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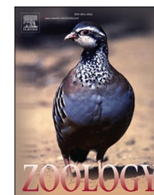

Inter and intra-island genetic structure and differentiation of the endemic Bolle's Laurel Pigeon (*Columba bollii*) in the Canary archipelago

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ABSTRACT

Islands provide excellent settings for studying the evolutionary history of species, since their geographic isolation and relatively small size limit gene flow between populations, and promote divergence and speciation. The endemic Bolle's Laurel Pigeon *Columba bollii* is an arboreal frugivorous bird species distributed on laurel forests in four islands of the Canary archipelago. To elucidate the population genetics, we genotyped ten microsatellite loci using DNA obtained from non-invasive samples collected across practically all laurel forest remnants, and subsequently grouped into eight sampling sites. Analyses including F-statistics, Bayesian clustering approaches, isolation by distance tests and population graph topologies, were used to infer the genetic diversity and the population differentiation within and among insular populations. Additionally, we evaluated the effect of null alleles on data analysis. Low genetic diversity was found in all populations of Bolle's Laurel Pigeon, with no significant differences in diversity among them. However, significant genetic differentiation was detected among all populations, with pigeons from La Palma and El Hierro exhibiting the closest affinity. Bayesian clustering supported population separation between islands, and also detected fine-scale structure within the Tenerife and La Gomera populations. Our results suggest that, despite columbids have a high movement ability, they can show signature of genetic divergence among populations, particularly on oceanic islands. Geological history of the islands and distribution range of habitats could have close influence on the evolutionary trajectories of these birds. This approach can provide practical tools to implement appropriate conservation measures for range-restricted species and their habitat.

1. Introduction

Oceanic islands are exemplary settings for understanding evolutionary and ecological processes (Losos and Ricklefs, 2009). Their biotas often exhibit demographic, behavioural, physiological, life history, morphological and ecological traits that differentiate them from their mainland ancestors (Lomolino, 2016). The isolation and ecological simplicity of these islands, but also other geological, geographic and climatic factors are considered fundamental drivers of speciation and divergence (Matthews and Triantis, 2021; Whittaker et al., 2023). As a result, island ecosystems are home to a wide diversity of unique species and habitats. Some islands have been designated as global biodiversity hotspots, with the Macaronesian archipelagos of Azores, Madeira, Canaries and Cabo Verde in the Atlantic Ocean constituting one such region (Fig. 1a; Myers et al., 2000; Cartwright, 2019; Florencio et al., 2021).

The Canary Islands are composed by eight islands located ca. 100 km off the coast north-western Africa. The central-eastern islands (Gran Canaria, Fuerteventura and Lanzarote) and La Gomera emerged between 24 and 10 Ma (Carracedo and Troll, 2021). Tenerife had a more complex origin, since around 11.6–3.2 Ma it was comprised of three separate proto-islands (Roque del Conde, Teno and Anaga) that finally merged into a single island between 1.9 and 0.2 Ma (Ancochea et al., 1990; Thirlwall et al., 2000). Finally, La Palma and El Hierro emerged within the last 3.5 and 1.1 Ma, respectively (Guillou et al., 1996; Carracedo et al., 2001). The repeated volcanic activity, formation of new territories, large-scale landslides and sea level variations (Carracedo, 1994), would have led to isolation, local extinctions and (re)colonization of the species communities, consequently shaping their evolutionary trajectories (see Gübitz et al., 2005; Mairal et al., 2015; Puppo

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et al. 2015; Noguerales et al., 2024).

The endemic Bolle's Laurel Pigeon (*Columba bollii*) is part of the guild of frugivorous birds in the laurel forests of Tenerife, La Gomera, La Palma, and El Hierro (Martín and Lorenzo, 2001). It shares natural range with the also endemic White-tailed Laurel Pigeon (*C. junoniae*), the Madeira Laurel Pigeon (*C. trocaz*) is its sister species, and both share a common ancestor with the European Wood Pigeon (*C. palumbus*) (Dourado et al., 2014). According to historical references and osteological remains uncovered in some archaeological sites, some pigeon species may have also inhabited the other islands of the archipelago (Alcover and Florit, 1989; Rando and Perera, 1994; Martín et al., 2013). However, before they could be conclusively identified at the species level, their populations vanished, likely due to the extensive deforestation of the laurel forests (Parsons, 1981; Martín Osorio et al., 2011). Although currently protected, the evergreen laurel forests occupy only ~ 11.8 % (10,181 ha) of their original range in the Canary archipelago (Del Arco Aguilar et al., 2010).

The loss and fragmentation of natural habitats are one of the main threats to biodiversity, as they often lead to the decline and extinction of many species by disrupting ecological processes and geographically segregating populations (Fahrig, 2003; Haddad et al., 2015; Wilson et al., 2016). This is particularly critical for island-endemic columbid species, which provide relevant ecosystem services, primarily as seed dispersers (Walker, 2007). Their large body, great flight capacity and ability to ingest fruits of various sizes to later defecate and/or regurgitate their seeds (endozoochory), make pigeons effective agents in the natural regeneration, spatial structuring, and enhancing connectivity among plant communities (Steadman and Freifeld, 1999; McConkey et al. 2004).

In the laurel forests, the close ecological relationship between *C. bollii* and fleshy-fruited plant species has been also evidenced. Pigeons move among forest patches in response to fruiting phenology and their food preferences. The spatio-temporal fluctuations in the local abundance of *C. bollii* populations (Martín et al., 2000), as well as the changes observed in the composition of its diet (Marrero, 2009; Marrero and Nogales, 2021), support these results. However, data on the status,

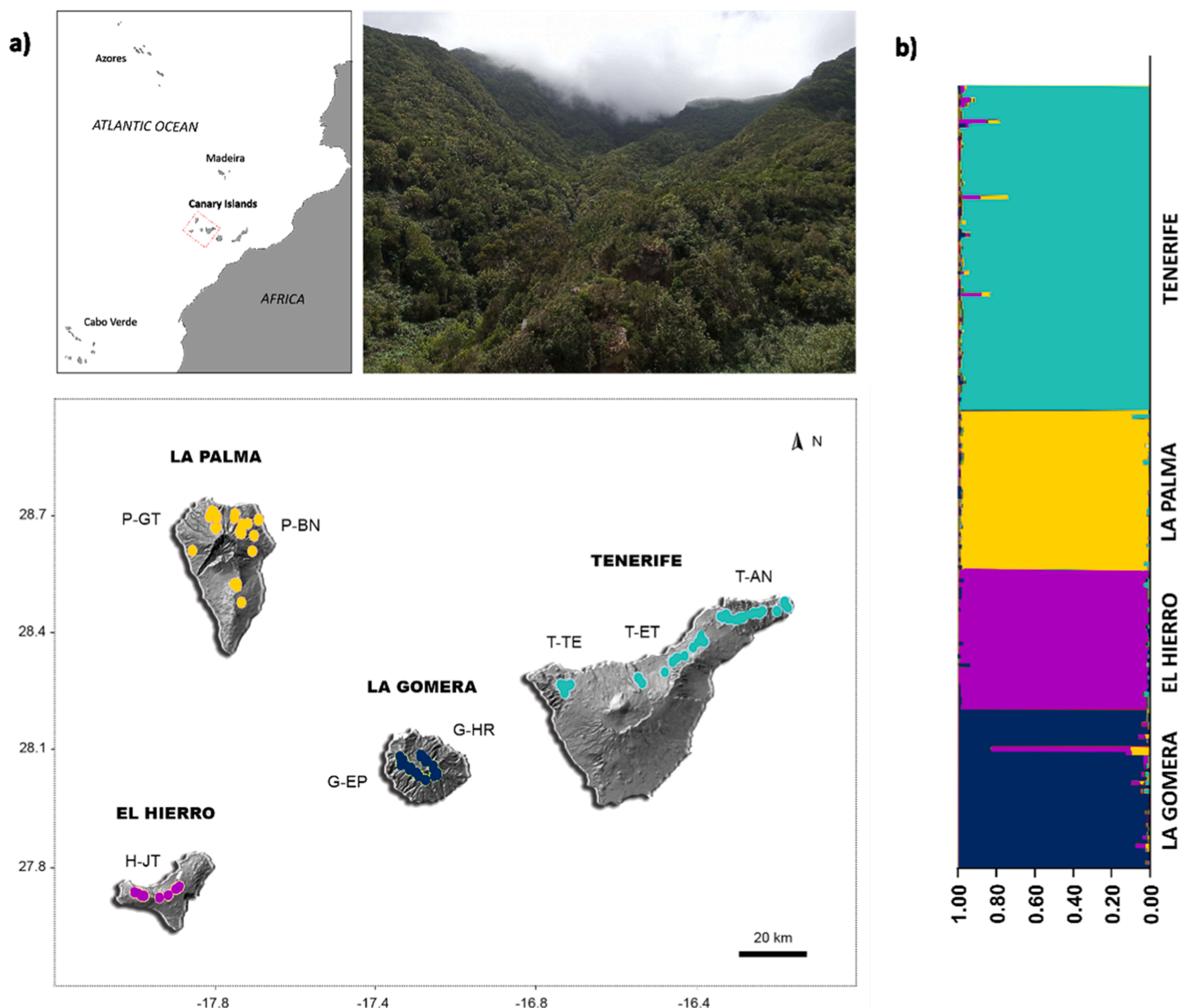

Fig. 1. (a) Map of the Macaronesia showing the location of the Canary Islands. The image in the middle shows the laurel forest, habitat of the Bolle's Laurel pigeon (*C. bollii*). Sampling sites are indicated with different coloured circles for each island. Abbreviations are listed in Table 1, (b) Results of the Bayesian analysis (STRUCTURE) by admixture model showing four clusters as the highest Delta k value for the populations of this pigeon species. Each bar shows a single individual, with each colour representing the proportion of a given genetic cluster to an individual's genotype.

trends and movements of the populations of this species are still scarce (see Martín et al., 2000). In such situations, where direct observational data on the species is limited, population genetic analysis can provide information about basic features of wildlife populations, such as demographic history, gene flow, genetic diversity and genetic differentiation of populations (Woodruff, 2001; Rollins et al., 2006; Hughes et al., 2008; Hohenlohe et al., 2021). The study of genetic variation patterns in *C. bollii* populations would reveal novel insights on the population structure, movement and connectivity within and between populations across laurel forests that still persist on the different islands.

Here we used microsatellite (SSRs) markers applied to DNA derived from moulted feathers of *C. bollii* collected in the field. Despite the growing advances in population genetics, microsatellites remain a relevant tool, providing highly informative results comparable to those obtained from other genetic markers (see Vieira et al., 2016; Hauser et al., 2021). Furthermore, non-invasive samples minimize the stress and disturbance to the birds and provide a suitable source of DNA (Presti et al., 2013). The goals of this study were to: (i) assess patterns of diversity and genetic differentiation between populations, (ii) determine genetic structure among populations of *C. bollii* within and among islands, (iii) evaluate rates of gene flow and therefore, effective dispersal among populations, and (iv) examine the implications of our findings for conservation management.

2. Methods

2.1. Study area and sample collection

Fieldwork was conducted in 2007–2008 and 2011 across all laurel forest remnants within the distribution range of *C. bollii.* Although the laurel forest has been reduced, modified or otherwise almost extirpated on all the islands, fragmentation is clearly evident on Tenerife, where three main relict forests can be identified: Anaga (T-AN), in the north-east of the island, La Esperanza-Tigaiga (T-ET) in the north, and Teno (T-TE) in the north-west (Fig. 1a). For the sampling of La Gomera and La Palma, we considered that the deep ravine areas in the north of these islands could act as barriers. We therefore sampled across two potentially divided areas on each island: Hoya del Tión-Los Roques (G-HR) in the north and Epina-Los Pajaritos (G-EP) in the south of La Gomera, and Barlovento-Niquiomo (P-BN) in the east and Garafía-Tinizara (P-GT) in the north-west of La Palma. Samples collected on El Hierro were treated as from a single population, Jinama-Tina de las Casillas (H-JT), since most of the laurel forest lies on a slope with a smoother relief (Fig. 1a). Across these eight sampling sites, moulted feathers of pigeons were collected and stored in individual paper envelopes or plastic bags under dark dry conditions. Feathers from birds killed by predators were also opportunistically sampled. In all cases, only fresh samples were analysed. Finally, GPS coordinates for each sample were recorded.

2.2. DNA extraction and sexing test

Individual feathers were chosen for genomic DNA extraction based on two criteria. First, large feathers were selected as they provide more DNA than smaller feathers (Bayard de Volo et al., 2008). Second, tail feathers were chosen as this allowed us to accurately resolve between *C. bollii* (whose feathers are blackish with a broad pale-grey subterminal band) and the sympatric *C. junoniae* (brownish tail feathers with a creamy-white terminal band). This visual diagnostic character avoids the additional expense associated with prior species identification of samples (Marrero et al., 2008). DNA extraction was performed using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions with slight modifications. All surfaces and laboratory supplies were routinely cleaned with 10 % bleach and then rinsed in sterile double-distilled water to avoid contamination among samples (Eggert et al., 2005). The entire calamus of each tail feather, including the blood clot of the superior umbilicus (Horváth et al. 2005), was cut transversely into small pieces and incubated with agitation overnight at 56ºC in 180–200 µl of ATL buffer (lysis solution) and 20 µl of proteinase K (20 mg/ml). DNA concentration and purity were determined for each extract using a Nanodrop spectrophotometer (NanoDrop 8000, Thermo Scientific, Wilmington, DE). PCR was performed with primers 18S-F/18S-R (Wu et al., 2007), to check whether DNA extracts were amplified satisfactorily. This primer pair generates a 256-base-pair (bp) fragment from the 18S ribosomal gene in both male and female Columbidae, which also served as internal control for sex determination (Wu et al., 2007). Amplifications were carried out in 10 µl total volume, containing 10–15 ng of template DNA, 0.5 µM of each primer, 10x CoralLoad PCR buffer and 5 µl of TopTaq Master Mix 2x (Qiagen). An initial denaturation cycle of 94°C for 6 min was followed by 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 45 s. All reactions ended with a final extension cycle at 72°C for 10 min. PCR products were visualised on 2 % agarose gels stained with ethidium bromide.

The conserved primers P8/P2 based on the CHD-Z and CHD-W genes (Griffiths et al., 1998) were used to determine sex. The reaction mixture was similar to those of 18S-F/18S-R, but including 20 mg/ml BSA and additional $MgCl_2$ (2.0 mM). PCR conditions were 94ºC for 3 min, 40 cycles of 94 ºC for 30 s, 55 ºC for 45 s and 72 ºC for 45 s, followed by 72 ºC for 10 min and a final step of 20 ºC for 60 s. PCR products were resolved on a 4 % agarose gel stained with ethidium bromide. Negative controls were included in all amplification runs. Due to the lack of preserved specimens of both sexes of *C. bollii*, we used muscle tissue from carcasses of *C. trocaz* (its closest relative), sexed by gonadal analysis, as reference positive control. Two PCR repetitions were performed when was necessary to ensure the correct sex assignment of each feather sample.

2.3. Microsatellite markers and genotyping

Since no information on the genomic sequence of *C. bollii* is available, we used the conserved microsatellite markers developed by Dawson et al. (2010) that can be used across a wide range of bird species. All single-plex PCR reactions were performed in a final volume of 2 µl with 0.2 µM of each primer, 1 µl Qiagen Multiplex PCR Master Mix, and approximately 12 ng of template DNA. The general PCR conditions were 15 min at 95°C, 40 cycles of 30 s at 94°C, 90 s at 58°C and 60 s at 72°C, followed by a final extension step at 60°C for 30 min. Products were diluted (1/200) and analysed on an ABI 3730 capillary sequencer (Applied Biosystems). Alleles were scored using GENEMARKER 2.4.0 (SoftGenetics) and GENEMAPPER 4.0 (Applied Byosystems). In order to detect contamination, a negative control was included in the amplifications. For some samples, re-extraction, ethanol precipitation and/or dilution of template DNA were also required to solve PCR inhibition problems.

2.4. Population genetics analyses

The reliability of genotyping results was estimated using a rarefaction method implemented in the R package "vegan" (Oksanen et al., 2013), to correct sample size differences. To assess genetic diversity within populations, the mean number of alleles per locus (A), number of private alleles (Ap), and observed (H_O) and unbiased expected heterozygosities (H_E) for each locus and population were calculated using GENALEX 6.5 (Peakall and Smouse, 2012). The software FSTAT 2.9.3.2 (Goudet, 2002) was used to calculate allelic richness (Ar) and fixation index (F_{IS}). Deviations from Hardy-Weinberg (HWE) and linkage equilibrium for each locus and population were estimated in GENEPOP 4.0 (Raymond and Rousset, 1995), using the exact test with default values for the Markov chain parameters (1000 dememorisation steps, 100 batches and 1000 iterations per batch). A sequential Bonferroni correction for multiple testing was applied to adjust p-values (Rice, 1989). The microsatellite genotyping from non-invasive samples containing low quantities of amplifiable DNA can result in heterozygote

deficits caused by the presence of null alleles (Broquet and Petit, 2004). Thus, the frequency of null alleles (f_D) for each locus was estimated using the EM algorithm of Dempster et al. (1977) with 10,000 bootstrap repetitions implemented in the program FREENA (Chapuis and Estoup, 2007). To check the effects of null alleles on our genetic diversity results, genotypes were corrected according to the estimated false homozygotes, and H_O, H_E, F_{IS} statistics and HWE test recalculated (Chapuis and Estoup, 2007; Oddou-Muratorio et al., 2009; Sun et al., 2012).

Pairwise F_{ST} and D_{est} estimators quantified genetic divergence among populations over loci. The measure of genetic fixation, F_{ST}, is calculated as the ratio of the variance in allele frequencies among populations to the overall variance (Weir and Cockerham, 1984). Additionally, D_{est} measures genetic differentiation among populations corrected for sampling bias using allele-diversity (Jost, 2008), which is thought to be more appropriate for microsatellite data sets (Bird et al., 2011). F_{ST} values were estimated in GENALEX 6.5 (Peakall and Smouse, 2012) while D_{est} was calculated in the "DEMEtics" package for R (Gerlach et al., 2010). Statistical significance and 95 % confidence intervals were tested by 10,000 bootstrap repetitions. FREENA was also used to obtain pairwise F_{ST} corrected for null alleles, $F_{ST}^{\{ENA\}}$ (Chapuis and Estoup, 2007). Hierarchical analyses of molecular variance (AMOVA) with 10,000 permutations were calculated using ARLEQUIN 3.5.1.3 (Excoffier and Lischer, 2010) to assess genetic variance at two levels: among islands, and among populations within islands. Based on the Cavalli-Sforza and Edwards distances (D_c), genetic relatedness among samples was depicted by an unrooted Neighbour-Joining (NJ) tree using POPULATION 1.2.32 (Langella, 2002), and visualised in FIGTREE 1.4.2 (Rambaut, 2007). To identify spatial patterns of genetic variation, a Principal Component Analysis (PCA) of the microsatellite individual-genotype matrix was performed using PCAGEN (Goudet, 1999).

A Mantel test was performed on log-transformed genetic and geographical distances in IBDWS 3.23 with 10,000 permutations (Jensen et al., 2005). The chord distance (D_c) of Cavalli-Sforza and Edwards (1967), calculated by the INA method in FREENA, was also used as a measure of genetic distance between populations, due to its limited sensitivity to the presence of null alleles (Chapuis and Estoup, 2007). Geographical distances were calculated (in km) from UTM coordinates using Geographic Distance Matrix Generator 1.2.3 (Ersts, 2013).

Population structure was determined using a Bayesian algorithm-based method in STRUCTURE 2.3.4 (Pritchard et al., 2000, 2010). The prior population information model was used, considering each island as a defined population. However, the admixture model was also applied at an initial point of the analysis, as recommended by the authors (Pritchard et al., 2010). Ten independent runs of K (number of clusters) for 1–10 possible populations were performed, using correlated allele frequencies between populations. Burn-in period and Markov Chain Monte Carlo (MCMC) repetitions were 50,000 and 300,000 respectively. STRUCTURE HARVESTER 0.6.93 (Earl and vonHoldt, 2012) was used to establish the value of K that best fits the data set deriving from the Evanno method (Evanno et al., 2005). First generation migrants were estimated in GENECLASS2 (Piry et al., 2004). The likelihood that an individual belongs to the reference population was calculated using the ratio of the population where the individual was sampled (L_home) to the highest likelihood observed in all sampled populations (L_max), described by Paetkau et al. (2004). The computation method was based on frequencies (Paetkau et al., 1995) and the Monte Carlo resampling algorithm was run on 10,000 simulated individuals, taking into account all loci and a type I error of 0.01.

Finally, Population Graph analysis was also used to explain genetic population structure. This is a multivariate graph-theoretical approach in which nodes (populations) are connected in a network by the shortest path of the edges, named conditional genetic distance (cGD). The edge width represents the degree of dependence of evolutionary trajectories between populations, while node sizes indicate allelic richness within populations (Dyer and Nason, 2004). The significance level was adjusted for $P = 0.05$. In a simulation analysis, Klütsch et al. (2012) found that null alleles influence population graph topologies, independently of their frequency in the populations. For that reason, the null allele corrected data set was also evaluated. Evidence of isolation by distance was also examined using the genetic covariance. We developed this analysis by "popgraph" package in R (Dyer, 2013).

3. Results

3.1. DNA extraction and sexing

DNA was extracted from 186 field-sampled moulted tail feathers: Tenerife (77; T-AN: 37, T-ET: 19 and T-TE: 21), La Gomera (38; G-EP: 23 and G-HR: 15), La Palma (38; P-BN: 21 and P-GT: 17) and El Hierro (H-JT: 33). The integrity of DNA obtained from feathers was demonstrated by the 100 % amplification success rate for the 18S ribosomal DNA. Molecular sexing reactions provided a reliable DNA profile allowing for chromosomal sex identification of *C. bollii* (Fig. 2): Amplicons from females consisted of two different sized bands (~ 350 and ~ 370 bp), while males showed only the larger band (~ 370 bp). We were able to sex 65.6 % of total samples, of which 54.1 % were identified as females and 45.9 % as males.

3.2. Microsatellite analysis and genetic diversity

Of the 34 markers tested, 10 yielded polymorphic products within *C. bollii* (TG01–077, TG01–124, TG01–148, TG02–078, TG02–120, TG03–002, TG04–012, TG07–022, TG13–017 and TG22–001), with allelic ranges similar to that described by Dawson et al. (2010) for other avian species. The rarefaction analysis demonstrated that sample sizes were clearly adequate to detect the cumulative allelic richness of each population (Fig. S1).

A total of 60 alleles were identified across 10 microsatellite loci (Table 1), with a mean per locus of 3.85 and an allelic richness of 3.57 for all islands, estimated from 23 diploid individuals as a minimum sample size. The maximum and minimum numbers of unique alleles were found on Tenerife (10) and El Hierro (3), respectively. The average observed and unbiased expected heterozygosities were low, ranging from 0.233 to 0.270 and 0.325–0.374, respectively. The deficiency in the proportion of observed heterozygotes with respect to the expected is also reflected by positive fixation indices (F_{IS} for all islands = 0.272, 95 % CI = 0.195–0.358). Similar results were obtained at population level, except for the number of private alleles, which was higher in G-HR (7) and absent in P-BN and G-EP (Table 1). No significant difference was found in the genetic diversity measures (Ar, H_O, H_E and F_{IS}) between islands ($P > 0.05$ for paired t-test) or populations. Genetic diversity values per locus are shown in Table S1. Significant deviations from HWE after Bonferroni correction were detected in some loci but were not

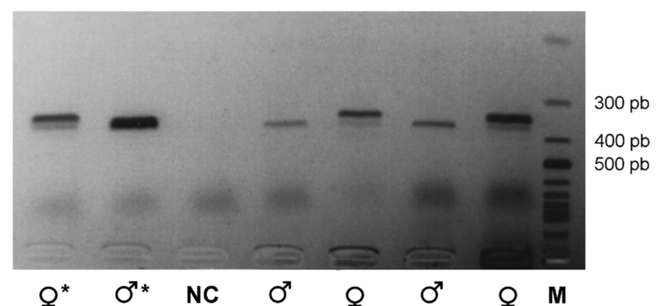

Fig. 2. Feather sexing test of Bolle's Laurel pigeon (*C. bollii*) using PCR products amplified for CHD-Z and CHD-W genes by primer sets P2/P8. Females (♀) have two bands and males (♂) one band. M is a 1Kb DNA marker, NC the negative control and the asterisk indicates the positive controls.

Table 1
Summary statistics of genetic diversity derived using ten microsatellite loci for Bolle's Laurel Pigeon (*Columba bollii*) populations in the Canary Islands. N = number of samples, A = mean number of alleles per locus, Ar = mean allelic richness, Ap = number of private alleles, H_O = observed heterozygosity, H_E = unbiased expected heterozygosity and F_{IS} = fixation index. [C] Statistics corrected for null alleles.

Island/ Population	N	A	Ar	Ap	$H_O/{}^CH_O$	$H_E/{}^CH_E$	$F_{IS}/{}^CF_{IS}$
El Hierro	**33**	**3.70**	**3.60**	**0.30**	**0.26/ 0.43**	**0.37/ 0.43**	**0.32/ −0.02**
H-JT	33	3.70	3.12	0.30	0.26/ 0.44	0.37/ 0.43	0.32/ −0.02
La Palma	**38**	**3.90**	**3.55**	**0.40**	**0.27/ 0.40**	**0.34/ 0.40**	**0.21/ 0.01**
P-BN	21	3.10	2.79	0.00	0.27/ 0.38	0.32/ 0.37	0.18/ −0.02
P-GT	17	3.50	3.25	0.30	0.27/ 0.4	0.36/ 0.43	0.25/ 0.01
La Gomera	**38**	**3.60**	**3.20**	**0.70**	**0.23/ 0.40**	**0.33/ 0.41**	**0.29/ 0.03**
G-EP	23	3.00	2.71	0.00	0.21/ 0.38	0.31/ 0.38	0.32/ 0.01
G-HR	15	3.20	3.11	0.70	0.28/ 0.39	0.36/ 0.40	0.23/ 0.03
Tenerife	**77**	**4.20**	**3.29**	**1.00**	**0.25/ 0.44**	**0.34/ 0.44**	**0.27/ 0.00**
T-AN	37	3.60	2.86	0.30	0.21/ 0.47	0.35/ 0.47	0.42/ 0.01
T-ET	19	2.80	2.62	0.10	0.30/ 0.41	0.33/ 0.38	0.08/ −0.08
T-TE	21	3.20	2.94	0.30	0.29/ 0.39	0.34/ 0.40	0.17/ 0.01
All islands	**186**	**3.85**	**3.57**	**0.60**	**0.25/ 0.42**	**0.35/ 0.42**	**0.27/ 0.01**

Sampling localities are indicated as H-JT: Jinama-Tina de las Casillas in El Hierro, P-BN: Barlovento-Niquiomo and P-GT: Garafía-Tinizara in La Palma, G-EP: Epina-Los Pajaritos and G-HR: Hoya del Tión-Los Roques in La Gomera, and T-AN: Anaga, T-ET: La Esperanza-Tigaiga and T-TE: Teno in Tenerife.

equally represented on all islands (Table S1). On Tenerife, six loci were out of HWE, while for La Palma, El Hierro and La Gomera only one to four were not at equilibrium. At population level, most of the deviation derived from T-AN samples on Tenerife, with five loci in disequilibrium. The departure from HWE was not locus specific. A monomorphic locus (TG07–022) was also detected for La Palma. There was no significant pattern of linkage disequilibrium ($P > 0.05$) for pairs of loci.

The frequency of null alleles per locus per island ranged from 0 to 0.291, with a mean frequency of 0.084. TG02–078 showed the highest mean frequency of null alleles ($\hat{r}_D = 0.155$) and TG03–002 the lowest ($\hat{r}_D = 0.037$), although these values varied according to island (Table S1). The correction of the data set for null alleles increased heterozygosity values (H_O = 0.396–0.436 and H_E = 0.403–0.429), reduced the overall heterozygote deficit (F_{IS} = 0.007, 95 % CI −0.010–0.025), and all islands/populations and loci were in Hardy-Weinberg equilibrium.

3.3. Genetic population differentiation

Genetic divergence, estimated as pairwise F_{ST} and D_{est} between islands, ranged from 0.008 to 0.020 for F_{ST}, and from 0.0008 to 0.018 for D_{est}. All pairwise comparisons were significant after sequential Bonferroni correction, except those involving samples from La Palma and El Hierro (Table 2). Both estimators gave similar results, however, pairwise $F_{ST}^{\{ENA\}}$ suggests that null alleles underestimated the overall differentiation between islands (Table 3). When the impact of null alleles was estimated on pairs of populations, the genetic divergence was more evident (Table S2). Highly significant differences (P<0.01) were found between La Gomera populations (G-EP and G-HR) and those from El Hierro (H-JT) and La Palma (P-BN and P-GT), while T-AN and T-ET were generally different from the other insular populations. The global F_{ST} was 0.015 (P = 0.001, 95 % CI 0.006–0.025) but the overall differentiation excluding null alleles was slightly higher with $F_{ST}^{\{ENA\}} = 0.024$ ($P < 0.001$, 95 % CI 0.011–0.039).

AMOVA results as a weighted average over loci revealed that 1.64 % ($F_{CT} = 0.016$, $P = 0.001$) of the total genetic variation was attributable to the variability among islands, 0.39 % ($F_{SC} = 0.003$, $P = 0.613$) to the variability between populations within islands and 97.98 % (F_{ST} = 0.020, $P = 0.004$) was accumulated within populations within islands. Clustering analysis using the NJ algorithm from D_c genetic distances showed that samples from La Gomera and Tenerife clustered together in their respective branches, while those from La Palma were mainly associated with El Hierro (Fig. 3a). The first two PCA axes performed on the allele frequencies of eight populations explained about 60 % of the genetic variability among populations. This also implied that populations from geographically close islands did not cluster together, although populations from La Palma were closer to the El Hierro population on the PC1-axis (Fig. 3b). However, the Mantel test of isolation by distance showed a positive relationship between geographical and genetic distances using D_C ($r = 0.182$, $P = 0.178$) and cGD ($r = 0.432$, $P = 0.004$) for the eight populations.

3.4. Population structure

The two models run in STRUCTURE 2.3.4 were largely consistent in the number of genetic clusters (K) estimated. The admixture model gave K = 4 as the most probable number of clusters and secondary peaks with larger standard deviations at K = 3, 6 and 9. Similarly, K = 4 was clearly supported by the prior population information model, when four islands were assumed (Fig. 1b). The genetic structure analysis within each of the four clusters showed support for K = 1 for La Palma and El Hierro, K = 2 for La Gomera and K = 3 for Tenerife (Fig. S2).

Table 3
Pairwise $F_{ST}^{\{ENA\}}$ (below diagonal) and their significance (above diagonal) between the populations of Bolle's Laurel Pigeon (*Columba bollii*) on the Canary Islands. 95 % confidence intervals are shown.

	El Hierro	La Palma	La Gomera	Tenerife
El Hierro		n.s.	***	***
La Palma	0.00 (0.00–0.01)		***	***
La Gomera	0.05 (0.01–0.10)	0.04 (0.01–0.08)		***
Tenerife	0.03 (0.01–0.04)	0.02 (0.01–0.03)	0.02 (0.00–0.04)	

***P < 0.001, n.s. = not significant

Table 2
Pairwise F_{ST} (below diagonal) and D_{est} values (above diagonal) between the four genetic clusters of Bolle's Laurel Pigeon (*Columba bollii*) in the Canary Islands. 95 % confidence intervals are shown.

	El Hierro	La Palma	La Gomera	Tenerife
El Hierro		0.00 (−0.01–0.01) n.s.	0.02 (0.01–0.03)*	0.02 (0.01–0.03)*
La Palma	0.01 n.s.		0.01 (0.01–0.02)*	0.01 (0.01–0.02)*
La Gomera	0.02*	0.02*		0.01 (0.01–0.02)*
Tenerife	0.02**	0.01*	0.01*	

*P < 0.05, **P < 0.01, n.s. = not significant

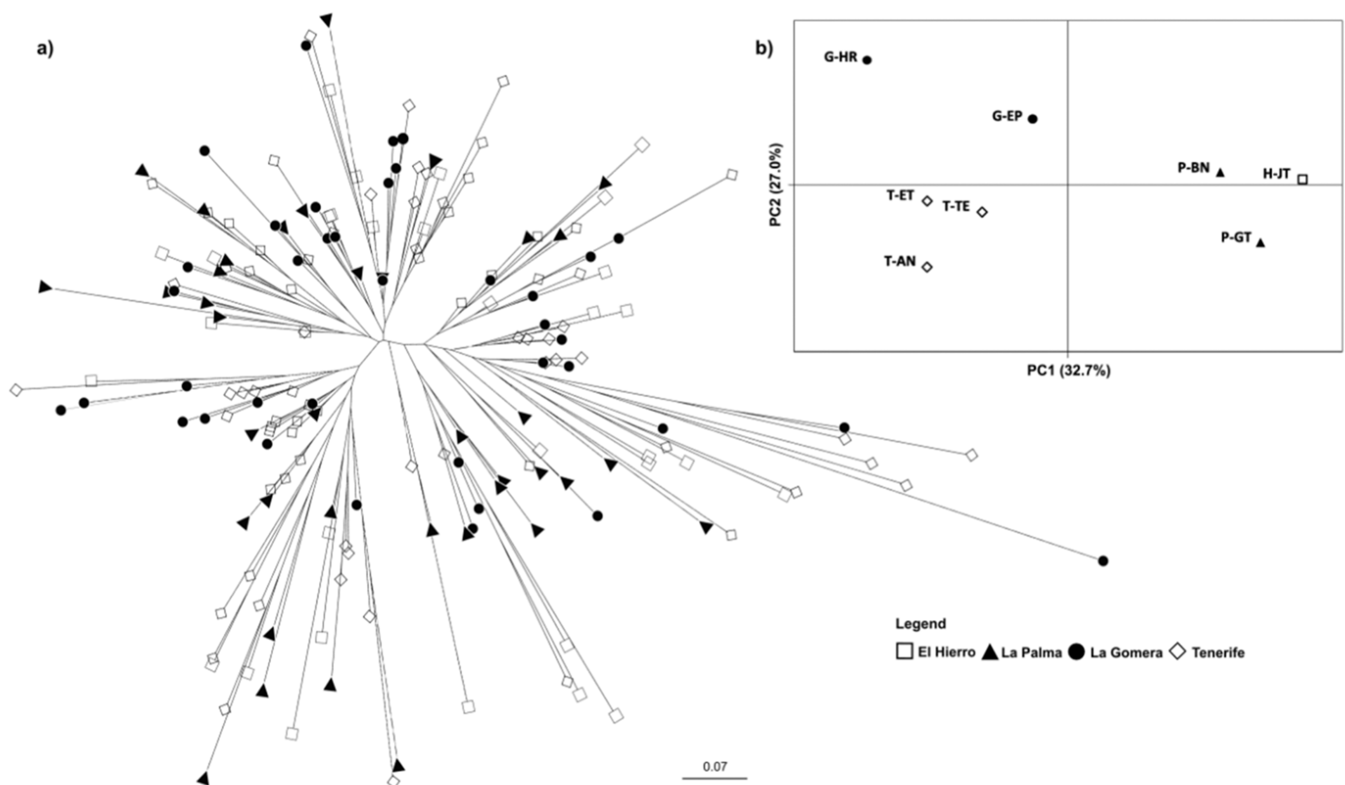

Fig. 3. Clustering of Bolle's Laurel pigeon (*C. bollii*) based on Neighbor Joining (NJ) and Principal Component Analysis (PCA). (A) Unrooted NJ tree inferred from Cavalli-Sforza genetic distances. Scale bar represents branch lengths. (B) PCA plot of averaged first (PC1) and second (PC2) principal component scores for the eight pigeon populations. Island distribution is shown with different colours and symbols. Sampling localities are indicated as H-JT: Jinama-Tina de las Casillas in El Hierro, P-BN: Barlovento-Niquiomo and P-GT: Garafía-Tinizara in La Palma, G-EP: Epina-Los Pajaritos and G-HR: Hoya del Tión-Los Roques in La Gomera, and T-AN: Anaga, T-ET: La Esperanza-Tigaiga and T-TE: Teno in Tenerife.

To evaluate the effect of null alleles on the STRUCTURE analysis, TG02–078 (with the highest frequency of null alleles) was deleted from the genotype data set and all the analyses rerun. The overall data then showed that the optimal number of clusters was three using the admixture model, but four using the prior population information model. The same pattern of sub-clusters within islands was obtained (results not shown). GENECLASS2 test was consistent with the STRUCTURE results because most individuals were assigned to the population in which they were sampled. Nevertheless, four possible first-generation migrants were detected; two of them from La Palma to El Hierro and La Gomera, and two from Tenerife to La Gomera and La Palma. No evidence of recent movements within islands was detected, possibly due to low power as a result of small sample size. However, these results mainly reflect affinities among individuals, rather than true migration events.

The Population Graph analysis showed 14 edges connecting the eight populations ($p_{edge} = 0.5$), which represent the best-fit model to the total among-population covariance structure (Fig. 4). The lower the number of edges, the higher the conditional genetic distance between nodes. The highest degree of connectivity was shown by T-TE, while T-AN was the most isolated population. Network topology also suggests that spatial genetic structure may depend on other variables besides geographic distances among populations, since populations geographically distant from each other do not always present higher genetic differentiation. This is shown by the thickness of the edges in Fig. 4. Analysis using the data set corrected for null alleles resulted in a different network structure (Fig. S3): a lower number of edges ($p_{edge} = 0.393$) reflected the separation of La Palma and El Hierro populations from those of La Gomera, with conditional independence of Tenerife populations through the links of P-BN and P-GT with T-AN and T-TE, respectively. In any case, the Population Graph revealed that La Palma and El Hierro populations constituted a module or complete graph subset (Fig. S3).

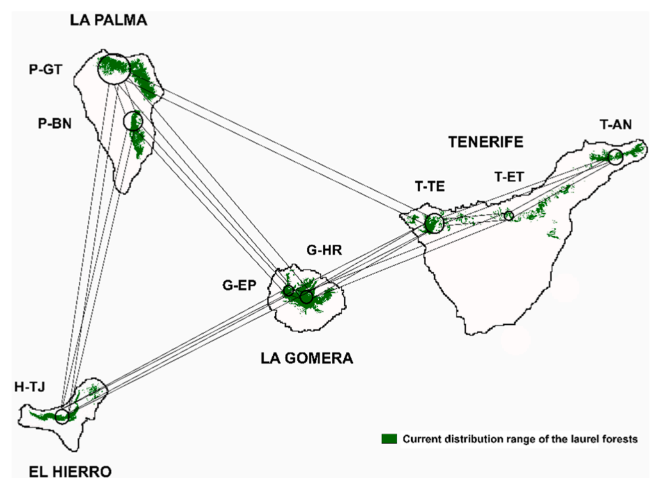

Fig. 4. Population graph showing the connectivity network among Bolle's Laurel pigeon (*Columba bollii)* populations. Circles represent the central location of sampled populations (size is proportional to within-population allelic richness). The edge set indicates the genetic structure conditionally dependent among populations (line width shows the strength of genetic covariance). Sampling localities are indicated as H-JT: Jinama-Tina de las Casillas in El Hierro, P-BN: Barlovento-Niquiomo and P-GT: Garafía-Tinizara in La Palma, G-EP: Epina-Los Pajaritos and G-HR: Hoya del Tión-Los Roques in La Gomera, and T-AN: Anaga, T-ET: La Esperanza-Tigaiga and T-TE: Teno in Tenerife. *The laurel forest areas comprise diverse plant communities that reflect varying environmental conditions. Taken from Arco et al. (2010).

4. Discussion

4.1. Genetic diversity

Genetic diversity estimated for *C. bollii* based on microsatellite markers was low. While strong selective constraints may preclude comparisons of microsatellite data across species evolutionary separated, our results showed similar an expected heterozygosity (average across the four islands, $H_E = 0.35$) to the value found for *C. janthina janthina*, endemic to islands in East Asia ($H_E = 0.31$; Ando et al., 2011). In contrast, the genetic diversity of *C. bollii* was clearly lower than that of *Zenaida galapagoensis*, endemic of Galapagos islands ($H_E = 0.56$–0.65; Santiago-Alarcón et al., 2006) and *Patagioenas squamosa*, the Caribbean-endemic pigeon ($H_E = 0.77$; Cambrone et al., 2021), but higher than that found for the critically endangered *C. janthina nitens*, endemic to the Ogasawara islands ($H_E = 0.12$; Ando et al., 2014). However, the presence of null alleles may have underestimated our results; although the mean frequency of null alleles was relatively low (< 0.1), the average heterozygosity increased after correcting the genotype data for false homozygotes. In addition, all islands/populations and loci were in HWE using the corrected data set. These results suggest that null alleles may be the main cause of heterozygote deficiencies and departures from HWE. Their potential bias should, therefore, be evaluated in population genetics despite their relatively low frequency. However, other factors such as the Wahlund effect (unresolved population substructure), inbreeding or selection near, or at, microsatellite loci might have similar effects on the heterozygosity estimation, so these factors should not be ruled out as contributing to departures from HWE (Dakin and Avise, 2004).

4.2. Population differentiation

Genetic variability within *C. bollii* populations was very similar in all sites (see Table 1 and S1), suggesting that the populations share a common origin through a metapopulation structure. AMOVA results also support this. However, the global F_{ST} and pairwise genetic differentiation values did reveal significant genetic structuring in the entire sample and between most islands and populations. Although $F_{ST}^{\{ENA\}}$ indicated a higher level of genetic differentiation than F_{ST} and D_{est}, the differentiation patterns inferred from these three estimators were largely consistent: La Palma and El Hierro populations were genetically more similar to each other than to the La Gomera and Tenerife populations. Moreover, AMOVA showed that although most of the genetic variation was explained by within-population variance, weak but significant genetic structure was explained by variation among islands.

These results imply that *C. bollii* populations do not comprise a single panmictic unit over their whole range, but rather of at least three insular groups: La Palma-El Hierro, La Gomera and Tenerife. These results could reflect that *C. bollii* populations once formed a genetically homogeneous population that became differentiated due to physical barriers (open sea and highly fragmented habitat), or, alternatively, to a single colonisation event occurring on a single island, followed by stepwise dispersal to neighbouring islands.

The metapopulation structure suggested for *C. bollii* pigeons implies a complex interaction between spatially separated individuals via dispersal. Migrants could also be able to recolonise depleted and/or new areas, a phenomenon that has probably occurred throughout their evolutionary history. Moreover, we also suggest that this population structure tends to reflect the relationship between the islands' geological ages and the evolution of pigeon populations. According Valente et al. (2017), the arrival of *C. bollii* may have occurred during the Pleistocene (2.14 Myr; 95 % CI: 1.42–2.91 Myr), when most of the islands of the archipelago had emerged. Therefore, the genetic traits of *C. bollii* could represent ancestral evolutionary responses to the geological history of archipelago, highlighting La Gomera and Tenerife (more precisely T-TE area) populations as the oldest lineages, given the greater number of private microsatellite alleles detected.

While available information on the geological origin of the islands provides important context for understanding the evolutionary history of *C. bollii* populations, the proposed genetic differentiation scenarios (homogeneous ancestral population vs gradual colonization) cannot be conclusively evaluated with current data. Molecular clock techniques and phylogeographic analysis would allow a more precise estimation of divergence times between island populations and a detailed examination of genetic differentiation patterns.

Population genetic analysis in several native Canarian passerines have showed a similar genetic differentiation pattern - with a close relationship between El Hierro and La Palma populations - to that found in Bolle's pigeons (Illera et al., 2012, 2016; Valente et al., 2017). The main explanation for this result could be related to the recent divergence of lineages, combined with differences in evolutionary rates in neutral genetic loci (Marthinsen et al., 2008). The genetic differentiation in island populations can be linked to morphological variations as described in the Azores woodpigeon, *C. palumbus azorica* (Andrade et al., 2020), as well as in some Macaronesian songbirds (eg. Dietzen et al., 2006; Päckert et al., 2006; Illera et al., 2007, 2018). This phenotypic differentiation has been suggested to be a response to insularity. Although yet not explored in *C. bollii*, slight variations in colour and morphology were noted in the sampled tail feathers (P. Marrero, pers. obs.), which could indicate underlying differences between populations that should be confirmed.

4.3. Isolation by distance

The Mantel test indicated that genetic differentiation can be only partially explained due to isolation by distance. This supports the idea that factors other than geographical proximity, such as barriers to gene flow, selection, or other demographic process, may also be contributing to the observed genetic patterns of these populations. The potential relationship between habitat characteristics, such as the size, fragmentation, and quality of the ecosystems, and the observed genetic differentiation would be further explored to fully understand the drivers behind the genetic structure (Orsini et al., 2013; Binks et al., 2019). In fact, the central-eastern islands of the archipelago once harboured pigeon populations, now extinct due to the drastic destruction of laurel forests (Alcover and Florit, 1989; Rando and Perera, 1994). Although some of these islands, such as Gran Canaria, still conserve small and impoverished laurel forest remains (Del Arco Aguilar et al., 2010), no population of wild extant laurel pigeons has yet been established there.

4.4. Population structure of the Bolle's Laurel Pigeon

The populations of *C. bollii* clustered together similarly in the NJ tree, PCA and Bayesian analyses. STRUCTURE and GENECLASS2 produced the same general assignment pattern, even when the microsatellite locus with the highest null allele frequency was excluded from the analysis (see Carlsson, 2008). Four genetic groups corresponding to the four islands were identified, although La Palma and El Hierro populations may belong to the same cluster according to results from the corrected data set. This result would indicate that both populations share a similar genetic composition, which could be related to a common origin, gene flow between them, or a recent genetic divergence that has not yet been reflected in a clear differentiation. The STRUCTURE analysis also revealed fine-scale structuring within Tenerife and La Gomera, that should be assessed through more exhaustive sampling. Nevertheless, some degree of contemporary gene flow between pigeons from different islands was detected by GENECLASS2.

Genetic structuring of populations was corroborated by the Population Graph analysis. The connectivity network revealed moderate and differential gene flow, confirming that genetic divergence between populations is not only because of the spatial distances. The graphical topology generated by the null allele corrected data set yielded fewer

edges, implying that the null alleles lead to overestimating the connectivity between populations, in accordance with the findings of Klütsch et al. (2012).

4.5. Implications for conservation

This study provides a useful first step in gaining the scientific data required to inform appropriate conservation measures for *C. bollii*. The species is now listed as Least Concern on the IUCN (2024), however, threads of ongoing habitat loss and fragmentation, invasive predation, introduction of potential diseases, as well as illegal hunting and poisoning, still persist.

From the genetic data obtained here, we conclude that the similar genetic diversity found in the pigeon populations may indicate gene flow among them, but not enough to eradicate such population differences. These genetic differences are likely to reflect both the ancestral origins and ecological relationships that should be preserved. Although our results indicate that the pigeon has long-distance flight ability, which has been recently confirmed by some field observations of *C. bollii* individuals in forestal areas of Gran Canaria (Martín et al., 2020), the actual range and frequency of dispersal remain unknown. The same lack of information applies regarding data on population size, reproduction, movement patterns and demographic trends including spatiotemporal variation and local adaptations in *C. bollii*. Therefore, the major aim of any management action on Bolle's pigeons should begin with careful field studies leading to protect ecological diversity and evolutionary processes across the geographical range of the species, as well as to maintain the natural network of genetic connections between their populations (see Snyder et al., 1996; Crandall et al., 2000; Theodorou et al., 2009).

Finally, comprehensive conservation efforts focused on protecting and restoring the species' laurel forests will be essential to safeguard its future. Insights from our population genetic studies of *C. bollii* provides a base on which to investigate the role that large-bodied frugivores, like pigeons, may have played in shaping the range of the plant communities, but also the biogeography and evolution of certain fleshy-fruited plant species (Jordano and Godoy, 2002; Corlett, 2017). Understanding these complex relationships will be crucial in informing targeted conservation strategies to protect both *C. bollii* and the laurel forests on which it depends.

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CRediT authorship contribution statement

Rosa Fregel: Writing – review & editing, Supervision, Funding acquisition. **David S. Richardson:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Patricia Marrero:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.zool.2024.126209.

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