35 ± 0.23	0.51 ± 0.23	0.31	991
Fogo	38	61	4.36 ± 1.55	3.83 ± 0.44	0.30 ± 0.23	0.52 ± 0.24	0.41	476

Sample size (*N*), number of alleles (*N_a*), mean number of alleles (Mean *N_a*), observed (*H_O*) and expected (*H_E*) heterozygosities, allelic richness (AR), and inbreeding coefficient (*F_{IS}*) are given per population. Size (square kilometer) per population studied is also shown.

We analyzed the genetic relatedness of populations in relation to geographic distance (i.e., isolation by distance), comparing pairwise genetic distance matrices ($F_{ST}/(1 - F_{ST})$) with log transformed geographic distances (Rousset 1997) using Mantel tests with 10,000 random permutations. Geographical distances were obtained using the approach of Illera et al. (2007).

Finally, we performed an approximate Bayesian computation (ABC) analysis (Beaumont et al. 2002) to characterize the divergence between the two main genetic groups identified by the DAPC. Microsatellites used in this study include loci with complex repeat motifs or allele sizes incompatible with their assumed repeat motif (e.g., dinucleotide microsatellites with alleles differing by three base pairs). This prevents the use of current implementations of coalescent samplers (such as IM, IMA, or Migrate; Kuhner 2009) to make inferences on population demographic history, because they assume microsatellites to mutate under the stepwise mutation model (or similar models) and they require knowing the number of repeats for each allele (which, in our case, could not be determined). The ABC approach allows us to model microsatellite evolution, including mutations that change the number of repeats, but also single nucleotide indels (SNI; as in DIYABC, Cornuet et al. 2008), that is, modeling the allele size variation in base pairs. Mutations that change the number of repeats were simulated following the generalized stepwise mutation (GSM) model that allows for changes of more than one repeat unit. In addition, loci with complex motifs could change

their allele size with changes in any of their repeat unit sizes. This procedure models the size of alleles measured in base pairs, which is the information available for the real data, and requires two additional mutational parameters: the probability that a mutation causes an SNI (P_{SNI} , with a uniform prior between 0 and 0.1) and the geometrical parameter for the GSM model if the mutation changes the number of repeats (α_{GSM} , with a uniform prior between 0 and 0.5). The divergence between groups was described by an isolation with migration (IM) model, which is characterized by five parameters: the scaled mutation rates for each of the populations (present plus ancestral ones), θ_1 , θ_2 , and θ_A (where $\theta_1 = 4N_1\mu$, $\theta_2 = 4N_2\mu$, $\theta_A = 4N_A\mu$, and N_1 , N_2 , and N_A are the effective population size of genetic group 1, group 2, and ancestral population, and μ is the mutation rate per generation); the scaled time of divergence, $\tau = t/4N_1$ (where t is the time measured in number of generations); and the scaled migration rate among the populations, $M = 4N_1m$ (where m is the migration rate). Priors for demographic parameters are specified in Table 2. They were chosen as to be uniform over a range wide enough to include values of the parameters usually found in the literature (Pinho and Hey 2010). Genetic data are only informative on the order of magnitude of parameter, θ ; thus, a log-uniform prior distribution was used to optimize the exploration of parameter space. One million simulations under this model were run on the program MS (Hudson 2002), which simulates DNA under the infinitely many site model; mutations simulated with MS were transformed

Table 2. Prior and posterior probabilities distributions for the demographic parameters of the spectacled warbler used in the approximate Bayesian computation analysis.

	Prior	Isolation with Migration		Isolation ($M = 0$)	
		Posterior Median	95% HPD	Posterior Median	95% HPD
$\theta_1 = 4N_1\mu$	Log-uniform (10^{-3} , 10^3)	3.66	1.83–7.94	2.68	0.43–17.45
$\theta_2 = 4N_2\mu$	Log-uniform (max [10^{-3} , $\theta_1 \times 10^{-2}$], min [10^3 , $\theta_1 \times 10^2$])	0.37	0.14–1.35	1.03	0.26–3.93
$\theta_A = 4N_A\mu$	Log-uniform (max [10^{-3} , $\theta_1 \times 10^{-2}$], min [10^3 , $\theta_1 \times 10^2$])	3.71	0.05–294.49	0.64	0.12–3.49
$\tau = 4N_1t$	Uniform (0, 100)	49.38	2.75–97.05	0.02	0.01–0.04
$M = 4N_1m$	Uniform (0, 100)	26.46	11.44–60.34	n.a.	n.a.

HPD, highest posterior density; n.a., not applicable.

into microsatellite data applying the GSM described above, and summary statistics (mean expected heterozygosity, number of alleles, and variance of allele size [measured in base pairs]) for each group and total sample and mean G_{ST} and mean and variance of $\delta\mu^2$ (using allele size measured in base pairs, Goldstein et al. 1995, between groups) and ABC steps (rejection of 99% of simulations plus non-linear local regression as in Blum and François 2010) were calculated with a custom script in R (Supplementary Information), using R package abc (Csilléry et al. 2012). In addition, a set of one million simulations with $M = 0$ (isolation model, I) was also simulated to test for the presence of gene flow with the ABC model selection procedure described by Beaumont (2008).

MORPHOLOGICAL ANALYSES

Measurements of wing length, tarsus length, tail length, culmen length, head length, bill width, and bill height and weight were taken for all individuals (Table S2; see Illera et al. 2007 for further details). However, weight was excluded from analyses because it can fluctuate greatly among days within individuals.

We performed a discriminant analysis to determine the occurrence of population structure based on morphological traits measured across the 14 populations. We also conducted a multivariate analysis of variance (MANOVA) to detect differences between the two subspecies described (i.e., islands vs. mainland) or between the groups identified by the genetic markers, where morphological traits were included as dependent variables and group as fixed factor. In addition, taking advantage of the impressive latitudinal gradient of 3580 km sampled, we performed a principal component analysis to synthesize the data into a set of compound axes with the final objective of assessing the latitudinal effect (longitudinal variation is comparatively negligible) on morphology.

Finally, we evaluated whether patterns of morphological differentiation were correlated with neutral genetic variation and geographic distances. If significant variation at morphological

traits and neutral genetic markers occurs, it suggests that both molecular and morphological differentiation increases with the geographic distance (isolation by dispersal limitation), as determined by a shared population history. Alternatively, the absence of such significant relationships would suggest that variation in morphological traits is not exclusively accounted by drift (Clegg et al. 2002b; Orsini et al. 2013). We performed a partial correlation between two dissimilarity matrices named genetic (F_{ST} or D_{EST} pairwise values) and morphologic (Euclidean pairwise distances) conditioned on a third matrix (geographical distance).

ACOUSTIC ANALYSES

Songs were recorded at the same dates and localities where birds were trapped, except in Morocco where we were not able to record any males. For each population, we recorded 12–34 males (Table S3). Males were recorded using a Marantz PMD660 (Marantz Europe, Eindhoven, The Netherlands) portable recorder and a Sennheiser ME 67 (Sennheiser electronic GmbH & Co. KG, Wedemark Wennebostel, Germany) microphone and then analyzed with the software Avisoft SASLabPro (Avisoft Bioacoustics, Glienicke, Germany) using a fast Fourier transform algorithm. Details on recording and sound analyses can be found in Palmero et al. (2012).

We considered 11 acoustic parameters that describe song complexity and the spectral and temporal patterns of song structure in the study species (Table S3; Palmero et al. 2012): song phrase duration (DurPhr); the minimum (Fmin) and maximum (Fmax) frequency of the song phrase; the dominant frequency in the song phrase (Fdom); the duration of the first syllable (DurSyl); the minimum (FminSyl) and maximum (FmaxSyl) frequency of the first syllable; trill syllable duration (DurSylTr); trill syllable dominant frequency (FdomTr); number of different original syllables (i.e., matching portions without similarity in timing, frequency, and shape) per phrase (OrSyl); and overall number of syllables per phrase (TotSyl). Several songs (from five to 10)

were recorded for each individual. Thus, the individual song parameters considered in this study represent the average values of individual songs. We previously showed that the variables used in this study varied more between than within individuals (Palmero et al. 2012), being therefore adequate to detect variability among populations. The only exception was (F_{dom}), which we showed that it could not be the case (Palmero et al. 2012). However, excluding this variable from the analyses did not change our results (data not shown); hence, we used all variables to maximize the statistical power of tests.

We performed a discriminant analysis with the 11 variables across the 13 populations to distinguish groups based on acoustic features. In addition, we performed a MANOVA between the groups identified by the genetic DAPC analysis. We did not perform an additional MANOVA analysis between the subspecies described (i.e., islands vs. continent) because only a limited number of individuals in Spain were recorded for *S. c. conspicillata*. Finally, we evaluated if acoustic differentiation was correlated with neutral genetic variation and geographic distances, in a similar way we performed with morphology. Thus, we calculated Euclidean distances in the acoustic features using the mean values of each population. We tested the relationships between acoustic and genetic matrices (conditioned by the geographical distances) with partial Mantel tests. Data were standardized and log transformed and significance was assessed with 10,000 random permutations. Statistical analyses were carried out with the software SPSS version 19.0, except Mantel and partial Mantel tests that were performed with the software R version 2.14.0 (R Development Core Team 2010).

Results

Overall, a total of 420 individuals were collected, bled, and measured from 14 populations, and song was recorded in 269 males, between 2010 and 2011 (Table 2; Tables S2 and S3).

MITOCHONDRIAL

We found a total of 25 haplotypes for 147 individuals analyzed (Table 3). Sequences have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database with the following accession numbers: KF517352–KF517375. DnaSP identified 28 polymorphic sites, albeit only nine were parsimony informative. Haplotype diversity reached the highest values in the continental areas ($h = 0.891$ and 0.844 , for the Iberian Peninsula and Morocco, respectively) and the lowest value in Cape Verde ($h = 0.351$). However, nucleotide diversity was highest in the Canary Islands ($\pi = 0.0022$), followed by the mainland populations ($\pi = 0.0017$ and 0.0016 , for the Iberian Peninsula and Morocco, respectively), reaching the lowest level in Cape Verde ($\pi = 0.0012$). Molecular variance analysis showed

a significant level of genetic differentiation among populations ($F_{\text{ST}} = 0.42$, $P < 0.0001$) and among populations within the same group ($F_{\text{SC}} = 0.37$, $P < 0.0001$), but we did not find a significant effect among the groups originally assigned (i.e., archipelagos and the continent; $F_{\text{CT}} = 0.09$, $P = 0.68$). Most variation was attributable to within populations (57.53%) and among populations within groups (33.48%). Similar results were obtained after grouping populations according to their taxonomy, that is, one group with the island and another group with the mainland populations ($F_{\text{ST}} = 0.38$, $P < 0.0001$; $F_{\text{SC}} = 0.42$, $P < 0.0001$; $F_{\text{CT}} = -0.08$, $P = 0.5$).

The phylogenetic network showed a clear difference between the Israeli sequence (the most distant population) and remaining individuals sampled. We did not obtain a clear genetic structure separating archipelagos and continental areas (each inhabited by a different subspecies) or within archipelagos (Fig. 1). The three Macaronesian archipelagos plus the continental areas of the Iberian Peninsula and Morocco shared the most common haplotype. Finally, the haplotype obtained in Senegal was shared with the Iberian Peninsula, Madeira, and the Canary Islands.

MICROSATELLITES

The 14 populations showed a moderate level of overall genetic differentiation ($F_{\text{ST}} = 0.107$, $D_{\text{EST}} = 0.096$). Values ranged from 0.025 to 0.221 and 0.005 to 0.117, for F_{ST} and D_{EST} , respectively. La Gomera and Maio showed the highest levels of genetic differentiation for both F_{ST} (0.221) and D_{EST} (0.112), meanwhile Spain and Morocco reached the lowest ones ($F_{\text{ST}} = 0.025$, $D_{\text{EST}} = 0.005$) in comparison to the other islands (Table S4). AMOVA results showed significant genetic variation among populations ($F_{\text{ST}} = 0.12$, $P < 0.001$), among populations within groups ($F_{\text{SC}} = 0.11$, $P < 0.001$), but nonsignificant variation among groups defined a priori (i.e., the three Macaronesian archipelagos plus the continental group; $F_{\text{CT}} = 0.01$, $P = 0.13$). Similar results were obtained after considering only two groups based on taxonomy, that is, Macaronesia versus mainland ($F_{\text{ST}} = 0.39$, $P < 0.001$; $F_{\text{SC}} = 0.11$, $P < 0.001$; $F_{\text{CT}} = 0.003$, $P = 0.31$). Most variation was found within populations (87.61%), but almost 11% was attributable to differentiation among populations within groups and only 1.54% among groups. Mantel tests revealed a significant pattern of isolation by distance ($r = 0.26$, $P = 0.036$), suggesting that genetic differentiation increases with increasing distances between populations (Fig. 2).

Clusters identified by the DAPC and STRUCTURE analysis (Figs. 3, S1, and S2) had no correspondence to the subspecies classification and little correspondence to geography for any value of K . Nevertheless, the two main genetic groups identified by the analysis with $K = 2$ in DAPC and STRUCTURE are consistent in both methods (Fig. S3) and for any value of K and seem to identify the most important structuring of the genotypes. Evaluation of the

Table 3. Diversity estimates obtained from the mitochondrial cytochrome *b* gene for the spectacled warbler populations.

Populations	Cytochrome b						
	Sample Size	Haplotypes	Haplotype Diversity	SD	Nucleotide Diversity	SD	Theta (θ)
Total	147	25	0.632	0.044	0.00165	0.00018	0.00512
Iberian Peninsula	11	8	0.891	0.092	0.00168	0.00034	0.00240
North Africa	10	6	0.844	0.103	0.00159	0.00036	0.00178
Macaronesia	124	19	0.643	0.047	0.00186	0.00019	0.00281
Madeira Islands	24	3	0.554	0.052	0.00113	0.00011	0.00081
Madeira	11	2	0.182	0.144	0.00018	0.00014	0.00034
Porto Santo	13	1	0.000	0.000	0.000	0.000	0.000
Canary Islands	69	17	0.722	0.055	0.00224	0.00025	0.00334
Lanzarote	10	6	0.911	0.062	0.00316	0.00040	0.00248
Fuerteventura	10	6	0.867	0.085	0.00212	0.00052	0.00213
Gran Canaria	10	5	0.822	0.097	0.00301	0.00064	0.00319
Tenerife	9	5	0.861	0.087	0.00289	0.00061	0.00258
La Gomera	10	2	0.200	0.154	0.00020	0.00015	0.00035
La Palma	9	2	0.222	0.166	0.00089	0.00067	0.00148
El Hierro	11	2	0.436	0.133	0.00044	0.00013	0.00034
Cape Verde	31	5	0.351	0.107	0.00117	0.00041	0.00201
Maio	10	2	0.200	0.154	0.00020	0.00015	0.00035
Santiago	10	3	0.511	0.164	0.00160	0.00049	0.00142
Fogo	11	2	0.327	0.153	0.00131	0.00062	0.00137

Haplotype and nucleotide diversities are given with their SDs. Total: sequences from Senegal and Israel are also considered. Sites with alignment gaps or missing data were excluded.

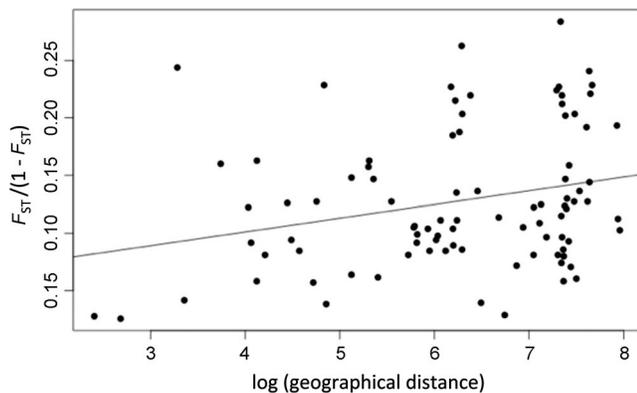


Figure 2. Isolation by distance relationships for the spectacled warbler for the 14 populations analyzed. Pairwise genetic ($F_{ST}/(1 - F_{ST})$) values were plotted against pairwise geographical distance (log transformed).

number of clusters (Fig. S4) shows that six or more cluster do not significantly explain additional population structure for both DAPC and STRUCTURE. From now on, we will focus on the two main clusters, as defined by DAPC results as they are more clearly defined with that method. Individuals from the smaller group (cluster 1) come mainly from Maio, Fogo, and Gran Canaria, and individuals from the larger group (cluster 2) from the remaining

populations, but there is no clearly geographical division between them (Fig. 3). Individuals were assigned on average to each cluster with high probability (cluster 1 = 0.81, cluster 2 = 0.94; Table S5).

Estimates of the demographic parameters from the ABC are presented in Table 2. Despite ABC analyses providing significant evidence for gene flow between the two genetic groups ($P(I) \approx 0$, $P(IM) \approx 1$; Figs. S5 and S6), there was no information about divergence time (almost identical prior and posterior probability distributions; Fig. S5). Regarding the effective population size, cluster 1 and 2 seems to be similar in size, but ancestral size was poorly estimated.

MORPHOLOGY

The number of females captured per population was very limited due to their limited response to the playback callings used to lure birds. This unbalanced sampling could produce bias in our morphological results and, therefore, the morphological analyses were performed only with male data, as was done also for acoustic data.

We measured 326 males across the 14 populations studied (Table S2). Discriminant analyses provided an overall limited power of discrimination of populations, thus only 39.9% of original cases were correctly classified to their group (Fig. 4A).

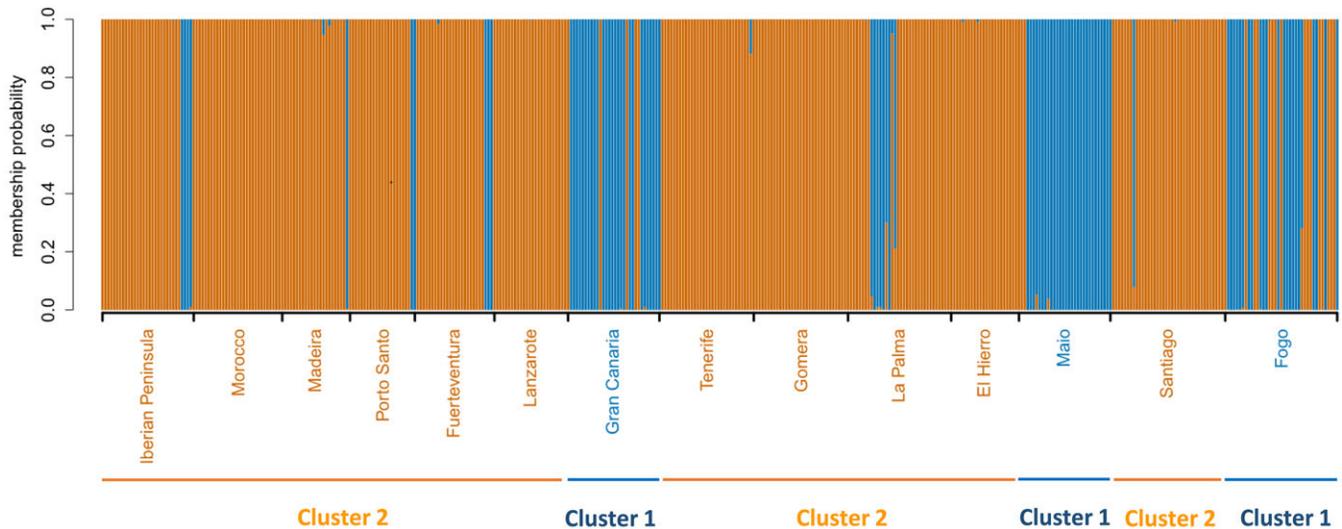
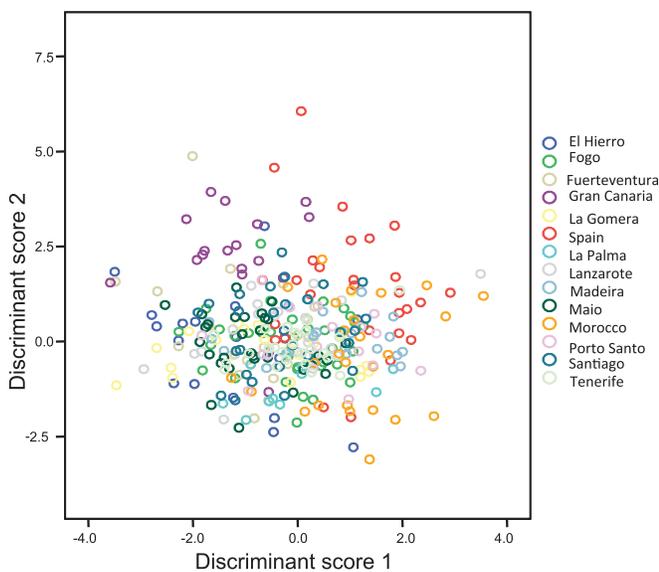


Figure 3. Assignment probabilities for each spectacled warbler individual to the clusters from DAPC analysis. Individuals from the same sampling locality (see Fig. 1) are represented consecutively in groups. The most likely number of clusters was obtained for $K = 2$.

A Morphology



B Acoustic

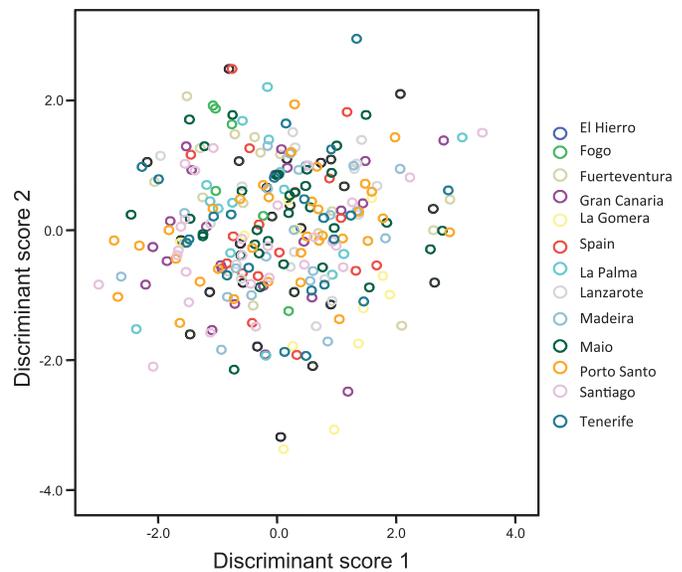


Figure 4. Plots of the first two axes obtained in two discriminant analyses. (A) Plot performed on seven morphological traits (Table S2) of the spectacled warbler in Macaronesia (14 populations). (B) Plot performed on 11 acoustics variables (Table S3) of the spectacled warbler in Macaronesia (13 populations). Both plots show the absence of population structure based on morphological and acoustic traits.

The MANOVA showed significant differences between the two groups identified by the DAPC analysis ($F_{7,314} = 5.39$, $P < 0.001$; Fig. 5A), associated with differences in tail length ($F_{1,321} = 9.33$, $R^2 = 0.028$, $P = 0.002$), bill width ($F_{1,321} = 13.33$, $R^2 = 0.04$, $P = 0.001$), bill length ($F_{1,321} = 4.13$, $R^2 = 0.013$, $P = 0.043$), and bill head ($F_{1,321} = 9.65$, $R^2 = 0.029$, $P < 0.01$). However, the MANOVA analysis of the two subspecies (i.e., islands vs. continent) did not show significant differences between the two groups ($F_{7,318} = 2.03$, $P = 0.051$; Fig. 5B).

Three principal components with eigenvalues higher than one, explaining 68.3% of the variation, were obtained. The first component (PC1) represented the overall size, with positive weighting for all variables and explaining 33.14% of the variance, PC1 was hereafter referred to as “body size.” The second component (PC2) explained 18.72% of the variance and showed a high negative weighting for head and bill length, thus we consider it a factor of “head shape.” Finally, PC3 (16.43% of variance) was mostly associated with positive weighting with

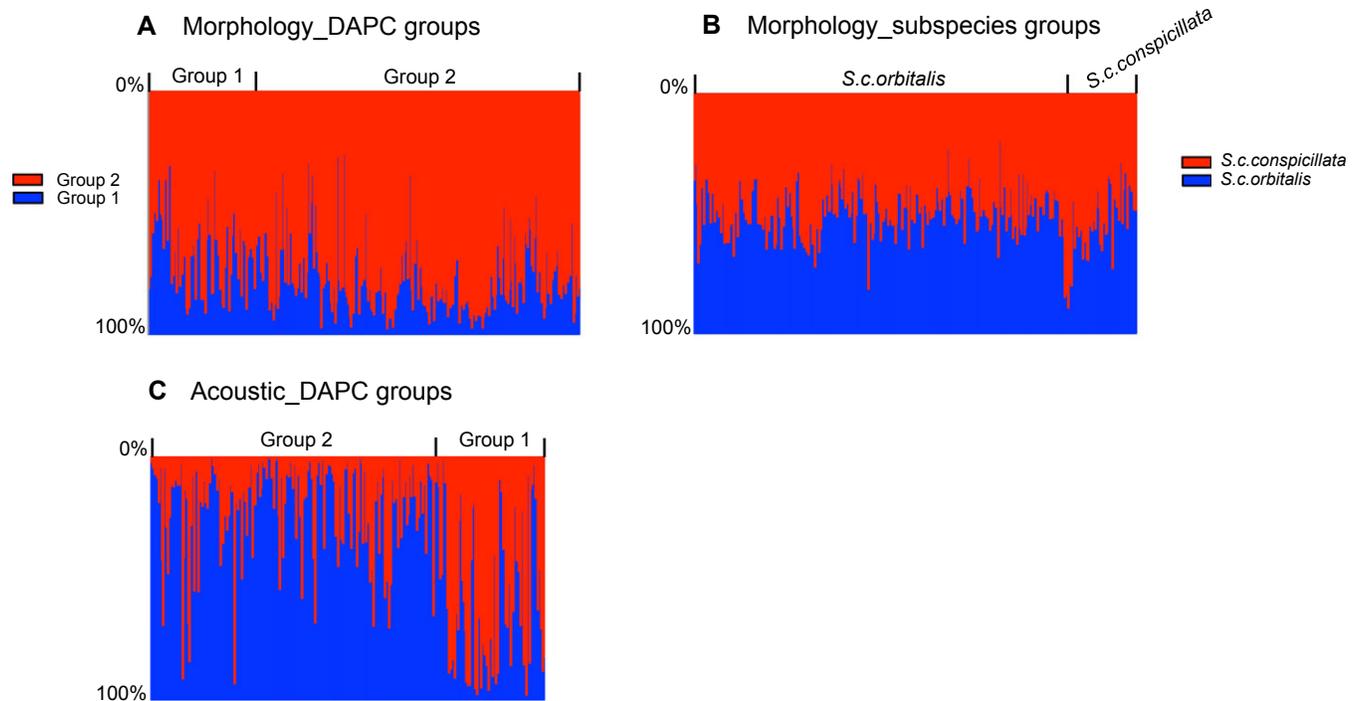


Figure 5. Bar plot of assignment probabilities on morphological and acoustic variables for each spectacled warbler individual obtained with a discriminant analyses: (A) considering seven morphological traits and the two DAPC clusters, (B) considering seven morphological traits and the two subspecies, (C) considering 11 acoustic variables and the two DAPC clusters. DAPC clusters obtained better discrimination power than subspecies (i.e., continent vs. islands), albeit it was overall very limited.

bill width and bill height measures and is here referred to as “bill shape.”

We found a weak but significant effect of latitude on some morphological traits. Thus, latitude was positively associated with body size ($r^2 = 0.08$, $n = 320$, $P < 0.001$), bill shape ($r^2 = 0.05$, $n = 326$; $P < 0.001$), and relative wing length ($r^2 = 0.07$, $n = 325$, $P < 0.001$), but it did not affect head shape ($r^2 = 0.003$, $n = 326$, $P = 0.178$). Thus, the largest, thickest billed, and longest winged birds were found toward the north.

Finally, partial Mantel tests did not reveal significant relationships among geographic distance, morphology, and neutral markers, considering both F_{ST} and D_{EST} pairwise comparisons ($r = 0.1$, $P = 0.15$).

ACOUSTICS

We analyzed the song of 269 males across the 13 populations recorded. Mean values of each variable per population are shown in Table S3.

Discriminant analyses correctly classified 26.2% of the original group cases, suggesting a low power of discrimination of groups (Fig. 4B). The MANOVA analyses showed significant differences between the two groups identified by DAPC ($F_{11,258} = 7.76$, $P < 0.001$; Fig. 5C). Significant differences were observed in the duration of the first syllable ($F_{1,268} = 5.42$, $R^2 = 0.02$,

$P = 0.021$), maximum frequency of the first syllable ($F_{1,268} = 10.99$, $R^2 = 0.039$, $P = 0.001$), trill syllable dominant frequency ($F_{1,268} = 11.78$, $R^2 = 0.042$, $P = 0.001$), and in the number original syllables per phrase ($F_{1,268} = 26.88$, $R^2 = 0.091$, $P < 0.001$). We did not to find any pattern of isolation by distance between song and geographic distances ($r = 0.10$, $P = 0.24$), or significant relationships between morphology, song, and genetic divergences among populations ($r = 0.15$, $P = 0.24$).

Discussion

The study of the colonization and diversification processes of island forms has contributed much to our understanding of biological diversity. Indeed some of them constitute classical examples of adaptive radiation (e.g., Grant and Grant 2008; Losos 2009; Lerner et al. 2011). Based on these precedents, we expected to find a burst of differentiation in the geographically isolated populations of spectacled warblers in Macaronesia, especially in the acoustic traits that show a much faster rate of changing (Grant and Grant 2008; Parker et al. 2012). However, our results provide evidence for only slight genetic, morphological, and acoustic divergence in the spectacled warbler in Macaronesia. This result is striking because we studied an extensive number of discrete entities defined by wide sea boundaries and isolated by long flight

distances for a small passerine, and we addressed multiple traits at the genetic and phenotypic levels. Microhabitat and habitat structure changes even between the closest islands (e.g., Illera et al. 2006), and such circumstances should have produced variation in song characteristics and acoustic adaptation to the habitat (Marler and Slabbekoorn 2004; Grant and Grant 2008), as well as local adaptation in morphological traits associated with foraging and maneuverability, such as bill, wing, and tail measures (Grant and Grant 2008). Therefore, we expected to find more pronounced difference among isolated (even if recently, early Holocene, founded) populations in both acoustic and morphological traits. However, the results observed in this study make it plausible to conclude that this species is not radiating in Macaronesia.

The parsimony network analysis did not reveal any clear allopatric lineages among the mainland areas and the Macaronesian archipelagos or within archipelagos, which is supported by the relatively low differentiation detected among groups with the AMOVA analysis. Similar mitochondrial genetic structure was obtained with the other two *Sylvia* species (i.e., *S. atricapilla* and *S. melanocephala*) inhabiting Macaronesia, which was related to a recent colonization process coinciding with the ending of the last glacial period in Europe (Pérez-Tris et al. 2004; Dietzen et al. 2008). The lack of allopatric lineages suggests a scenario of either very recent isolation or ongoing gene flow. The exception is Porto Santo, a small island of the Madeira archipelago that did not share its single haplotype with any other population (Fig. 1), such a pattern being compatible with a founder effect and isolation from the remaining populations. However, results obtained with microsatellites provided evidence for genetic structure in two groups, with individuals from both groups co-occurring in some islands. Another unexpected finding is that the two groups identified with the microsatellite data do not correspond with the ranges of the two subspecies (mainland and Macaronesia) described so far. DAPC identified a small cluster of three islands, Gran Canaria, Maio, and Fogo (i.e., one Canary and two Cape Verde islands), and another with the 11 remaining populations (Fig. 3). These results, together with the lowest values of both haplotype and nucleotide diversity in Cape Verde and the four haplotypes shared by the Cape Verde islands (two shared only with the Canary Islands and the remaining shared with the Canaries and other populations), lead us to hypothesize a double colonization of the Cape Verde islands, the Canaries being the most plausible origin of the southward expansion. Thus, the islands of Maio and Fogo could have been colonized in a separate wave from that, which colonized Santiago, and the source would likely be Gran Canaria. This expansion event from north to south is consistent with the most common colonization pathway pattern inferred in other land bird species in the region, which is favored by the dominant north trade winds (Illera et al. 2012).

The overall lack of genetic differentiation between distant archipelagos (and the mainland) deserves some interpretation. Some lines of evidence suggest that gene flow is the most likely cause to occur. The haplotype obtained in Senegal was shared with the Iberian Peninsula, Madeira, and the Canary Islands (Fig. 1). Because the spectacled warbler in the Canary and Madeira archipelagos is considered a year-round sedentary species, the Senegal bird could be a migrant or wintering individual from continental populations. Such migratory behavior makes plausible that some continental birds arrive regularly in the Macaronesian islands (Martín and Lorenzo 2001), and it is not possible to rule out that some of them breed there. The pattern of isolation by distance observed (Fig. 2) could arise from a directional history of colonization, gene flow being limited and influenced by the proximity of neighboring populations (e.g., Illera et al. 2007; Clegg and Phillimore 2010; Hoeck et al. 2010). Nonetheless, the general lack of differentiation despite wide geographic separation suggests some ongoing gene flow.

The structure of two clusters was also associated with phenotypic and acoustic divergence. This result is consistent with a system where phenotypic and acoustic divergence is driven by a shared population history. Because we could not estimate whether the degree of divergence was higher in morphology and song than in microsatellites, the lack of a positive correlation in the partial Mantel tests per se should not be treated as an evidence for selection. However, the fact that the discriminant analyses could not correctly assign morphology or song to source populations, it appears that no “local” song and morphology have evolved in each island, or even archipelago. This is in spite of the great local differences in habitat (and especially climate), which are supposed to strongly promote the evolution of local adaptations. Indeed, the latitudinal cline of body size (i.e., northern birds are bigger than southern ones) indicate that deterministic forces, such as climate-driven natural selection on body size or phenotypic plasticity, played (although weakly) some role in morphological differentiation (Clegg et al. 2002b). Morphological traits could be influenced by differences in the migration behavior of the study populations (in our system continental populations, the northern ones, are migratory; Cramp 1992). Such condition could be associated with specific changes in morphology (e.g., Pérez-Tris and Tellería 2001; Baldwin et al. 2010) and bias our results. Nevertheless, these results remained consistent even when removing the continental populations from analyses (data not shown). Finally, although we acknowledge that the inclusion of more individuals in our analyses would have provided higher power in our analyses, we feel, however, confident with the sample sizes presented here, which are comparable and consistent with previous studies (e.g., Clegg et al. 2002b; Clegg and Phillimore 2010; Spurgin et al. 2014). Overall, such a system provides an invaluable opportunity to carry out studies on physiological adaptations, at the

individual and population levels, in discriminating how these differences associated with latitude might provide the initial divergence on which selection operates even in the presence of gene flow.

Song variation is regularly invoked to be one of the most important traits promoting differentiation among allopatric and sympatric bird populations (Price 2008; Grant and Grant 2010; Laiolo 2012; Wilkins et al. 2013). This is because in oscine passerines the song has a strong cultural component (Price 2008) and sources of variation can be numerous, resulting from errors in the learning process, random cultural drift, acoustic adaptation to different sound transmission qualities of the habitat, and song character displacement among coexisting species (Nicholls et al. 2006; Kirschel et al. 2009; Grant and Grant 2010; Weir et al. 2012). Song can also vary as a consequence of morphological variation, with body size variation being an important driver of song frequency shifts (Hall et al. 2013). Notably, song is among the first phenotypic characters that differ in recently separated passerine taxa, and can be responsible for creating a reproductive barrier between incipient species (Edwards et al. 2005). Therefore, on the basis of the above-mentioned body of evidence, and taking account (1) the complexity of spectacled warbler songs (Palmero et al. 2012, 2014) and (2) the fact that island populations are isolated by long distances along a wide latitudinal gradient (3580 km), we did expect to find stronger acoustic substructure among warbler populations. Contrary to expectations, we failed to detect such strong structure among the populations studied and only observed an incipient divergence between the two genetic clusters identified. Moreover, we did not find a correlation among song acoustic properties and biometric parameters in spite of considering several morphological measurements that may affect song spectrotemporal output (e.g., bill shape, body size; Podos et al. 2004; Hall et al. 2013). The pattern of acoustic variation observed (i.e., neither matching morphological nor genetic traits) does not permit differentiating the occurrence of local selective forces (natural or sexual selection) or stochastic differentiation due to founder effects or drift. Therefore, due to our inability to disentangle the contribution of each process involved (or not involved), we can only stress the lack of acoustic differentiation in our system and discuss on the potential causes of such findings. Habitats with dense vegetation (like forest) have been suggested to drive local song variation because their structural complexity engenders a strong selection on long-range communication systems (Weir et al. 2012), and divergence can be so faster as to occur in less than 50 years (Parker et al. 2012). The spectacled warbler inhabits nonforest habitats throughout its whole range distribution (Cramp 1992). The simple structural complexity of this habitat, which likely does not cause strong habitat-driven selection, together with the recent colonization of Macaronesia and some movements of individuals among populations, may all have

slowed down the action of local habitat as driver of selection (Weir et al. 2012). These historical and population contingencies may also have hampered the diverging action of climate-driven selection that does promote acoustic variation in open habitat sedentary passerines (Laiolo 2012).

We recognize that a more detailed study on the acoustic behavior of the species by means of playback experiments, as well as the measurements of environmental variables, would have helped to target more directly the role of sexual or natural selection on song variation (Tietze et al. 2011). We do not exclude, for instance, that males and females could exhibit differences in the discrimination of songs from other populations inhabiting slightly different microhabitats (Tobias and Seddon 2009; Podos 2010; Seddon and Tobbias 2010), and this behavior may limit our understanding of the effect of vocal divergence on mate recognition.

Overall, the generally smaller differences found between populations within those taxa characterized by low diversification rate have probably limited the interest of evolutionary biologists, identifying the processes that gave rise to their differentiation. However, such studies provide insights on incipient differentiation processes and on the origin of the phenotypic diversity or the causes of its absence. We addressed here the question of what drives differentiation in a widespread island bird species with an “a priori” early differentiation process, distributed along an extraordinary natural laboratory of discrete geographical entities, by using a multidisciplinary approach involving sexual signals, morphology, and genetics. Contrary to our expectations, and despite having studied a resident and common breeding passerine in three oceanic archipelagos showing very little migratory behavior, we have strikingly found less differentiation than would be expected by drift, selection, or founder effects in acoustic, morphological, and genetic traits (Clegg et al. 2002b; Clegg and Phillimore 2010; Monceau et al. 2013; Spurgin et al. 2014). Importantly, this species provides an unexpected example of lack of diversification in a songbird occurring in oceanic islands. Our results suggest that local differences in song parameters may not readily evolve (culturally or biologically) as a result of isolation and that impressively wide climatic gradients may engender only weak morphological clines. Further studies on physiological adaptations, breeding biology, and habitat selection, along with playback and common-garden experiments are now required to disentangle the reasons behind such lack of differentiation for this island warbler.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.q42p7. Data accessibility: mtDNA sequences—Genbank accessions KF517352–KF517375. Final DNA sequence assembly and ABC Script R uploaded as online Supplemental Material.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Assignment probabilities for each spectacled warbler individual to the clusters from DAPC analysis.

Figure S2. Assignment probabilities for each spectacled warbler individual to the clusters obtained from STRUCTURE analysis.

Figure S3. Individual assignment posterior probabilities estimated from DAPC and STRUCTURE for the spectacled warbler.

Figure S4. Estimated modal values of ΔK (for STRUCTURE analysis) and BIC (for DAPC analysis) for the spectacled warbler.

Figure S5. Prior and posterior probability density distribution of the demographic parameters from the isolation with migration model inferred with ABC in the spectacled warbler.

Figure S6. Prior and posterior probability density distribution of the demographic parameters from the isolation model inferred with ABC in the spectacled warbler.

Table S1. Microsatellite loci used with the spectacled warbler.

Table S2. Mean values (\pm SE) to each morphological trait in the spectacled warbler per population.

Table S3. Mean values (\pm SE) to each acoustic feature measured in the spectacled warbler per population (data not standardized).

Table S4. Microsatellite pairwise F_{ST} (below the diagonal) and Jost's D_{EST} (above diagonal) values obtained in the spectacled warbler.

Table S5. Proportion of spectacled warbler individuals per population assigned to each of the two clusters identified by DAPC without using prior information.