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Contrasting patterns of genetic differentiation in Macaronesian lineages of *Ilex* (Aquifoliaceae)

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Islands offer an interesting framework in which to study the effect of geographical isolation on population genetic differentiation. For plant species with high dispersal abilities, however, oceanic barriers may not represent a factor promoting strong population structure. In this work, we analysed seven nuclear microsatellite loci in *Ilex* (Aquifoliaceae), a bird-dispersed plant group, to infer patterns of genetic differentiation among Macaronesian taxa: *I. canariensis*, *I. perado* ssp. *lopezlilloi*, *I. perado* ssp. *platyphylla* (Canary Islands) and *I. perado* ssp. *azorica* (Azores). In agreement with current taxonomic classification, our results revealed a high genetic differentiation between *Ilex* lineages (*I. canariensis* and the *I. perado* complex), and also supported previous hypotheses that these are the result of independent dispersal events to the islands. In contrast, genetic differentiation between *I. perado* ssp. *azorica* and the two subspecies from the Canaries was high, suggesting that taxonomic revision may be necessary. Levels of genetic variation at microsatellite loci in ssp. *azorica* were, in addition, the lowest reported among Macaronesian bird-dispersed taxa. Lastly, low genetic differentiation was observed between subspecies occurring on the same island (sspp. *platyphylla* and *lopezlilloi*). In summary, our results revealed contrasting patterns between Macaronesian *Ilex* lineages: *I. canariensis* displayed moderate population structure across islands, whereas the *I. perado* complex showed strong differentiation among populations sampled on different islands. Thus, the Macaronesian *Ilex* taxa show that long-distance dispersal syndromes (ornithochory) do not always ensure genetic connectivity across large areas in island systems. Plant groups that successfully colonized the islands on multiple occasions may have found barriers to gene flow within certain lineages. *Botanical Journal of the Linnean Society*, 2013, **173**, 258–268.

ADDITIONAL KEYWORDS: Canary Islands – genetic diversity – *Ilex canariensis* – *Ilex perado* ssp. *azorica* – *Ilex perado* ssp. *lopezlilloi* – *Ilex perado* ssp. *platyphylla* – long-distance dispersal – Macaronesia – microsatellites.

INTRODUCTION

The genus *Ilex* L. (Aquifoliaceae) is a cosmopolitan taxon which includes > 400 species of trees and shrubs widely distributed in the tropics, subtropics and temperate zones of both hemispheres (Mabberley, 1993; Manen *et al*., 2010). In the Macaronesian region, *Ilex* is represented by two currently recognized species (Arechavaleta *et al*., 2010; Silva *et al*., 2010): *I. canariensis* Poir. (Canary Islands and Madeira) and *I. perado* Aiton, a complex of four subspecies distributed in different Macaronesian archipelagos: *I. perado* ssp. a*zorica* (Loes) Tutin (*I. azorica* auct.) (Azores), *I. perado* ssp. *perado* Aiton (Madeira) and *I. perado* ssp. *platyphylla* (Webb & Berthel.) Tutin and *I. perado* ssp. *lopezlilloi* (G. Kunkel) A.Hansen & Sunding (Canary Islands). Previous phylogenetic analyses have indicated that these two species are the result of a minimum of two independent colonization events from mainland ancestors (Cuénoud *et al*., 2000; Manen *et al*., 2010). Nevertheless, our knowledge of the patterns of differentiation in Macaron-

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esian *Ilex* lineages is based on evidence obtained from a few (one or two) samples included in genus-wide phylogenetic analyses (e.g. Manen, Boulter & Naciri-Graven, 2002; Selbach-Schnadelbach *et al*., 2009; Manen *et al*., 2010). A lack of studies addressing relationships in the *I. perado* complex, for instance, precludes further inference on the evolutionary history of *Ilex* in the Macaronesian islands.

One feature that probably explains multiple events of colonization in plant species, as inferred for *Ilex* in island regions, is the presence of fleshy fruits dispersed by birds (e.g. Cuénoud *et al*., 2000). Results from recent population genetic studies have suggested that long-dispersal syndromes, such as ornithochory, could be responsible for the low level of genetic structuring found in island species with widespread distributions (García-Verdugo *et al*., 2010b; Ferreira *et al*., 2011; Rumeu *et al*., 2011). In the case of *Ilex*, the widespread occurrence of *I. canariensis* throughout the Canaries and the presence of *I. perado* populations on several islands (Arechavaleta *et al*., 2010) points towards frequent events of successful seed dispersal over oceanic barriers. However, the restricted distribution of some subspecies, with their low population numbers, has been a reason to include some of these taxa on the IUCN red list of threatened taxa (IUCN, 2010). Understanding the levels and apportionment of genetic diversity within and among populations is therefore important for the conservation of these island endemics, especially because their island ranges may make them more vulnerable to extinction than continental taxa (Sosa, 2001; Frankham, Ballou & Briscoe, 2002; González-Pérez *et al*., 2009a, b; Sosa *et al*., 2010a). Thus, information on the genetic structure and relationships among *Ilex* taxa may have implications for classification and genetic conservation (González-Pérez *et al*., 2009a; 2009b).

The general aims of this study were: (1) to investigate the patterns of genetic variation in Macaronesian *Ilex* taxa; and (2) to evaluate whether levels of genetic differentiation as inferred from molecular data are in agreement with the current taxonomic treatment.

MATERIAL AND METHODS

Study taxa

Four taxa encompassing the limits of distribution for *Ilex* in the Macaronesian region were considered in the present study. *Ilex canariensis* (Canary holly) is a laurel forest tree that has been catalogued as 'near threatened' by the IUCN (IUCN, 2010). It is easily distinguishable from *I. perado* by its smaller leaves with bluntish points, its entire margins and the presence of stalked clusters of flowers in the upper leaf axils (Loizeau *et al*., 2005). In the Canarian archipelago, this species occurs on five of the islands: El Hierro, La Palma, La Gomera, Gran Canaria and Tenerife (Arechavaleta *et al*., 2010). *Ilex perado* ssp. *lopezlilloi* (hereafter, *lopezlilloi*) is endemic to the island of La Gomera (Canary Islands), and has been classified as 'critically endangered' (IUCN, 2010) because of the small number of individuals known in the wild. It was also catalogued as 'endangered' by the Canarian Government (BOC, 2010) and has also been included on the Spanish Red List of Endangered Plants (Moreno *et al*., 2008). The distribution area of *lopezlilloi* is small, and wild individuals are only found within the Garajonay National Park. However, conservation strategies recently adopted by this National Park (La Gomera) have reinforced the number of individuals by means of asexual propagation (Bañares *et al*., 2004). Morphologically, the few known individuals exhibit intermediate characteristics between *I. perado* ssp. *platyphylla* and *I. canariensis*, having, in general, cuneate leaves and longer floral pedicels.

Ilex perado ssp. *platyphylla* (hereafter *platyphylla*) has been classified as 'vulnerable' according to IUCN categories (IUCN, 2010), and has also been included on the Spanish Red List of Endangered Plants (Moreno *et al*., 2008). It is endemic to the Canary Islands (Kunkel, 1977), and populations have been reported for the islands of La Palma, La Gomera and Tenerife (Arechavaleta *et al*., 2010). Lastly, *I. perado* ssp. *azorica* (hereafter *azorica*) is endemic to the Azores archipelago (Silva *et al*., 2010), and has been classified as 'near threatened' according to IUCN criteria (IUCN, 2010). It is characterized by obovate, acute or obtuse leaves, which are relatively small compared with those displayed by taxa of the *I. perado* complex. A common feature for all of these *Ilex* taxa is that their fruits are known to be dispersed by frugivorous birds, especially blackbirds (*Turdus merula*), European robins (*Erithacus rubecula*) and two endemic fruit pigeons, Bolle's pigeon (*Columba bollii*) and the laurel pigeon (*Columba junoniae*) (Arévalo, Delgado & Fernández-Palacios, 2007).

Sampling and genotyping

The total sample size included 233 individuals from 15 populations on five islands (Fig. 1, Table 1). For *I. canariensis*, population sampling represented the distribution of the species on each island of the Canarian archipelago, with the exception of the island of El Hierro, which was not sampled. All known individuals of *lopezlilloi* were sampled, whereas *platyphylla* was represented from each island where the species occurs sympatrically with *I. canariensis* (La Gomera and Ten-

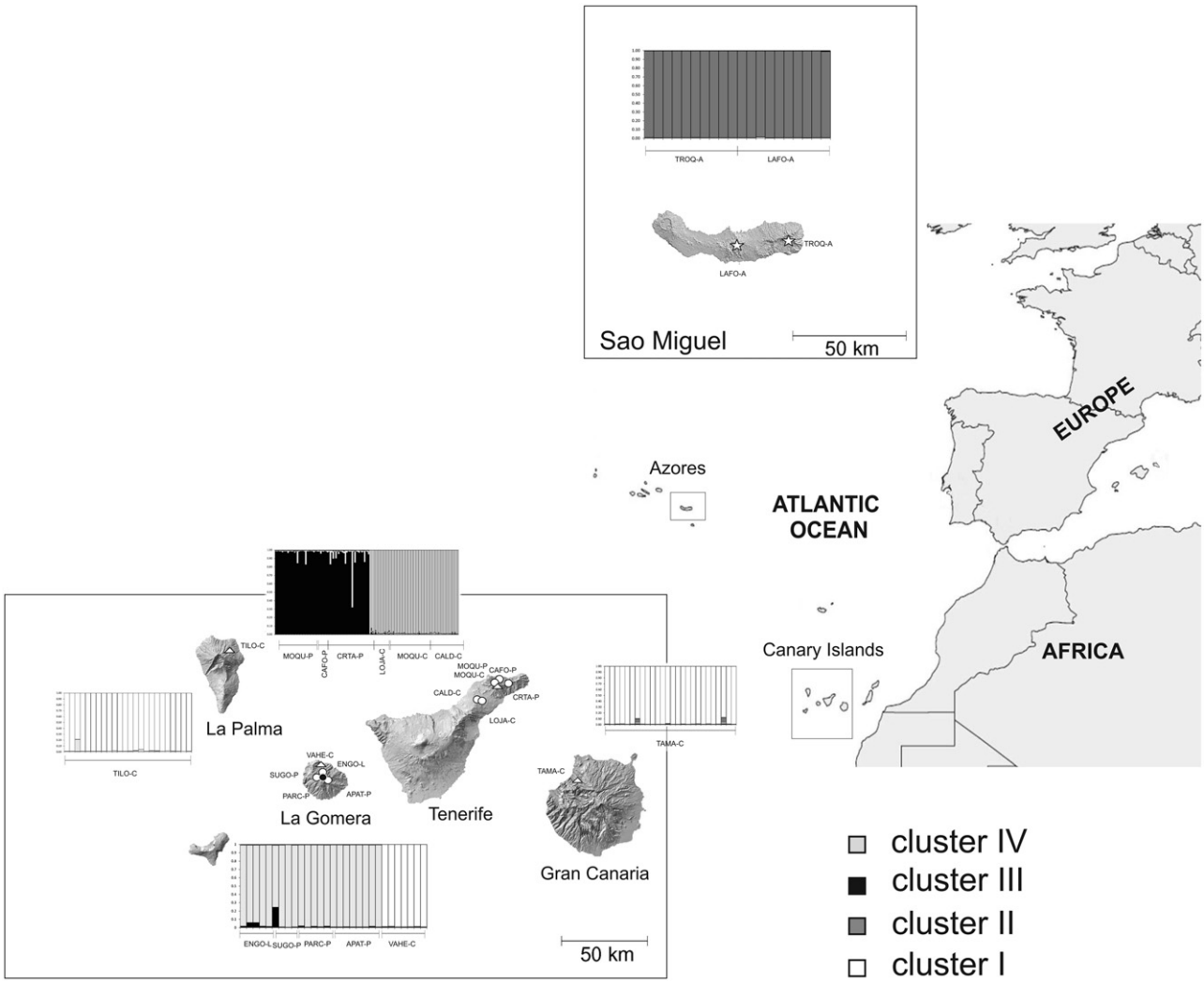

Figure 1. Geographical distribution of *Ilex* populations sampled in this study: *I. canariensis* (△), *I. perado* ssp. *azorica* (☆), *I. perado* ssp. *platyphylla* (○) and *I. perado* ssp. *lopezlilloi* (•). Graphs next to the islands display results for genetic structure as inferred with STRUCTURE ($K = 4$). Each bar represents an individual and each inferred group is represented by a different colour. Populations are coded as in Table 1.

erife). Unfortunately, samples of *Ilex perado* ssp. *perado* from Madeira could not be obtained for this study.

For DNA analysis, silica-dried leaf samples collected from each individual were ground in a mixer-mill (RETSCH MM 301). DNA was extracted following a modified 2 × cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). However, DNA from *I. canariensis* was extracted following the protocol of Dellaporta, Wood & Hicks (1983), because of the high concentration of metabolites detected in this species. In both cases, 150 μL of each total DNA sample was purified using a Genelute PCR Clean-Up Kit (SIGMA). Five primer pairs described for *I. leucoclada* Makino by Torimaru *et al*. (2004) (ILE04–ILE10, ILE03–ILE38, ILE03–ILE53, ILE03–ILE01 and ILE04–ILE06) and two primer pairs recently isolated for the same species (ILE05–ILE81 and ILE03–ILE86b; T. Torimaru, pers. comm.) were labelled with fluorochromes (6-FAM, VIC, NED and PET) and used for PCR amplification. Each 25-μL PCR contained approximately 20 ng of DNA, 10 pmol of each primer and a PCR Master Mix (Reddy-Mix, ABgene, Surrey, UK) that included 0.625 units of Taq DNA polymerase, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween20, 2.5 mM $MgCl_2$ and 0.2 mM of each deoxynucleoside triphosphate (dNTP). Amplifications were carried out using the following thermal cycling conditions: 3 min denaturation at 94 °C; 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 56 °C and 45 s elongation at 72 °C; followed by 5 min elongation at 72 °C. Detailed protocols are fully available

Table 1. Location, sample size and genetic indices for populations of *Ilex* taxa considered in this study

Population	Island	*N*	*G/N*	*A*	NA	NE	H_O	H_E	*%P*	F_{IS}
I. perado ssp. *lopezlilloi*										
ENGO-L	La Gomera	5	0.60	2.14	15	0	0.600	0.486	100	–0.273^{ns}
I. perado ssp. *platyphylla*										
SUGO-P	La Gomera	4	1.00	2.29	16	0	0.393	0.418	100	0.070^{ns}
PARC-P	La Gomera	5	1.00	2.43	17	0	0.429	0.391	85.7	–0.111^{ns}
APAT-P	La Gomera	8	0.38	1.57	11	0	0.515	0.302	57.1	–0.807^{ns}
MOQU-P	Tenerife	28	0.96	3.29	23	1	0.457	0.493	100.0	0.071^{ns}
CAFO-P	Tenerife	10	1.00	2.71	19	0	0.486	0.534	100.0	0.088^{ns}
CRTA-P	Tenerife	31	0.52	3.00	21	1	0.493	0.531	100.0	0.073^{ns}
Overall		86		3.86	27	7	0.475	0.575	100.0	
I. perado ssp. *azorica*										
TROQ-A	São Miguel	10	0.90	2.71	19	4	0.171	0.249	57.1	0.323†
LAFO-A	São Miguel	10	1.00	2.71	19	4	0.214	0.278	71.4	0.239†
Overall		20		3.57	25	13	0.193	0.263	71.4	
I. canariensis										
VAHE-C	La Gomera	7	1.00	2.57	18	3	0.561	0.493	85.7	–0.240^{ns}
LOJA-C	Tenerife	11	1.00	3.43	24	0	0.570	0.607	85.7	0.070*
MOQU-C	Tenerife	31	1.00	3.57	25	1	0.667	0.541	85.7	–0.210†
CALD-C	Tenerife	23	1.00	3.86	27	2	0.596	0.492	85.7	–0.221^{ns}
TILO-C	La Palma	24	1.00	3.43	24	3	0.667	0.525	100.0	–0.283^{ns}
TAMA-C	Gran Canaria	26	1.00	3.29	23	3	0.410	0.406	85.7	–0.043‡
Overall		122		6.29	44	27	0.578	0.534	100.0	–0.240^{ns}
Total		233		10.14	71	47	0.482	0.734	100.0	

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

N, number of sampled individuals; *G*/*N*, clonal diversity; *A*, average number of alleles per locus; NA, total number of alleles; NE, number of private alleles; H_O, observed heterozygosity; H_E, unbiased expected heterozygosity; *%P*, percentage of polymorphic loci; F_{IS}, inbreeding coefficient. ns, non-significant.

from the Bank of Molecular Markers of the Macaronesian Flora webpage (http://www.banmac.ulpgc.es). PCR products were detected using an ABI 3130XL Genetic Analyser and fragment sizes were determined using GENESCAN v. 2.02 and GENOTYPER v. 1.1 (Applied Biosystems, Inc.). We identified allele peak profiles at each locus and assigned a genotype manually to each individual, considering its diploid nature (Selbach-Schnadelbach *et al*., 2009).

DATA ANALYSIS

Data from allele scoring were entered into TRANSFORMER 4 software (Caujapé-Castells *et al*., 2011), which generates data formatting for other programs. Standard measures of genetic diversity at the population level were calculated using POPGENE 1.32 (Yeh *et al*., 1997) and included: proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), observed heterozygosity (H_O) and unbiased sampled heterozygosity (H_E; Levene, 1949). Clonal diversity was calculated as *G*/*N*, where *G* is the number of different multilocus genotypes and *N* is the number of sampled individuals in the population. To investigate potential deviations from Hardy–Weinberg equilibrium, exact tests were performed in GENEPOP V4 (Rousset, 2008). Tests were run for each pair of loci and for each population. For all tests, a sequential Bonferroni correction for multiple comparisons was applied (Rice, 1989).

In order to analyse the levels of genetic differentiation among *Ilex* populations, pairwise F_{ST} estimates between populations (Wright, 1951) were calculated using GENEPOP V4 (Rousset, 2008). In addition, a minimum spanning tree (MST) was generated following Gower & Ross (1969). To investigate the spatial representation of the samples, the resulting tree was superimposed on a plot obtained from a principal coordinate analysis (PCoA) using NTSYSpc (Rohlf, 2000).

Following our hierarchical sampling design (taxa/island, populations), allele frequency data were analysed using a nested analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992). Differ-

ent hierarchical levels were considered, depending on the subset of data analysed, including 'among taxa/islands', 'among populations within taxon/islands' and 'within populations'. The software GENALEX version 6.4 (Peakall & Smouse, 2006) was used to run each AMOVA.

Population structure was also inferred using the Bayesian clustering procedure implemented in STRUCTURE 2.2 (Falush, Stephens & Pritchard, 2007) to identify the K (unknown) genetic clusters of origin of the sampled individuals and to assign individuals to the inferred clusters. The most likely value of K was assessed by comparing the likelihood of the data for different values of K. Populations and individuals were assigned to one cluster if their proportion of membership (q_i) to that cluster was ≥ 0.05. We assumed an admixture model and independent allele frequencies. A number of independent runs for each value of K (1–10) were performed.

RESULTS

GENETIC DIVERSITY ESTIMATES

The amplification of seven polymorphic microsatellites in four *Ilex* taxa revealed 71 alleles. The number of alleles per locus ranged from six (locus ILE03-86b) to 14 (locus ILE04-06) (Supporting Information, Table S1). The average number of alleles (A) ranged from 1.57 to 3.86 and the expected heterozygosity (H_E) varied from 0.249 to 0.607 across populations (Table 1). Multilocus genotypes congruent with clonality were only found in some *I. perado* populations. The lowest clonal diversity at the population level was found in *platyphylla* (APAT-P), which resulted in a small number of alleles (A) and percentage of polymorphic loci (P) (Table 1). *Ilex canariensis* showed the highest levels of genetic variability ($A = 6.29$; $H_E = 0.534$), whereas *azorica* displayed the lowest ($A = 3.57$; $H_E = 0.263$). Exact tests suggested that most of the analysed populations, with the exception of those of *azorica*, were at Hardy–Weinberg equilibrium. Thus, 83% (67 of 81 possible tests) of comparisons were not significantly different from Hardy–Weinberg expectations after Bonferroni corrections (Supporting Information, Table S2). Populations showing substantial levels of clonality generally show negative F_{IS} values, as a consequence of fixed heterozygosity among clonal plants (results not shown).

ALLELIC FREQUENCIES AND GENETIC DIFFERENTIATION

Of the 71 alleles detected, none was shared by all populations. At a taxon level, no taxon-diagnostic alleles were found. However, two alleles with a high frequency were present exclusively in *azorica*. Also, four privative alleles were detected in *I. canariensis*. No exclusive alleles were detected for *lopezlilloi* (Table 1).

Pairwise F_{ST} estimates ranged from virtually zero (between populations of *azorica*) to 0.668 (between populations of *azorica* and *platyphylla*) (Table 2). The most remarkable result was that the average F_{ST} value among *platyphylla* populations (sampled across two islands) was 0.252, whereas the same average value for *I. canariensis* (sampled across four islands) was substantially smaller, equalling 0.095 (Table 2). The minimum genetic differentiation between taxa was found between *lopezlilloi* and *platyphylla* (average $F_{ST} = 0.108$).

The first two axes of the PCoA accounted for a relatively high proportion of the total variance (45%) (Fig. 2) and revealed three clearly differentiated clusters. The first cluster was constituted by all populations of *I. canariensis*, regardless of their island of origin. The second was formed by the two *azorica* populations and the third grouped *platyphylla* and *lopezlilloi* populations together. Two subgroups were also evident in the third cluster, in which samples from different islands were differentiated (Fig. 2). The MST superimposed on the PCoA showed that *azorica* was intermediate between *I. canariensis* and *I. perado* ssp. *platyphylla/lopezlilloi*.

Similar results were obtained when applying Bayesian analysis. Using the total dataset (233 individuals, seven microsatellite loci, 15 populations) and testing values of K from 1 to10, the probability of the data reached its maximum when $K = 4$ (Supporting Information, Fig. S1), which suggests that the total sample can be split into four distinct genetic clusters. The first group included individuals of *I. canariensis*. All *azorica* individuals were assigned to cluster II. The third inferred cluster included those individuals of *lopezlilloi* and *platyphylla* sampled on the island of La Gomera. Finally, the last group was formed by *platyphylla* individuals from the island of Tenerife. Populations of *lopezlilloi* and *platyphylla* from the island of La Gomera were not separated into different clusters, regardless of the number of K considered in the analysis.

The hierarchical analysis of variance (AMOVA) revealed that a high percentage of genetic variation detected in Macaronesian *Ilex* was contained between taxa (36.38%) (Table 3). Within the *I. perado* complex, AMOVA revealed that 28.38% of the variation was accounted for at the 'among subspecies' level. However, this analysis did not detect significant differentiation (1.07%) between *lopezlilloi* and *platyphylla*. In contrast, when we considered the geographical origin of the populations, significant genetic variation between Tenerife and La Gomera was detected (23.02%) (Table 3).

Table 2. Pairwise F_{ST} values between *Ilex* populations sampled in this study. Population codes are detailed in Table 1

	ENGO-L	SUGO-P	PARC-P	APAT-P	MOQU-P	CAFO-P	CRTA-P	TROQ-A	LAFO-A	VAHE-C	LOFA-C	MOQU-C	CALD-C	TILO-C
SUGO-P	0.037^{ns}													
PARC-P	0.088^{ns}	0.020^{ns}												
APAT-P	0.203*	0.276†	0.363‡											
MOQU-P	0.258‡	0.330‡	0.365‡	0.391‡										
CAFO-P	0.223‡	0.275‡	0.336‡	0.387‡	0.046*									
CRTA-P	0.184‡	0.205‡	0.248‡	0.305‡	0.062‡	0.056†								
TROQ-A	0.571‡	0.634‡	0.649‡	0.668‡	0.405‡	0.465‡	0.380‡							
LAFO-A	0.558‡	0.616‡	0.634‡	0.652‡	0.404‡	0.453‡	0.380‡	0.000^{ns}						
VAHE-C	0.510‡	0.532‡	0.558‡	0.622‡	0.451‡	0.435‡	0.431‡	0.662‡	0.638‡					
LOFA-C	0.440‡	0.444‡	0.478‡	0.561‡	0.424‡	0.371‡	0.395‡	0.586‡	0.556‡	0.049†				
MOQU-C	0.450‡	0.479‡	0.495‡	0.547‡	0.436‡	0.395‡	0.420‡	0.585‡	0.569‡	0.046‡	0.024^{ns}			
CALD-C	0.484‡	0.515‡	0.529‡	0.575‡	0.464‡	0.426‡	0.442‡	0.621‡	0.606‡	0.159‡	0.078†	0.036†		
TILO-C	0.467‡	0.488‡	0.501‡	0.558‡	0.457‡	0.432‡	0.432‡	0.606‡	0.590‡	0.042‡	0.093‡	0.043†	0.113‡	
TAMA-C	0.552‡	0.566‡	0.578‡	0.613‡	0.491‡	0.502‡	0.469‡	0.652‡	0.638‡	0.155‡	0.196‡	0.155‡	0.126‡	0.123‡

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.
ns, non-significant.

DISCUSSION

Levels of genetic diversity in Macaronesian Ilex taxa

The analysis of seven nuclear microsatellite loci revealed moderate levels of genetic variation in Macaronesian *Ilex* taxa (Table 1; see Nybom, 2004 for typical values in narrow endemics). Genetic diversity estimates obtained in this study are slightly lower than those reported for other Macaronesian diploid taxa showing potential for long-distance dispersal (Table 4). The most remarkable exception to this pattern was *I. perado* ssp. *azorica*, for which the expected heterozygosity values were the lowest reported in the literature for the Macaronesian species analysed with nuclear microsatellite markers to date (Table 4, cf. López de Heredia *et al*., 2010; Sosa *et al*., 2010b). Levels of genetic diversity should be interpreted in the context of the interactions among factors related to population dynamics (Hamrick & Godt, 1989; Nybom, 2004). As these species are long-lived and strictly outcrossing (dioecious), one could expect populations to display high levels of variation at microsatellite markers (e.g. Nybom, 2004). Low variation within Azorean island populations, however, could be explained by a small number of island colonizers (founder effect) and subsequent genetic drift (Frankham, 1997). The heterozygote deficiency observed in these populations (Table 1, Table S2) supports the idea of intense inbreeding, probably as a result of small population sizes. Thus, low levels of differentiation between populations in *I. perado* ssp. *azorica* suggest extensive gene flow within islands, but our results on genetic diversity levels also seem to indicate that dispersal across oceanic barriers could have been a much rarer process. A comprehensive sampling of the area occupied by this taxon, comprising all but one of the Azores (Silva *et al*., 2010), would be needed to better characterize the patterns of differentiation in the archipelago.

Similar levels of genetic diversity between the narrowly distributed *I. perado* ssp. *lopezlilloi* and the more widespread *I. perado* ssp. *platyphylla* are also unexpected. Considering its small population size, one would expect the *lopezlilloi* population to exhibit low levels of genetic diversity. Our results suggest that this critically endangered taxon experienced a relatively recent reduction in the number of wild individuals, which may explain the moderate levels of genetic variation despite low effective population sizes (Aguilar *et al*., 2008). A complementary explanation, however, is that both Canarian subspecies constitute a single genetic pool, and active gene flow from *platyphylla* to *lopezlilloi* would therefore account for the levels of genetic diversity detected, as well as

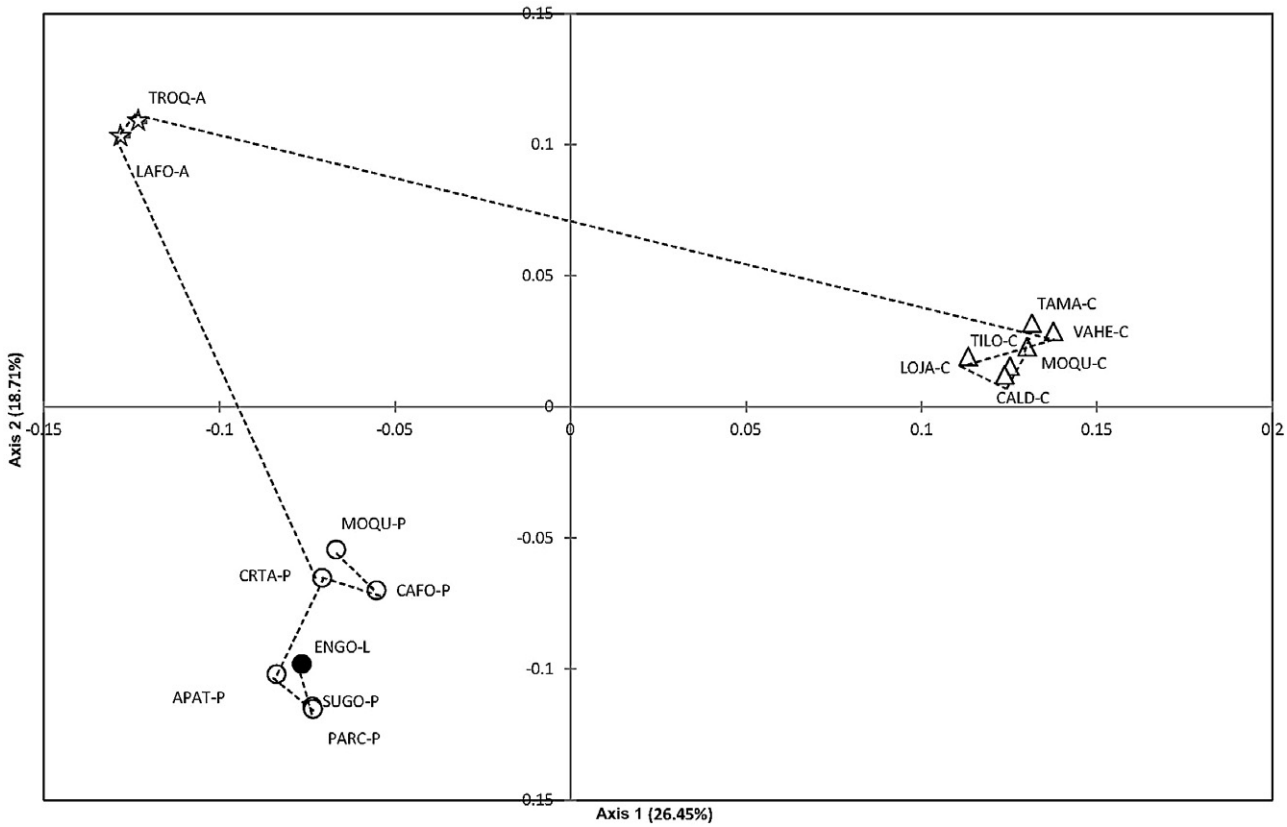

Figure 2. Graphical representation of principal coordinate analysis results and minimum spanning tree based on microsatellite data for populations of *Ilex canariensis* (△), *I. perado* ssp. *azorica* (☆), *I. perado* ssp. *platyphylla* (○) and *I. perado* ssp. *lopezlilloi* (•).

Table 3. Analysis of molecular variance (AMOVA) results at three hierarchical levels for different combinations of *Ilex* taxa or islands

Source of variation	d.f.	Sum of squares	Percentage of variation	Fixation indices
Among *Ilex* taxa	3	346.4	36.4	0.364*
Among populations within *Ilex* taxa	11	114.9	8.2	0.129*
Within populations	451	802.3	55.4	
Among *I. perado* ssp.	2	78.3	28.4	0.284*
Among populations within *I. perado* ssp.	6	60.6	12.6	0.177*
Within populations	213	339.8	59.0	
Between *lopezlilloi* and *platyphylla*	1	7.0	1.1	0.011^{ns}
Among populations within ssp.	5	59.7	18.4	0.186*
Within populations	175	304.7	80.5	
Between Tenerife and La Gomera	1	36.0	23.0	0.230*
Among populations within islands	4	23.7	6.2	0.081*
Within populations	166	289.4	70.8	

*$P < 0.001$.
d.f., degrees of freedom; ns, non-significant.

Table 4. Genetic variation indices obtained from microsatellite analyses of bird-dispersed Macaronesian taxa

Taxon	NL	*A*	H_o	H_E	Reference
Ilex canariensis	7	6.29	0.58	0.53	This work
Ilex perado ssp.*azorica*	7	3.57	0.19	0.26	This work
Ilex perado ssp. *platyphylla*	7	3.00	0.49	0.53	This work
Ilex perado ssp. *lopezlilloi*	7	2.14	0.60	0.48	This work
Morella faya Aiton	6	9.30	0.57	0.67	González-Pérez *et al*. (2009a)
Morella rivas-martinezii A.Santos	6	6.50	0.49	0.56	González-Pérez *et al*. (2009a)
Olea europea ssp. *guanchica* P.Vargas *et al*.	6	9.44	0.63	0.63	García-Verdugo *et al*. (2010a)
Phoenix canariensis Hort. ex Chabaud	8	11.00	0.51	0.60	Saro *et al*. (unpubl. data)
Sambucus palmensis Link	5	6.80	0.55	0.50	Sosa *et al*. (2010a)
Sorbus aria (L.) Crantz	9	4.22	0.99	0.68	Sosa *et al*. (2010b)

NL, number of loci; *A*, average number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

the low genetic differentiation observed among populations of both taxa (Table 3).

Patterns of differentiation and taxonomy in Macaronesian Ilex

Our analyses revealed contrasting patterns of population differentiation between the two currently recognized *Ilex* spp. *Ilex canariensis* showed a pattern typically described in other bird-dispersed Macaronesian species, i.e. moderate to high levels of genetic diversity and weak population structure across islands (García-Verdugo *et al*., 2010b; Ferreira *et al*., 2011; Rumeu *et al*., 2011). On the contrary, our analyses detected high levels of differentiation among islands within the *I. perado* complex (Tables 2 and 3), whereas levels of genetic variation ranged from low (*I. perado* ssp. *azorica*) to moderate (*I. perado* ssp. *platyphylla* and *I. perado* ssp. *lopezlilloi*; Table 4). Life-history traits, such as longevity or pollination/seed dispersal syndromes, do not account for the contrasting patterns of differentiation detected in this study, as these features are common to both lineages. Evidence for clonal propagation, however, was only found in *I. perado* populations, which limits the levels of genetic diversity within populations (Table 1), and may represent an efficient strategy for population establishment in this taxon (García-Verdugo *et al*., 2013). In addition, phylogenetic studies have shown that these two lineages are not closely related within the genus, which has been interpreted as evidence for independent colonization events (e.g. Cuénoud *et al*., 2000; Manen *et al*., 2010). Species-specific traits related to habitat colonization, in addition to different phylogeographical histories, may therefore explain the patterns detected in this study. Substantial genetic differentiation between both *Ilex* lineages ultimately supports current taxonomic classification and the genetic consequences expected for two lineages resulting from independent dispersal events to the islands (e.g. Selbach-Schnadelbach *et al*., 2009).

Following current taxonomic classification, one unexpected result, however, concerns the high degree of differentiation detected between *I. perado* ssp. *azorica* and the two Canarian *I. perado* subspecies (Tables 2 and 3). The occurrence of similar taxa in different Macaronesian archipelagos is often associated with low phenotypic differentiation (Brochmann *et al*., 1995; García-Verdugo *et al*., 2010a). Based on morphological grounds, similar island forms of the same genus occurring on different archipelagos have been frequently classified by taxonomists as geographical races (subspecies) instead of different species. However, some studies have shown that morphological similarity between taxa could merely reflect the response to similar environments, as phenotypic similarity between island subspecies is often associated with strong molecular differentiation (Sahuquillo & Lumaret, 1999; García-Verdugo *et al*., 2010a). In addition, a growing number of examples suggest that Macaronesian taxa with vicariant Canarian–Azorean distributions, such as *Hedera* L. (Valcárcel, Fiz & Vargas, 2003), *Picconia* DC. (Ferreira *et al*., 2011), *Juniperus* L. (Rumeu *et al*., 2011) or *Prunus* (García-Verdugo *et al*., 2013), are the result of independent dispersal events to each archipelago from mainland ancestors. High genetic differentiation between *I. perado* ssp. *azorica* and the Canarian *I. perado* complex is in agreement with a third independent event of colonization of *Ilex* to the Macaronesia (Azores). Although the MST tree (Fig. 2), the high number of private alleles (Table 1) and the Bayesian analysis support this hypothesis, further phylogenetic analyses would be needed to test this possibility, as alternative explanations (e.g. ancient dispersal of Azorean ancestors from the Canaries, followed by strong geographical isolation) cannot be ruled out with our current results. In any case, such

a high degree of differentiation supports the idea of strong isolation between Azorean and Canarian populations, which warrants taxonomic revision of the *I. perado* complex.

Lastly, the low genetic differentiation detected among the two Canarian subspecies of *I. perado* is in agreement with the results obtained by Werner, Ros & Fernández (2007). These authors did not find evidence of clear differentiation between both subspecies using internal transcribed spacer (ITS) sequences and inter simple sequence repeat (ISSR) markers. In contrast, Jaén-Molina *et al*. (2010) found differences between these two taxa using *rbcL* and *matK* sequences. Although the scope of these latter results remains to be determined, it is feasible that the differences detected by these authors could be caused by the fact that the specimens used in their analyses were sampled on different islands, and therefore genetic variability could be a result of differences between islands. However, our results and those of Werner *et al*.(2007) were based on nuclear markers, whereas those of Jaén-Molina *et al*. (2010) were based on plastid data. Discrepancies between our results and those reported by Jaén-Molina *et al*. (2010) may be a result of the complex evolutionary history of the genus, in which introgression and hybridization seem to have produced intricate patterns of differentiation in each genome, as discussed elsewhere (Selbach-Schnadelbach *et al*., 2009; Manen *et al*., 2010).

In summary, our results show contrasting patterns of differentiation among *Ilex* taxa and provide evidence for further taxonomic revision and formulation of hypotheses on the number of introductions of *Ilex* into Macaronesia. To clarify the phylogeographical patterns in Macaronesian *Ilex* further, future studies should include some specimens of *I. perado* ssp. *perado* from Madeira and a more complete representation of *I. perado* ssp. *azorica* using a phylogenetic approach. This study shows that the potential for efficient seed dispersal, as exemplified by recurrent events of dispersal of *Ilex* to the islands, is not necessarily repeated within archipelagos. Gene flow limitations would potentially arise, despite such potential to overcome the geographical isolation imposed by oceanic barriers, triggering diversification processes within certain lineages.

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SUPPORTING INFORMATION

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

Figure S1. (a) Posterior probability of the data (PPD) against the maximum number of clusters (*K*) considered; (b) increase in PPD given *K* (Evanno *et al*., 2005).

Table S1. Genetic variability indices by locus analysed.

Table S2. F_{IS} estimates for each locus and population analysed for the study of *Ilex* taxa. Population codes are the same as those detailed in Table 1.