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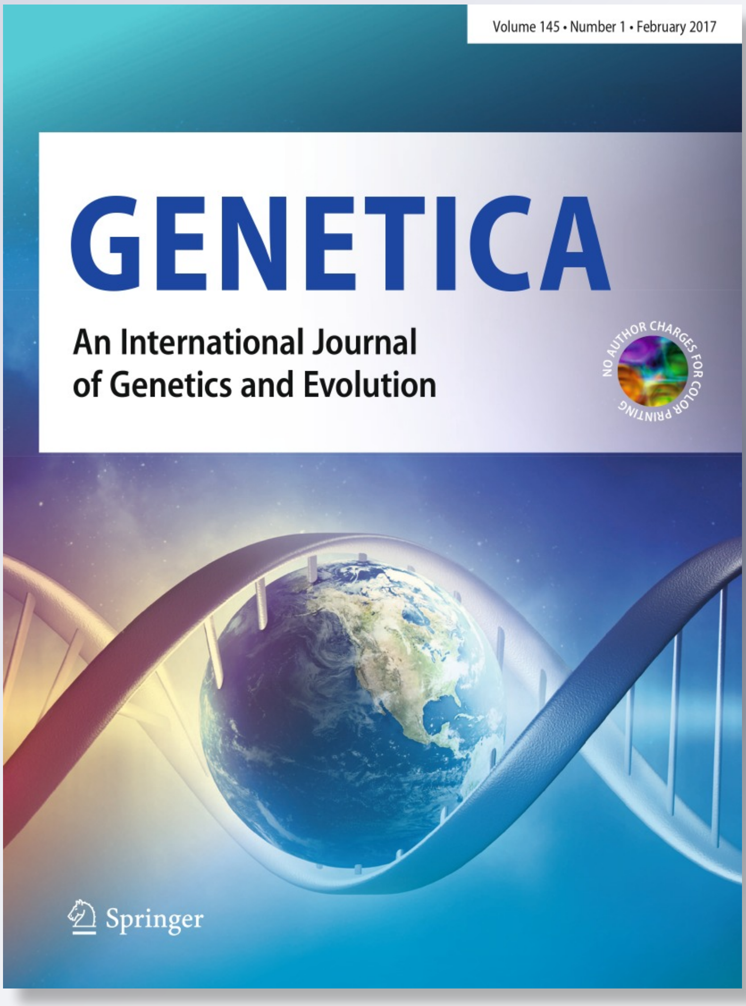

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ORIGINAL PAPER

Surviving in isolation: genetic variation, bottlenecks and reproductive strategies in the Canarian endemic *Limonium macrophyllum* (Plumbaginaceae)

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Abstract Oceanic archipelagos are typically rich in endemic taxa, because they offer ideal conditions for diversification and speciation in isolation. One of the most remarkable evolutionary radiations on the Canary Islands comprises the 16 species included in *Limonium* subsection *Nobiles*, all of which are subject to diverse threats, and legally protected. Since many of them are single-island endemics limited to one or a few populations, there exists a risk that a loss of genetic variation might limit their long-term survival. In this study, we used eight newly developed microsatellite markers to characterize the levels of genetic variation and inbreeding in *L. macrophyllum*, a species endemic to the North-east of Tenerife that belongs to *Limonium* subsection *Nobiles*. We detected generally low levels of genetic variation over all populations ($H_T = 0.363$), and substantial differentiation among populations ($F_{ST} = 0.188$; $R_{ST} = 0.186$) coupled with a negligible degree of inbreeding ($F = 0.042$). Obligate outcrossing may have maintained *L. macrophyllum* relatively unaffected by inbreeding despite the species' limited dispersal ability and the genetic bottlenecks likely caused by a prolonged history of grazing. Although several factors still constitute a risk for the conservation of *L. macrophyllum*, the lack of inbreeding and the recent positive demographic trends observed in the populations of this species are factors that favour its future persistence.

Keywords Conservation · Genetic diversity · Islands · Macaronesia · Microsatellites · Sea lavender

Introduction

Representing about 4% of the total land surface of the Earth, islands are home to over 13% of all known vascular plant species in the world (Whittaker and Fernández-Palacios 2007), and approximately 25% of the endemic ones (Kreft et al. 2008; Caujapé-Castells et al. 2010). Such a remarkable contribution of islands to global biodiversity is largely due to evolutionary radiations, i.e., rapid and extensive processes of diversification from a single shared ancestral lineage. Radiations are particularly frequent in oceanic islands because they emerge from the sea devoid of life, so the availability of niches in absence of competing species provides multiple opportunities for successful colonization, establishment and diversification (Whittaker and Fernández-Palacios 2007). Oceanic islands are thus unparalleled "natural laboratories" for the study of evolution (e.g. Carlquist 1997; Hendry et al. 2006; Grant and Grant 2009) and prime targets for many recent and on-going conservation efforts (e.g. Kier et al. 2009; Caujapé-Castells et al. 2010; Courchamp et al. 2014).

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The Canarian archipelago, with a minimum distance of ca. 96 km from the Atlantic shore of Africa, consists of seven major oceanic islands and several islets of volcanic origin, some of them still active. The present archipelago started to emerge in a roughly east-to-west direction 20 million years ago, as the African plate moved eastwards over a hot plume of the mantle (Anguita and Hernán 2000; Fernández-Palacios et al. 2011). The Canaries are home to about 1300 species of vascular plants, 44.3% of which are endemic (Whittaker and Fernández-Palacios 2007; Reyes-Betancort et al. 2008). Over 20 plant genera have undergone remarkable radiations in the Canary Islands, either by adaptation to different habitats or by allopatric vicariance (e.g. *Echium*, Böhle et al. 1996; *Sideritis*; Barber et al. 2000; *Aeonium*; Mort et al. 2002; *Lotus*; Allan et al. 2004; *Micromeria*; Meimberg et al. 2006; *Limonium*; Lledó et al. 2011).

With over 400 species distributed worldwide (Boissier 1848), sea lavenders (genus *Limonium*, Plumbaginaceae) have diversified primarily in the Asian steppes and western Mediterranean region, including the Canary Islands. Within *Limonium*, the subsection *Nobiles* of section *Pterocladros* sensu Boissier (1848), hereafter referred to as *L.* subsec. *Nobiles*, comprises 16 Canarian endemic species that share a putatively monophyletic origin (Karis 2004; Lledó et al. 2005, 2011), representing one of the most remarkable, yet understudied, plant radiations in the Canarian archipelago. These 16 evergreen, woody species share four important floral traits. First, like most species in the genus, they have hermaphroditic flowers that display the pollen-stigma dimorphism typical of the Plumbaginaceae, i.e. one floral morph with A pollen and cob stigmata and the other floral morph with B pollen and papillate stigmata. Such dimorphism is putatively linked to a sporophytic, diallelic self-incompatibility system that inhibits the germination of A pollen on cob stigmata and of B pollen on papillate stigmata, making these plants obligate outcrossers (Baker 1948, 1953, 1966; Dulberger 1975). Although the self-incompatibility system has not yet been experimentally demonstrated for any species of *L.* subsec. *Nobiles*, preliminary evidence indicates that it occurs in the tested species (J. Pérez de Paz, Jardín Botánico Canario “Viera y Clavijo” – Unidad Asociada CSIC, personal communication). Second, each flower has a single ovule in the ovary, so it can produce only one seed. Third, the propagules of these plants are spikes that consist of one to six spicules, each surrounded by a group of three bracts that embrace from three to four flowers, which develop sequentially starting from the distal end of the spicule. The spikes, which detach from the inflorescence as single units from an abscission zone, act as diaspores bearing several seeds (Karis 2004). And fourth, each flower has a scarious, persistent calyx of a deep-blue to lavender colour. It has been suggested that these calyces enhance wind dispersal (Bañares et al. 2004), although experimental evidence in other *Limonium* species demonstrates that persistent calyces improve buoyancy for water dispersal (Boorman 1967; Koutstaal et al. 1987; Archbald and Boyer 2014), thus favouring hydrochory.

Most species in *L.* subsec. *Nobiles* are single-island endemics restricted to one or a few populations, often with a scarce number of individuals. All species in the subsection are catalogued with different degrees of threat in the Spanish Red List (Bañares et al. 2004; Moreno 2008), and several of them are severely endangered, with less than one hundred wild plants surveyed in populations subject to anthropic impacts. The main threats include grazing by alien vertebrates, tourism, economic and human demographic growth, habitat alteration and destruction, and competition with foreign plant species, all of which jeopardize the conservation of plant biodiversity in many islands worldwide (Caujapé-Castells et al. 2010). Considering that most *Limonium* species on the Canary Islands are confined to small populations in hardly accessible vertical cliffs and ledges, and that signs of demographic recovery have been observed in the populations where grazing pressure diminished in the last decades (Bañares et al. 2004), the presence of feral goats appears to be the most harmful remaining threat for these plants. Furthermore, the species of *L.* subsec. *Nobiles* are strongly affected by stochastic fluctuations in rainfall regimes, with dry summers negatively impacting on the subsistence of their populations (Bañares et al. 2004).

Given the importance of understanding evolutionary processes in the light of the ongoing global biodiversity crisis, scientists have emphasized the need for studies specifically aimed at assessing levels of standing genetic variation and gene flow within and between island populations (e.g. Franks 2010; Kisel and Barraclough 2010). Notably, random genetic drift can erode genetic variation in small, isolated populations, thus reducing their chances to adapt to changing conditions (Orr and Betancourt 2001). Moreover, isolated populations tend to be affected by inbreeding, which can result in a loss of fitness in the offspring, ultimately leading to extinction (Ellstrand and Elam 1993; Frankham 1998). Even though inbreeding depression can affect also outbreeding species when populations are small (Frankham 2005), obligate cross-fertilization plays a decisive role in limiting inbreeding depression in the short term. However, in dimorphic, self-incompatible species, as is presumably the case for those in *L.* subsec. *Nobiles*, decreasing population sizes entail an increased risk of skewed floral morph ratios. In extreme cases, one of the morphs can even be entirely absent, thus preventing reproduction in absence of pollen flow. Such situation has been documented, for example, in another Canarian endemic, i.e., the critically endangered *L. dendroides* of section *Limoniodendron*, where only either the cob or the pap morph has been observed in its wild populations of La Gomera (Suárez-García et al. 2009).

Limonium macrophyllum (Brouss.) Kuntze is one of 16 species in *L.* subsec. *Nobiles*. It is restricted to the north face of the Anaga basaltic massif in the north-east of Tenerife, and is listed as vulnerable by the International Union for the Conservation of Nature (IUCN) in the Spanish Red Book of Threatened Flora (Bañares et al. 2004). We developed *de novo* a set of eight polymorphic microsatellites, and used them to characterize the genetic variation and mating system of this endemic, putatively self-incompatible species, which is currently expanding after an alleviation of the grazing pressure maintained during centuries in the Canary Islands (Bañares et al. 2004). In addition, given the life-history traits shared by all species in *L.* subsec. *Nobiles*, our results could also be used to draw some general guidelines for the conservation of other endangered species from the same subsection that cannot be studied directly due to the extremely difficult accessibility of their populations. Beyond the implications for conservation, genetic diversity results may also contribute to understanding the relationships between island colonization, reproductive strategies and genetic variation. Specifically, in this contribution we aim at addressing the following questions: (1) What is the genetic diversity of the populations of *L. macrophyllum*? (2) How much gene flow occurs among the populations of this species? (3) Have the populations of *L. macrophyllum* experienced genetic bottlenecks? (4) What is its mating system and how inbred are its populations? (5) Does the joint dispersal of seeds of *L. macrophyllum* determine a spatial structure of its genetic variation over short scales?

Materials and methods

Study species

Limonium macrophyllum (Brouss) O. Kuntze ($2n=14$; Ardévol Gonzales et al. 1993) is a small (up to 0.8 m), sparsely branched shrub with evergreen, large, linear leaves usually disposed in a rosette at the tip of the stem or, in particularly large individuals, at the tip of the branches. The inflorescences, which develop from the center of the rosettes and are variable in number, are very conspicuous, due to the purplish blue colour of the flowers' persistent calyces. The flowers display the cob-A/pap-B stigma-pollen dimorphism typical of *Limonium* (Baker 1953). Flowers are pollinated mainly by the generalist bees *Eucera gracilipes* and *Amegilla quadrifasciata* (A. Reyes-Betancort, Instituto Canario de Investigaciones Agrarias, personal communication), although pollinator visits are not frequent, based on field observations by the first author. The flowering season of *L. macrophyllum* spans from March to June, and fruit maturation extends until August. Each spike bears between two and four spicules, each of them with three to four flowers. As in all other species of *L.* subsec. *Nobiles*, spikes become detached from the inflorescences after fruit maturation and act as diaspores dispersed mainly by gravity.

Limonium macrophyllum is endemic to the northeastern portion of Tenerife in the Canary Islands, with a distribution range spanning from the eastern to the western mountains of the Anaga massif (Bañares et al. 2004). It grows in rupicolous habitats, such as cracks and ledges with accumulation of humic soil on north-facing cliffs between 300 and 700 m above the sea. Those habitats are greatly influenced by trade winds that blow from the northeast towards the southwest and crash against the mountains, creating a mist belt that reduces insolation and provides a wet environment with mild temperatures year round. The two major factors threatening *L. macrophyllum* are the residual goat grazing that is still maintained in the area and the presence of competing alien species, mainly *Opuntia ficus-indica*. All five populations of *L. macrophyllum* reported in the literature are included in the Rural Park of Anaga.

Sampling

We sampled all five known populations of *L. macrophyllum* spanning the entire distribution area of the species (Table 1; Fig. 1). We repeatedly prospected a putative sixth locality (Roques de Afur) during our sampling season, but no *L. macrophyllum* plants could be found. All individuals were sampled at random within each population. In population CHA, besides the 40 individuals indicated in Table 1, we additionally sampled and recorded the positions on an *x, y* coordinates system of all 102 individuals located within a 30 × 6 m transect. This sampling allowed us to study the structure of genetic variation in *L. macrophyllum* over a short spatial scale. One voucher specimen for each of the five sampled populations was deposited in the Herbarium of the University of Zurich (Z).

Microsatellite development and genotyping

Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland), following the manufacturer's protocol, from one individual of *L. macrophyllum* sampled from population CHI. The extracted total genomic DNA was used by Genetic Marker Services (Brighton, UK) to develop a microsatellite-enriched library and design and test microsatellite primer pairs. Library enrichment involved incubating adapter-ligated, size-restricted DNA with the filter-bonded synthetic oligonucleotide repeats $(AG)_{17}$, $(AC)_{17}$, $(AAC)_{10}$, $(CCG)_{10}$, $(CTG)_{10}$ and $(AAT)_{10}$. Primers for nineteen microsatellite inserts

Table 1 Populations of *L. macrophyllum* sampled for this study and mean values of four genetic variation and inbreeding parameters

Acronym	Population	Latitude (N)	Longitude (W)	Altitude (m a.s.l.)	*N*	*A* ± SE	H_o ± SE	H_e ± SE	*F* ± SE
IZO	Pico de Izogue	28° 32′ 47.1″	16° 20′ 00.3″	581	34	2.375 ± 0.263	0.294 ± 0.074	0.309 ± 0.064	0.062 ± 0.087
CHI	Chinamada	28° 33′ 54.8″	16° 17′ 44.0″	560	35	2.375 ± 0.375	0.307 ± 0.073	0.330 ± 0.077	0.059 ± 0.099
MBR	Mesa del Brezal	28° 34′ 12.3″	16° 17′ 07.0″	594	37	2.375 ± 0.461	0.294 ± 0.088	0.314 ± 0.091	0.061 ± 0.053
TAB	Roque de Taborno	28° 34′ 00.7″	16° 16′ 00.5″	581	39	2.875 ± 0.766	0.405 ± 0.126	0.378 ± 0.115	-0.092 ± 0.145
CHA	Chamorga	28° 34′ 40.6″	16° 08′ 53.2″	588	40	2.125 ± 0.398	0.186 ± 0.067	0.210 ± 0.073	0.101 ± 0.091

N number of individuals sampled per population, *A* average number of alleles per locus, H_o observed heterozygosity, H_e expected heterozygosity under HWE, *F* Wright's (1943) coefficient of inbreeding, *SE* standard error of the mean

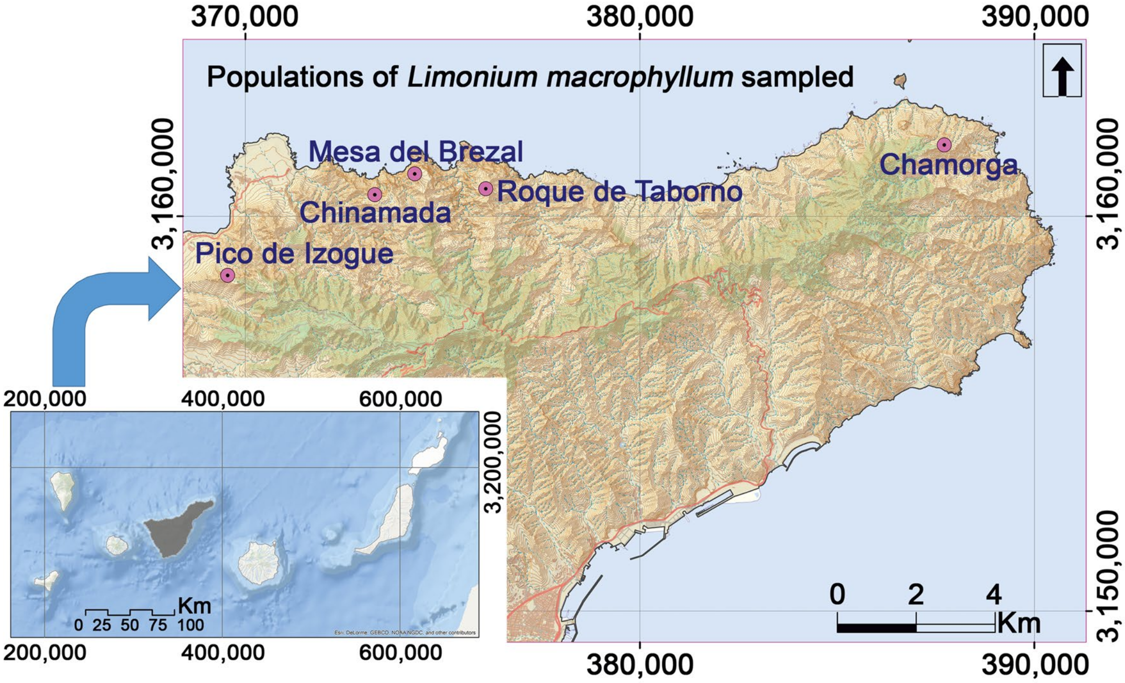

Fig. 1 Location of the five populations of the insular endemic *L. macrophyllum* sampled for this study in the northeastern coast of Tenerife in the Canary Islands

obtained by sequencing positive recombinant colonies were designed with Primer3 (Rozen and Skaletsky 2000). Unlabeled primer pairs were amplified in PCR runs under a range of conditions. Specifically, all reaction mixtures included 0.2 µl of each primer (10 mM), 0.5 µl of a mix of all four dNTPs (10 mM), 0.1 µl of GoTaq® DNA polymerase (Promega; 50 U/µl), 2 µl of 10× reaction buffer and 1 µl of ca. 10 ng/µl genomic DNA, combined with varying amounts of 50 mM $MgCl_2$ (0.3–2.0 µl) and sterilized water up to final volumes between 10 and 25 µl. A touchdown protocol and a gradient PCR protocol were used to identify the PCR profiles producing the best amplification products for each tested primer pair. The touchdown PCR profile consisted of 3 min of initial denaturation at 95 °C, followed by 26 cycles of 95 °C for 1 min, 64 – 59 °C (decreasing one °C each cycle over the first six cycles), 58 °C (7th to 16th cycles) or 57 °C (10 last cycles) for 1 min, and 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The gradient PCR profile consisted of 3 min of initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 1 min, 51–65 °C across the gradient for 1 min, and 72 °C for 1 min, with a final extension step of 72 °C for 5 min. PCR products were tested in agarose gels. The forward primers of the primer pairs that produced clean, putatively polymorphic amplification products were labeled with a fluorescent chromophore (6-FAM, NED, PET or VIC) and tested for polymorphism in 16 individuals of *L. macrophyllum* from populations CHA and CHI in an ABI 3130 × 1 Genetic

Analyzer (Applied Biosystems, Foster City, California, USA). PCR conditions for preliminary polymorphism testing were the same as those used for the genotyping of all samples (see below). Ultimately, eight microsatellite primers were selected for genotyping (Table 2).

Genomic DNA of all plants used for subsequent genotyping was extracted with the MagAttract 96 DNA Plant Core Kit (Qiagen, Hombrechtikon, Switzerand), following the manufacturer's instructions with some modifications. Specifically, as suggested by the manufacturer's troubleshooting guide, we used buffer AP1 (Qiagen, Hombrechtikon, Switzerand) instead of buffer RLT. DNA quality and concentration were tested with a NanoDrop ND-1000 V3.8.1 spectrophotometer, trial PCRs and fragment size analysis. Multiplexed PCRs were performed in 10 µl each containing 2 µl of 10× reaction buffer, 0.4 µl of $MgCl_2$ (50 mM), 0.5 µl of a mix of all four dNTPs (10 mM), 0.2 µl of the flourescent forward primer (10 mM), 0.2 µl of the reverse primer (10 mM), 0.1 µl of GoTaq® DNA polymerase (Promega; 50 U/µl), 1 µl of ca. 10 ng/µl genomic DNA, and sterilized water up to the final volume of 10 µl. PCR profiles consisted of 3 min of initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 1 min, 57–62 °C (depending on primer pair; see Table 2) for 1 min, and 72 °C for 1 min, and a final extension step of 72 °C for 5 min. The resulting fluorescent fragments were run in multiplexes on an ABI 3130 × l Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using the internal size standard LIZ500 (Applied Biosystems, Foster City, California, USA) and scored using GeneMapper 4.1 (Applied Biosystems, Foster City, California, USA).

We tested our microsatellite data for the incidence of genotyping errors due to large allele dropout, heavy stuttering or null alleles with MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). Additionally, the neutrality of the eight microsatellites used in this study was tested with BayeScan 2.1 (Foll and Gaggiotti 2008) and LOSITAN (Antao et al. 2008), both of them run under the default settings.

Data analysis

Genetic variation in *L. macrophyllum* was assessed by estimating the average number of alleles per locus (A) and the overall total heterozygosity (H_T). Genetic differentiation among populations was evaluated with F_{ST} (Weir and Cockerham 1984), R_{ST} (an analogue of F_{ST} for microsatellite loci that mutate following a stepwise model; Slatkin 1995), and an exact G-test (Goudet et al. 1996). The average number of reproductively successful migrants per generation (*Nm*) was estimated following Wright's (1943) formula $F_{ST} \approx 1/(1+4\ N\ m)$ and with the private allele method (Barton and Slatkin 1986). The observed heterozygosity (H_o), expected heterozygosity (H_e) under Hardy–Weinberg equilibrium (HWE), and Wright's (1943) inbreeding coefficient ($F=1-H_o/H_e$) as a measure of inbreeding were calculated for each locus in each population. The significance of the deviations from HWE was tested with an exact probability test (Guo and Thompson 1992). All analyses were carried

Table 2 Characterization of eight microsatellites newly developed for *L. macrophyllum*

Locus	GenBank accession number	Repeat motif	Size range (bp)	Primer sequences (5′–3′)	T_a (ºC)
Mac16	KR135426	$(TG)_9$	104–110	F: TCACATCAATGTAATGGGAGA R: TGGCCACTACTAAGAGCGTA	59
Mac38b	KR135427	$(TTG)_7$	127–149	F: TGCGCGTTAGAACACAGCTA R: CCGTGATTGCAGGAAATAAGA	59
Mac39	KR135428	$(GA)_9$	134–136	F: GGTTGAAGCTGCCAGAAAAG R: CCCCTTCCCTGTTTCTACCT	57
Mac65	KR135429	$(TG)_{12}$-GCG-$(TG)_{10}$	191–209	F: GGAAAATGCATCAAGAAACC R: TGACAATCAACACCCAATGT	62
Mac66	KR135430	$(TTC)_9$	155–162	F: TCTCTTTCCGCCCGATCCT R: ATTCCTCTCCGGCCCAATC	62
Mac67	KR135431	$(CAT)_{13}$	129–157	F: AGAAAATGGGGAAGGTTATGG R: CCACCTCCTGGTTCTCAGTG	62
Mac70	KR135432	$(CGA)_6$	164–169	F: TGCAGAATCAGAGGAAGGTT R: CAGGAGGTCGTCATTTCTACTC	57
Mac79	KR135433	$(CAG)_6$	122–125	F: CAGTTCCCCAGACAGTCGAT R: TGATGAGAGCCTTGTGGTTG	62

bp base pairs, *F* forward primer, *R* reverse primer, T_a annealing temperature out in Genepop 4.2 (Rousset 2008) except for H_T, which was calculated according to Nei (1973) and weighted for population sizes using a spreadsheet, and R_{ST}, which was calculated with the program FSTAT (Goudet 2002).

Contemporary gene flow between the populations of *L. macrophyllum* was further explored with GeneClass2 (Piry et al. 2004), a software devised to detect first-generation migrants. First, putative first-generation migrants were identified by running the analyses under two Bayesian criteria (Rannala and Mountain 1997; Baudouin and Lebrun 2001) and one frequencies-based method (Paetkau et al. 1995), each of them under three computation algorithms (Rannala and Mountain 1997; Cornuet et al. 1999; Paetkau et al. 2004). Default values were used for all settings in the analyses, except for the number of individuals simulated for probability computation, which was set to 10,000. Second, the likelihood for each individual to have originated from each population was estimated with an assignment test, which was carried out following the Rannala and Mountain (1997) method with probability computation disabled. Following the criteria used by Merwin et al. (2012), assignments of individuals to populations of origin were accepted as unambiguous only if the difference (δ) between the largest and the second largest log-likelihood for the assignments was higher than 1, because low levels of stringency (i.e., δ near 0) would increase the risk of assigning individuals as immigrants due to pollen flow between populations or to early backcrosses with recently arrived migrants.

We tested for recent genetic bottlenecks in the populations of *L. macrophyllum* with the software BOTTLENECK version 1.2.02 (Piry et al. 1999). We ran one-tailed Wilcoxon sign-rank tests for heterozygosity excess (Luikart et al. 1998), with 10,000 iterations under three microsatellite mutation models: the infinite allele model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM). The test under the TPM model was carried out with the default settings (i.e., a proportion of single-step mutations of 70% and a variance of 30 in the multi-step mutations).

We performed a Bayesian clustering procedure as implemented in STRUCTURE 2.3 (Pritchard et al. 2000) to infer genetic clusters in the five sampled populations. The analyses were run under the admixture model with 50,000 MCMC iterations after a burn-in of 5000 iterations. We tested *K* values from 1 to 8; each value of K was run 10 times under the admixture model with 50,000 MCMC iterations after a burn-in of 5000 iterations. We used the program STRUCTURE HARVESTER (Earl and vonHoldt 2012) to infer the correct *K* value using the statistics L(*K*) and Δ*K*.

Finally, we used the software Alleles in Space (Miller 2005) to determine the existence of non-random spatial patterns of genetic diversity in *L. macrophyllum* at small spatial scales. We performed an allelic aggregation index analysis (AAIA) over the 102 individuals sampled in the 30×6-m transect of the CHA population. This analysis tests the null hypothesis that each allele at a locus is distributed at random across the landscape (i.e., there is no aggregation or genetic structure; Miller 2005) relative to the aggregation of the individuals sampled in the field. In addition, we tested the correlation between genetic and geographic distances within the sampling transect with a Mantel test and with a generalized spatial autocorrelation analysis dividing the transect in ten distance classes.

The program Transformer-4 v2.0.1 (Caujapé-Castells et al. 2013) was used to enter the raw genotype data and generate all the input files required for GenePop, Alleles in Space, STRUCTURE and FSTAT. A genetic diversity digest including the geo-referenced genotype matrix used in this paper and other relevant information is deposited in the genetic diversity digest coded D-NMICR-116 and stored in the *Demiurge* information system (http://www.demiurge-project.org/).

Results

Quality of microsatellite data

The software MICRO-CHECKER detected no genotyping errors in any locus or population, with the exception of the possible presence of one or more null alleles in locus Mac70 in population IZO with a frequency fluctuating between 0.15 and 0.40, depending on the algorithm used, due to a significant excess of homozygotes. Assuming HWE (see below), a representative frequency of null alleles in a given locus and population should result in at least some null allele homozygotes. Since all 34 individuals sampled in IZO amplified for at least one of the alleles of Mac70, we believe that the frequency of null alleles in this locus and population must be very low. Consequently, we consider that the inclusion of this locus (which, in any case, does not have any null alleles in the other five populations) should produce only a minimal distortion on our results and, therefore, on the conclusions drawn from them. On the other hand, the neutrality tests carried out with BayeScan and LOSITAN did not detect any evidence of selection for any of the eight microsatellites (Online resource 1).

Genetic variation, genetic differentiation and bottlenecks

Population IZO was polymorphic for all eight loci tested, whereas CHI and MBR were polymorphic for six loci and TAB and CHA were polymorphic for only five loci (Online resource 2). The mean number of alleles per locus (Table 1) had its largest value in population TAB ($A = 2.875$) and its lowest value in population CHA ($A = 2.125$), whereas the other three populations showed the same intermediate value ($A = 2.375$). Averaged over all loci and populations, the value for A was 2.425. Population TAB had three private alleles, whereas populations CHI, MBR and CHA had one private allele each and population IZO (not considering null alleles) did not have any (Online resource 3). The average total heterozygosity for all populations was $H_T = 0.363$.

Genetic differentiation between populations weighted over all loci was 0.188 and 0.186, according to the global values of F_{ST} and R_{ST}, respectively. This genetic differentiation was highly significant according to the G-test across loci ($p < 0.001$). The average number of migrants per generation was $Nm = 1.080$ according to Wright's (1943) formula, and $Nm = 1.383$ following the private allele method. The combination of different methods and algorithms in GeneClass2 identified up to six putative first-generation migrants in the populations of *L. macrophyllum* (Online resource 4), three of which were above the stringency level of $\delta = 1$ for unambiguous assignment of the migrants (MBR-13, MBR-29 and TAB-57; Table 3).

The Wilcoxon's sign-rank tests detected a significant excess of heterozygosity in comparison to the heterozygosity expected from observed allele numbers, thus indicating a recent bottleneck in populations CHI and TAB under both the IAM and TPM models, and in population MBR under the IAM model (Table 4).

Levels of inbreeding

Most loci were in HWE in all five populations (Online resource 2), and only four loci showed a significant deviation from HWE: loci Mac67 in population CHA and Mac70 in population IZO, with a deficit of heterozygotes, and Mac16 and Mac65 in TAB, with an excess of heterozygotes. The inbreeding coefficient F averaged a value of 0.042 over all loci and populations.

Table 4 Significance of Wilcoxon's sign-rank tests indicating a genetic bottleneck in the studied populations of *L. macrophyllum*

Population	*N*	Significance of Wilcoxon's sign-rank test		
		IAM	SMM	TPM
IZO	34	0.156	0.629	0.191
CHI	35	0.008*	0.344	0.016*
MBR	37	0.039*	0.500	0.055
TAB	39	0.016*	0.078	0.016*
CHA	40	0.406	0.922	0.500

N number of individuals sampled, *IAM* infinite allele model, *SMM* stepwise mutation model, *TPM* two-phase model

$*p < 0.05$

Genetic structure and spatial genetic structure

Both the L(K) and the ΔK methods supported a best value of $K = 3$ in STRUCTURE, thus indicating that the five sampled populations of *L. macrophyllum* constitute three genetic clusters. Most individuals from population CHA are included in one of the clusters, whereas most individuals from populations CHI and MBR are included in a second cluster. Individuals from population IZO were assigned mostly to the two former clusters. Individuals of population TAB are included almost exclusively in a third cluster (Fig. 2).

According to the results of the AIAA, alleles are distributed spatially at random across the sampled transect ($R^{AVE} = 0.826$; $p = 0.071$). The Mantel test showed a non-significant correlation between genetic and geographic distances ($r = 0.151$; $p = 1.000$), and the spatial autocorrelation analysis also revealed a non-significant relationship between geographic and genetic distances at any distance class ($V = 0.024$; $p = 0.300$).

Table 3 Putative first-population migrants in the *L. macrophyllum* populations

Sample ID	Home population	Most likely source population (%)	Second most likely source population (%)	δ^1	δ^2
CHI-44	CHI	IZO (74.2%)	CHA (13.4%)	0.742	0.788
MBR-13	MBR	CHA (77.7%)	IZO (18.9%)	0.613	1.417
MBR-29	MBR	CHA (77.7%)	IZO (18.9%)	0.613	1.417
TAB-57	TAB	MBR (63.9%)	CHI (34.2%)	0.271	2.723
CHA-44	CHA	MBR (43.1%)	CHI (41.1%)	0.021	0.695
CHA-49	CHA	IZO (59.7%)	CHA (39.5%)	0.178	–

% probability that the individual sampled comes from that population, δ^1 difference in log-likelihood between the first and second most likely source populations, δ^2 difference in log-likelihood between the home population and the most likely source population

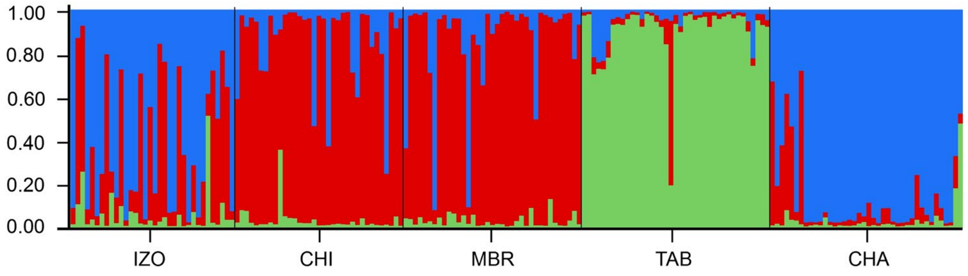

Fig. 2 Estimated membership coefficients (Q) of each *L. macrophyllum* individual sampled for each of the three genetic clusters (optimum $K=3$) identified with STRUCTURE. Each vertical bar represents one individual, and each colour represents one genetic cluster

Discussion

Genetic variation, differentiation and bottlenecks

Despite the general expectation of low genetic variation in oceanic island plants, because of founder effects upon colonization (Mayr 1942; Frankham 1997; but see; García-Verdugo et al. 2015), most Canarian plants harbor relatively high levels of total genetic variation, at least as estimated from allozyme data, especially compared to plants from other archipelagos (Francisco-Ortega et al. 2000; Pérez de Paz and Caujapé-Castells 2013). In contrast, especially considering the high allelic diversity typically displayed by microsatellites, overall genetic variation in *L. macrophyllum* ($A=2.425$; H_T = 0.363) can be regarded as rather low. In fact, the average A value obtained for this species is considerably lower than that calculated for other Canarian endemic plants investigated with microsatellite data (*Pinus canariensis*, Navascués and Emerson 2007; *Bencomia exstipulata* and *B. caudata*; González-Pérez et al. 2009; *Olea europaea* susbp. *guanchica*; García-Verdugo et al. 2010; *Sambucus palmensis*; Sosa et al. 2010; *Ruta microcarpa*; Meloni et al. 2013, 2015; *Ruta oreojasme*).

The low levels of genetic variation in *L. macrophyllum*, especially in populations CHI, MBR and TAB, are best explained by more recent bottlenecks caused by goat grazing in the mountains of Anaga. Goat grazing was practiced by Guanche aborigines after their arrival in the Canary Islands more than 2500 years ago (Rando et al. 1999), and by Europeans since their colonization of Tenerife in the fourteenth and fifteenth centuries AD. Goats feed on *L. macrophyllum* and other plants (especially in dry years), trample their seedlings, and compact and erode the soil. For these reasons, they have been identified as a major threat for many other endangered Canarian endemics (Bañares et al. 2004; Caujapé-Castells et al. 2010). Although some residual grazing still occurs in the villages of the Anaga mountains, the decrease of grazing practices resulting from migration from rural villages to urban areas during the past few decades enabled a positive demographic trend in populations of *L. macrophyllum* and other Canarian plants (Bañares et al. 2004), as also observed in other island systems upon grazer eradication (e.g. Riley et al. 2010). Genetic bottlenecks are detected in populations CHI, MBR and TAB, which are very close to villages where grazing activities used to occur until recently. By contrast, the absence of genetic bottlenecks in IZO and CHA is probably determined by their larger distances from any human population center.

The average number of alleles per locus and other variables used to estimate genetic variation tended to decrease from population TAB ($A=2.875$; H_o = 0.405; 3 private alleles), located in the middle of the species range, towards the populations at either extreme of the distribution area of *L. macropyllum*: CHA in the east ($A=2.125$; H_o = 0.186; one private allele) and IZO in the west ($A=2.375$; H_o = 0.294; zero private alleles). This result is congruent with the trend of declining genetic variation towards the range limits observed in other organisms (Eckert et al. 2008).

Populations of *L. macrophyllum* are significantly differentiated from a genetic point of view (G-test; $p<0.001$). Indeed, F_{ST} values above 0.15 are typically considered as an indication of significant differentiation among populations (Frankham et al. 2010) and, according to the qualitative guidelines suggested by Wright (1978), values between 0.15 and 0.25 indicate great differentiation. The average values of F_{ST} (=0.188) and R_{ST} (=0.186) obtained in our analyses fall within this range. Likewise, the estimated average number of migrants per generation is only slightly above one ($Nm=1.080$ following Wright's (1943) formula and $Nm=1.383$ according to the private allele method) and contemporary gene flow is not abundant among *L. macrophyllum* populations (Table 3). In fact, only three individuals (two from population MBR and one from population TAB) were assigned as first-generation migrants coming from another population as a result of a seed dispersal event, whereas three additional individuals (two from population CHA and one from population CHI) could not be unambiguously assigned as first-generation migrants, thus indicating that their genotype is likely the product of a pollen dispersal event or a recent back-cross with a migrant genotype.

Both the low migration rates and the few dispersal events detected by our microsatellite analyses reflect the limited dispersal ability of *L. macrophyllum*. Genetic differentiation and gene flow values similar to those of *L.*

macrophyllum have been reported for *L. dendroides*, another Canarian endemic restricted to La Gomera, which was investigated using allozymes (Suárez-García et al. 2009). Although *L. macrophyllum* seeds are small and the diaspores preserve the hydrochory-enhancing persistent calyces, there are no obvious adaptations for overland long-distance seed dispersal. Nevertheless, occasional dispersal between non-adjacent populations (Table 3) can be aided by particularly strong wind gusts during storms, and the role of human or animal dispersal (e.g. seeds embedded in mud attached to the hooves of feral goats) cannot be ruled out. At the same time, although the foraging behavior of the pollinators of *L. macrophyllum* is barely known, the few putative pollen-immigration events detected in our analyses (Table 3) and the low values of *Nm* obtained in this study suggest that the insects that pollinate *L. macrophyllum* rarely fly over long distances. It is not clear whether the low levels of gene flow detected in *L. macrophyllum* would suffice to prevent further genetic differentiation between populations and genetic drift in the long run. Theoretically, one migrant per generation is sufficient to prevent drift-mediated population differentiation (Wright 1931; Slatkin 1987), although it has also been suggested that higher values of *Nm* might be necessary to offset genetic drift (Lacy 1987; Mills and Allendorf 1996), especially in populations subjected to fluctuations in size (Vucetich and Waite 2000).

Spatial genetic structure

According to the Bayesian clustering analysis, all *L. macrophyllum* individuals sampled in this study can be classified in three genetic sclusters (Fig. 2). One of them corresponds almost exclusively to individuals sampled in population TAB, whereas the other two clusters include individuals from populations IZO and CHA, and individuals from populations IZO, CHI and MBR, respectively. The genetic bottlenecks likely experienced by CHI, MBR and TAB, together with the low rates of gene flow detected in our analyses, can provide a partial explanation for the observed genetic clustering. During the times of highest grazing activity, the populations of *L. macrophyllum* were probably decimated and restricted to steep outcrops and ledges inaccessible to goats, as currently observed in several other species of *L.* subsec. *Nobiles* (e.g., *L. benmageci* and *L. vigaroense* in Gran Canaria, *L. relicticum* in La Gomera, and *L. spectabile* and *L. perezii* in Tenerife). Small populations that have been subject to bottlenecks over long periods can lose part of their genetic variation through random genetic drift, and a lack of gene flow among them will increase differentiation among populations (Barrett and Kohn 1991). A fast demographic recovery upon cessation of most grazing activity will involve chiefly the genotypes that survived during the bottlenecks, thus resulting in genetically differentiated clusters. Although even low levels of gene flow (either via seeds or pollen) suffice to substantially reduce genetic differentiation among populations in a few generations (Barrett and Kohn 1991; Ingvarsson 2001), the spread of immigrant alleles can take some decades in woody plants like *L. macrophyllum* because of longer generation times.

Every diaspore of *L. macrophyllum* can carry a maximum of 16 seeds (Karis 2004). The joint dispersal of several seeds in a diaspore should produce a clustered spatial distribution of related genotypes, since the plants developed from those seeds would share at least the maternal genome. In addition, the diaspores of *L. macrophyllum* are mainly gravity-dispersed (Bañares et al. 2004) and, consequently, most of them remain clumped under the mother plant. As a result, a structuring of genetic variation over short spatial scales in *L. macrophyllum* populations could be expected. However, the analyses of spatial distribution of genetic variation in population CHA revealed that genetic distances do not increase significantly with geographic distances at a short scale.

There exist several possible, non-exclusive explanations for this result. First, as observed in the field by the first two authors, every flower lasts open only one day and, because of sequential ripening, there are rarely two or more flowers simultaneously open in the same spike. This blooming strategy minimizes the chance that two different ovules in the same diaspore are fertilized by the same pollen donor, thus diminishing genetic similarity among seedlings.

Another possibility is that only a small proportion of ovules are fertilized in every spike, so diaspores would carry only a few seeds. Therefore, siblings coming from the same diaspore would co-occur more rarely than if all flowers in the spike had produced a seed. Infrequent pollinator visits and low seed output observed in the field concur with this second hypothesis.

A third possibility is that competition for microsites or resources may prevent the development of more than one plant, even if two or more seeds from the same diaspore germinate in the same locality. Finally, the overlapping of seed shadows of different plants, which also reduces the genetic relatedness among individuals living in the same patch (Hamrick and Trapnell 2011), could also account for the lack of spatial autocorrelation of genotypes in the studied transect. Genetic studies using both nuclear markers (biparentally inherited) and chloroplast markers (maternally inherited) would be very helpful in further exploring the contribution of seed dispersal and pollen flow to the genetic structure of *L. macrophyllum* at small spatial scales.

Levels of inbreeding and mating system

Despite the restricted distribution, low number of populations and scarce gene flow between populations, the overall level of inbreeding in *L. macrophyllum* is very low ($F=0.042$). The concordance of most loci with HWE in most *L. macrophyllum* populations indicates that mating is chiefly random. The only departures from HWE occurred in one locus of population IZO and one locus of population CHA (the westernmost and easternmost populations, respectively), due to a deficit of heterozygotes (although, according to the micro-checker results, the apparent deficit of heterozygotes in IZO obeys to the presence of a null allele in locus Mac70), and in two loci of population TAB, due to an excess of heterozygotes (Online resource 2), consistent with the lower values of genetic variation in IZO and CHA and the higher values in TAB.

Our genetic results indicate low levels of inbreeding in *L. macrophyllum*, thereby suggesting that the cob-A/pap-B floral dimorphism observed in the species (Baker 1953) is most likely associated with a sporophytic self-incompatibility mechanism, as reported in other members of the Plumbaginaceae that display the same type of floral dimorphism (Baker 1966; Dulberger 1975; Vekemans et al. 1990; Richards 1997). Thus, both genetic and floral morphological observations support the conclusion that *L. macropyllum* is mostly allogamous, as found also in other *Limonium* species (e.g. Suárez-García et al. 2009). The floral dimorphism and likely self-incompatibility of species in *L.* subsec. *Nobiles* fit the pattern of more widespread outcrossing detected in the Canarian flora than in other volcanic archipelagos (Francisco-Ortega et al. 2000; Crawford et al. 2011). However, the predominant allogamy of Canarian species runs counter to the expectations of Baker's law (Baker 1955), according to which islands should harbour mostly selfing plants, because self-compatibility facilitates colonization after long-distance dispersal of single propagules to new habitats presumably devoid of the necessary pollinators. Additionally, the floral dimorphism and likely self-incompatibility of *L. macrophyllum* contrast with the monomorphism and apomixis—two traits that confer individual plants the ability to reproduce autonomously—displayed by many *Limonium* representatives of other island systems (Baker 1953; Erben 1979, 2005; Arrigoni and Diana 1999; Artelari and Georgiou 2002).

One factor put forward to explain the overrepresentation of outcrossing species on the Canary Islands is the relatively short distance to continental Africa, which improves the chance of repeated arrivals of potential mates (Francisco-Ortega et al. 2000). The minimum distance between mainland Africa and Fuerteventura, the island closest to the continent, is currently ca. 100 km, and that distance would have been as short as ca. 60 km during the Quaternary glacial maxima, when sea levels were lower (Fernández-Palacios et al. 2011). In addition, joint seed dispersal may have facilitated the colonization of the Canary Islands by the *L.* subsec. *Nobiles* lineage. Since its floral dimorphism is thought to be diallelically controlled (Dulberger 1975), the progeny of each mother plant consists of the same proportion of cob-A and pap-B plants. This genetic system, combined with the contemporaneous dispersal of multiple seeds in the same diaspore, increases the chance of simultaneous arrival of compatible mates after dispersal.

Finally, we cannot rule out the occasional occurrence of pseudo-self-incompatibility in *L. macrophyllum*. This mechanism, which allows for sporadic self-fertilization in mainly obligate outcrossers, has been observed in other self-incompatible island species, including the Hawaiian silverswords (*Argyroxyphium, Dubautia* and *Wilkesia*; Carr et al. 1986) and the Canarian *Tolpis* (Crawford et al. 2008, 2010). Further experiments aimed at understanding the ecological and evolutionary implications of joint seed dispersal and pseudo-self-incompatibility are necessary to explain the mechanisms that allowed species of *L.* subsec. *Nobiles* to radiate in the Canarian archipelago.

Implications for conservation

Although both genetic variation and inter-population gene flow as inferred from microsatellites are low, the populations of *L. macrophyllum* are overall not affected by inbreeding and show a positive demographic trend. There is, however, one major factor to consider for the future conservation of this species, namely the potential genetic bottlenecks that abrupt reductions in population sizes and low migration rates could cause (Vucetich and Waite 2000). Woody, long-lived plants with overlapping generations can maintain genetic variation for some generations during bottlenecks (Petit and Hampe 2006), but sustained bottlenecks in species with limited gene flow can lead to the depletion of genetic variation due to genetic drift, thus compromising the evolutionary potential to adapt to environmental changes. An additional consequence of low population sizes is biparental inbreeding, i.e. the inbreeding derived from mating with siblings and other genetically related individuals (Ellstrand and Elam 1993; Young and Brown 1999). Even though a lack of genetic structure at short spatial scales, as detected in *L. macrophyllum*, contributes to avoiding biparental inbreeding (Ellstrand and Elam 1993; Zhao et al. 2009), population sizes below ca. 100 individuals greatly increase the risk of biparental inbreeding even in outcrossing species (Ellstrand and Elam 1993).

The intensification of two of the current major threats for the Canarian endemic flora, namely introduced animals feeding on native plants and competition with exotic plant invaders (Francisco-Ortega et al. 2000; Caujapé-Castells et al. 2010), could decimate the populations of *L. macrophyllum* and, as explained above, imperil the long-term survival of the species. Although the populations of this species show signs of demographic recovery, both factors named above are probably holding back further population expansion. Furthermore, the impact of non-native grazing animals and plant invaders on populations of *L. macrophyllum* could increase under scenarios of climate warming forecasted for Tenerife by current climate models (Martín et al. 2012). Specifically, a higher incidence of hot, dry years would likely hinder seedling recruitment while increasing herbivory on adult *L. macrophyllum* plants by feral goats still present in the Anaga mountains, thus favouring the expansion of exotic xerophytes, such as *Opuntia ficus-indica* and *Pennisetum setaceum*, that do not represent suitable food sources for grazing animals. This selective grazing behaviour was observed by the first two authors during the exceptionally dry field season of 2012.

All species in *L.* subsect. *Nobiles* share several life history traits, including perennial habit, joint seed dispersal and cob-A/pap-B floral dimorphism. Therefore, our genetic results on *L. macrophyllum* can provide useful general insights into the conservation prospects of its related species. The low genetic variation and recent bottlenecks detected in *L. macrophyllum* predict potentially serious genetic erosion in species of the same subsection with only one known population (e.g., *L. benmageci* in Gran Canaria and *L. relicticum* in La Gomera; Bañares et al. 2004), or with small populations affected by overgrazing (e.g., *L. bourgeaui* in Fuerteventura and *L. spectabile* in Tenerife; Bañares et al. 2004). Although perennial habit, floral dimorphism linked to self-incompatibility and lack of genetic structure over short spatial scales may help to delay genetic erosion and inbreeding in such species, consistently small population sizes and low or absent gene flow will eventually result in genetic drift and biparental inbreeding (Ellstrand and Elam 1993). Whilst reintroduction of populations in suitable habitats is one possibility for the *in situ* conservation of *L. macrophyllum*, the detection of significant genetic structure (Fig. 2) should limit eventual reinforcement strategies to populations within each of the three groups resolved by our analyses.

It is important to preserve the current genetic variation of Canarian *Limonium* species because they can increase it only slowly through mutations or infrequent gene flow among populations. One of the main tenets of biological conservation is that habitat preservation is often the best strategy in order to guarantee the long-term survival of threatened species. Unfortunately, in the context of the current global economical crisis, biological conservation policies are not likely to be adequately funded. Therefore, apart from *ex situ* conservation of the most ailing species in seed banks or living collections, it is necessary that governments develop near-zero cost conservation programs that involve the participation of civilian society. For instance, in the case of *L.* subsec. *Nobiles*, volunteering programs aimed at monitoring the incidence of grazing on wild populations and eradicating alien plants would represent a significant step forward towards ensuring that the generations to come can also enjoy the extraordinary biological legacy of the Canary Islands.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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