SPECIAL ISSUE

The role of seed dispersal, pollination and historical effects on genetic patterns of an insular plant that has lost its only seed disperser

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ABSTRACT

Aim The genetic structure of many plant species is heavily dependent on their pollinators and seed dispersers, and can thus be altered if either of the associated mutualistic interactions is disrupted. In this study we assess the genetic diversity and structure and infer the seed/pollen gene-flow patterns among insular populations of *Daphne rodriguezii*, a shrub pollinated and dispersed by animals that has lost its only disperser (the lizard *Podarcis lilfordi*) in most of its populations.

Location The island of Menorca and the islet of Colom (Balearic Islands, Western Mediterranean).

Methods To assess the contribution of gene flow via pollen and seeds to the genetic structure of *D. rodriguezii* we used amplified fragment length polymorphisms (AFLPs; seeds and pollen) and plastid DNA sequences (cpDNA; seeds). We sampled individuals from all population nuclei of the species (12–19 adults per population): one population in Colom, where the plant–lizard interaction persists, and four in Menorca, where the seed dispersal mutualism disappeared with the extinction of the lizard.

Results The highest heterozygosity values were found in Colom and in its closest population (Favàritx), whereas values were lower in the smallest Menorcan populations, which also had higher relatedness among individuals. We found distinct genetic signals between AFLP and cpDNA analyses. While AFLP markers showed low differentiation between populations, cpDNA showed a clear differentiation between them.

Main conclusions Our results point to negative impacts of the disperser loss on genetic diversity and relatedness in the smaller and more isolated populations. They also suggest an old isolation by seeds, probably occurring well before the extinction of the lizard (*c.* 2000 years ago). Gene flow was maintained via pollination; however, the seed disperser loss may ultimately hinder pollinator-mediated gene flow, as a result of reduced probabilities of effective pollination among increasingly distant and scarce individuals.

Keywords

Amplified fragment length polymorphism, Balearic Islands, cpDNA, gene flow, mutualism disruption, plant–lizard interactions, seed disperser loss.

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INTRODUCTION

The current biodiversity crisis is increasingly threatening species interactions, especially on islands (Hansen & Galetti, 2009; Caujapé-Castells *et al.*, 2010; Kaiser-Bunbury *et al.*, 2010). The vulnerability of islands to the loss of biodiversity has been associated with their isolation and small size (Caujapé-Castells *et al.*, 2010), higher rates of anthropogenic habitat loss (Brooks *et al.*, 2002) and higher sensitivity to biological invasions and climate change (Fordham & Brook,

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2010; Kueffer *et al.*, 2010). In this situation, it is not enough to focus only on the conservation of species to preserve and restore biodiversity on islands – it is also necessary to maintain and restore the interactions between species (Bond, 1994; Kaiser-Bunbury *et al.*, 2010).

Plant–animal mutualisms such as pollination and seed dispersal are crucial for maintaining gene flow and population dynamics in plant populations (Slatkin, 1985; Herrera & Pellmyr, 2002). Gene flow in plants is achieved by the independent movements of seeds and pollen (Ennos, 1994; Petit *et al.*, 2005), and the relative contributions of seed and pollen dispersal agents depend on their movement ranges (García *et al.*, 2007). Interactions between any particular plant and animal are diffuse in most systems and their mutual dependence is weak (Waser *et al.*, 1996; Bascompte & Jordano, 2007). On islands, however, communities are often depauperate, and some studies show that plants are more specialized than on the mainland (Olesen & Jordano, 2002). Thus, a given plant species might be pollinated or dispersed by just a few or a single animal species (Traveset & Riera, 2005; Hansen & Müller, 2009). This increases the mutual dependence between interacting partners and, subsequently, their vulnerability to the extinction or decline of a mutualist partner.

On many islands, disruptions of seed-dispersal mutualisms owing to frugivore extinction or declines have led to decreasing recruitment and increasing isolation between populations of fleshy fruited plants (e.g. Traveset & Riera, 2005; McConkey & Drake, 2006; Hansen & Galetti, 2009; Wotton & Kelly, 2011). However, the long-term demographic and genetic consequences of the loss of vertebrate dispersal agents are still poorly understood. The disruption of seed dispersal may lead to the loss of genetic variability as a result of genetic drift (Hedrick, 2005), decreased fitness, and reduced potential to adapt to changing environments (Bijlsma & Loeschcke, 2005; Bakker *et al.*, 2010).

Daphne rodriguezii Texidor (Thymelaeaceae) is a threatened endemic shrub from the island of Menorca and an offshore islet (Balearic Islands, Western Mediterranean; Fig. 1). It has a tube-like corolla, and nectar is accessible mainly to long-tongued insects, such as butterflies and moths (Rodríguez-Pérez & Traveset, 2011; Sam Pons & A. Traveset, pers. obs.), which are usually strong fliers with relatively large movement ranges (Del Socorro & Gregg, 2001; Stevens *et al.*, 2010). The endemic lizard *Podarcis lilfordi* Günther (Lacertidae) is the only seed disperser of *D. rodriguezii* (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010). This lizard became extinct on the two larger Balearic Islands (Mallorca and Menorca), presumably after the introduction of carnivorous mammals *c.* 2000 years ago (Reumer & Sanders, 1984; Pérez-Mellado, 2002), but is abundant on some surrounding islets (Terrasa *et al.*, 2009; Fig. 1). *Daphne rodriguezii* and its sole disperser coexist only on Colom Islet (Fig. 1). Current population sizes and dynamics differ greatly between the population on Colom and those with no disperser (disrupted populations): seedling recruitment in disrupted populations is scarce, individuals are relatively older, and they are 50 times less abundant and 10 times less dense than on Colom (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010, in press). *Podarcis lilfordi* has a limited movement range compared with birds and mammals, with average seed dispersal distances of 28 m (range: 15–52 m; Santamaría *et al.*, 2007).
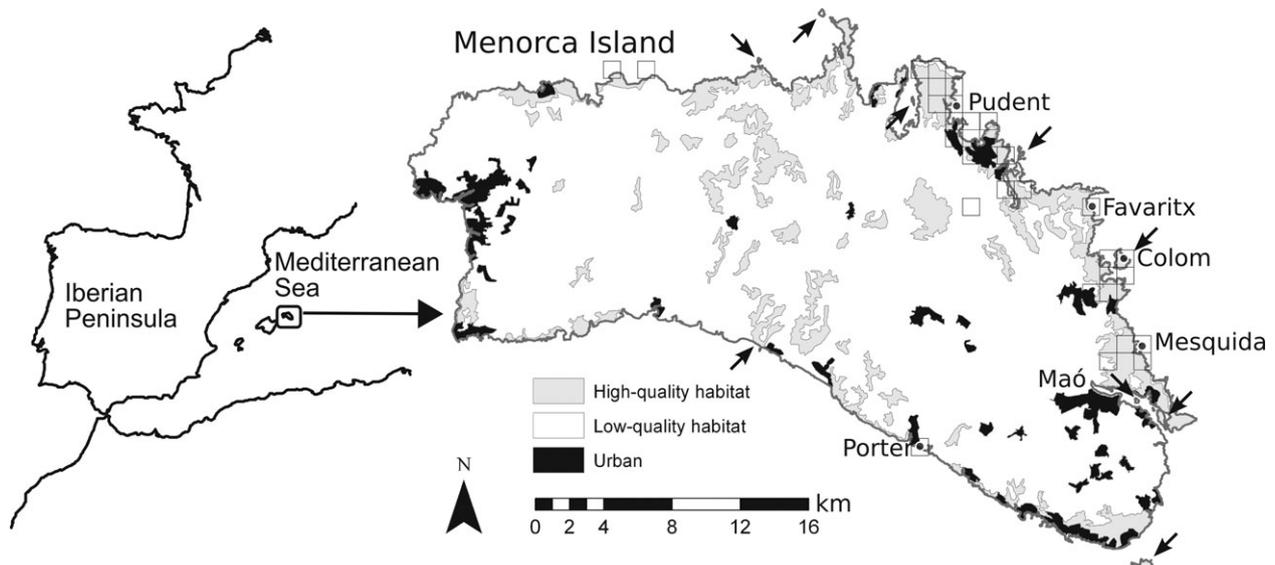

Figure 1 Map of Menorca and nearby islets showing the location of studied populations of *Daphne rodriguezii* (black dots). Squares show the confirmed presence of individuals within an area of 1 km × 1 km (BIOATLES Distribució d'Especies de les Illes Balears, 2006). Different colours indicate the quality of habitats for *D. rodriguezii* derived from the Corine land cover (Büttner & Kosztra, 2007): (1) high-quality habitat (i.e. dense and sparse shrubland) for successful regeneration of *D. rodriguezii* (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010), (2) low-quality habitat, which represents natural (i.e. woodlands, wetlands) and human-modified areas (i.e. arable and agricultural areas), and (3) urban areas. Small arrows represent the current islets where *Podarcis lilfordi* persists (modified from Terrasa *et al.*, 2009).

In addition to ecological factors, historical effects may also play a crucial role in determining genetic patterns, especially on islands (Affre et al., 1997). The Balearics are continental islands (Colom, 1957) that became isolated from the continent by the post-Messinian flooding of the Mediterranean Sea, after the opening of the Strait of Gibraltar (c. 5 Ma). This flooding also led to their separation into the western (Ibiza and Formentera) and eastern (Mallorca, Menorca and Cabrera) groups, which have remained separated (Gautier et al., 1994). Glaciation events during the last 2 Myr allowed new connections among the eastern islands (including between Colom and Menorca), which seemingly became completely isolated from each other only after the Last Glacial Maximum (LGM, c. 15,000 years ago) (Gràcia & Vicens, 1998; Tuccimei et al., 2000). Daphne is a pre-Pliocene genus of the Mediterranean flora, which was present in the area before the establishment of the current Mediterranean-type climate (Cardona & Contandriopoulos, 1979; Herrera, 1992). From the middle Pliocene (3.2 Ma), the tropical-like climate that predominated in the Mediterranean Basin during the early Pliocene changed progressively with the onset of the Mediterranean seasonality and its dry summers and the start of the Quaternary climatic oscillations (2.4 Map; Suc, 1984). These changes brought about important shifts in vegetation, with many taxa becoming extinct, others experiencing drastic range contractions, and some proliferating and coming to dominate under the new conditions (Suc, 1984).

In this study, we investigate the role of historical and ecological factors in determining genetic patterns in D. rodriguezii. We used two types of markers with different modes of inheritance: plastid DNA (cpDNA) and amplified fragment length polymorphism (AFLP) loci, which are assumed to be from across the whole plant genome, although mainly from the nuclear genome, as it is much larger than the plastid genome. The maternally inherited cpDNA reflects seed gene flow, while the biparentally inherited AFLP loci reflect both seed and pollen gene flow, revealing the total genetic distances between populations and/or individuals. Combining these two types of markers enabled us to tackle the following questions: (1) What are the contributions of gene flow via pollen and seeds to the genetic patterns in D. rodriguezii? (2) Has the loss of the seed disperser had an impact on the genetic patterns? (3) How are the genetic patterns affected by the palaeobiogeographical context? We expect contrasting patterns of genetic diversity and relatedness among individuals in the populations from Menorca and Colom, with reduced genetic diversity in Menorca as a result of genetic drift and increased inbreeding in the smaller populations there (Barrett & Kohn, 1991; Cruzan, 2001). We also expect some degree of genetic differentiation among populations in Menorca as a result of the lack of seed dispersal. Pollinators could maintain some gene flow, counteracting, at least partly, the genetic erosion associated with reduced population sizes. This would translate into contrasting patterns of differentiation in cpDNA and nuclear DNA (nDNA). The probability of pollen transfer decreases with decreasing population sizes and increasing geographic distances between individuals (Cruzan, 2001). Thus, the patterns of genetic diversity and relatedness among individuals are expected to be associated with population size and distance to other populations (especially to large populations, such as Colom in this system). The separation of Colom from Menorca, which became definitive after the LGM, might translate into some differentiation in plastid markers between them. No physical barriers for lizard movement between Menorcan populations seem to have existed. Thus, as lizard extinction was relatively recent, we expect less differentiation among Menorcan populations than between Colom and Menorca, although the limited movement range of lizards might have hindered connectivity in the long term.

MATERIALS AND METHODS

Study plant species

The genus Daphne (Thymelaeaceae) comprises 70 shrub species distributed mainly in Europe and temperate and subtropical Asia (Brickell & Mathew, 1976). All species bear fleshy fruits, and are dispersed by vertebrates. Daphne rodriguezii inhabits coastal shrublands on the island of Menorca (total area 700 km²), with minimum between-population distances of 5–20 km, and on the islet of Colom (60 ha) 270 m east of Menorca (Balearic Islands, Western Mediterranean; Fig. 1). It is an evergreen shrub that reaches up to 50 years of age (J. Rodríguez-Pérez, unpublished data) and 1.5 m in height, and is currently considered a vulnerable species by the IUCN (Peraza Zurita, 2011). It is a hermaphroditic, insect-pollinated species. Plant reproduction appears to be pollination-limited: very few pollinators have been observed (which has been attributed to the flowering time, the peak being in March when insects are still scarce) and the fruit set is consistently low (< 10%) but can be increased roughly fivefold by hand-pollination compared with open pollination (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2011). Daphne rodriguezii produces orange–red drupes from May until June, and seeds are dispersed only by the endemic lizard P. lilfordi (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010). According to the fossil record, P. lilfordi began to vanish from the large Balearic Islands, Mallorca and Menorca, about 2000 years ago, presumably after the human introduction of predators such as weasels (Mustela nivalis), pine martens (Martes martes), genets (Genetta genetta) and cats (Felis catus) (Reumer & Sanders, 1984; Pérez-Mellado, 2002). It is, however, abundant on some islets surrounding these large islands (Terrasa et al., 2009; Fig. 1) where these carnivores are absent. Daphne rodriguezii grows mostly under nurse plants such as Phillyrea latifolia L. ssp. media (L.) Fourn., Rosmarinus officinalis L., and Pistacia lentiscus L. In Colom, lizards facilitate seedling recruitment by defecating seeds under the nurse shrubs, whereas the lack of seed dispersal in Menorca restricts seedling recruitment to under conspecifics (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010), creating high-density clumps of seedlings and juveniles that may compete for

Table 1 List of studied populations of *Daphne rodriguezii* from the islet of Colom and from four localities in Menorca (Balearic Islands).

Population	Location long/lat	N	n	H_E	PL	PLO	Br	PLs	r
Colom	31SFE0924	18,000	17	0.111 ± 0.010	0.543	9	1.592	0.694	0.17
Pudent	31TEE9833	298	16	0.090 ± 0.009	0.425	5	1.499	0.559	0.35
Favàritx	31SFE0727	168	14	0.123 ± 0.011	0.457	5	1.567	0.613	0.11
Mesquida	31SFE0918	47	12	0.072 ± 0.008	0.279	5	1.387	0.387	0.42
Porter	31SEE9713	48	19	0.077 ± 0.009	0.342	5	1.376	0.437	0.50

Population names, location (UTM coordinates), population size (N) (Rodríguez-Pérez & Traveset, in press), sample size (n), expected heterozygosity (H_E), proportion of polymorphic loci (PL), number of private loci (PLO), band richness (Br), proportion of polymorphic loci estimated with standardized sample sizes (PLs), and relatedness (r) value are indicated.

resources with adult plants (J. Rodríguez-Pérez & A. Traveset, unpublished data). This dependence on nurse plants makes dispersal especially important in this species (Howe & Miriti, 2004). The lack of dispersers in Menorca precludes the re-colonization of suitable habitat patches when local extinctions occur, leading to reduced plant densities and long-term population declines (Rodríguez-Pérez & Traveset, in press).

Plant material and DNA extraction

In July 2004, we collected samples from 12 to 19 reproductive individuals at five localities of *D. rodriguezii* (Table 1): on Colom and at four localities in Menorca (Porter, Mesquida, Pudent and Favàritx). Owing to the strong spatial clumping of plants in Menorcan populations (J. Rodríguez-Pérez & A. Traveset, unpublished data), the sampling was stratified on two levels: we selected groups of reproductive individuals (up to 10 groups in the largest populations) separated by at least 20 m, and up to three individuals within each group (up to 5 m from each other). In order to be consistent, this distance pattern was repeated on Colom. Fresh new leaves were collected in the field and stored in silica gel. Total genomic DNA was extracted from *c.* 0.02 g leaf material (dried with silica gel) using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.

AFLP analyses

The AFLP procedure followed that of Gaudeul *et al.* (2000) with some modifications. 6-FAM – *Eco*RI + ACT and *Mse*I + CG, and VIC – *Eco*RI + AGG and *Mse*I + CG primer pairs were chosen. For each individual, 0.5 μL of each labelled selective PCR product was combined with 0.5 μL of GeneScan 500 LIZ (Applied Biosystems, Foster City, CA, USA) and 13.5 μL of formamide, and run on a capillary sequencer (ABI 3730; Applied Biosystems).

Scoring of amplified fragments (100–500 bp) as either present or absent for all individuals was implemented with GeneMapper 4.0 (Applied Biosystems Inc.) to construct a binary matrix. All electropherograms were examined visually, and only AFLP fragments that could be scored unambiguously were included in the analyses. For consistency, visual checks were performed by the same person. Invariable loci and individuals with missing data were excluded from further analyses.

To analyse population patterns of diversity, we computed two gene diversity indices (H_E, H_T; Hartl & Clark, 1997) as well as an estimation of private alleles (PLO) and proportion of polymorphic loci (PL) using GenAlEx 6.4 (Peakall & Smouse, 2006). We also used a rarefaction approach implemented in AFLPDIV 1.1 (Coart *et al.*, 2005) to standardize sample sizes and estimate band richness (Br) and the percentage of polymorphic loci (PLs) expected at each locus in each population. We estimated pairwise relatedness coefficients (following Lynch & Milligan, 1994) between each pair of individuals in the overall data set using AFLP-Surv 1.0 (Vekemans, 2002), and computed the average relatedness between individuals within each population.

Genetic differentiation among populations was estimated using pairwise F_{ST} values and pairwise genetic Nei distances (following Lynch & Milligan, 1994) as implemented in AFLP-Surv 1.0 (Vekemans, 2002). Analysis of tree topology and support was performed using estimated pairwise genetic Nei distances and the neighbour-joining (NJ) algorithm of the Neighbor and Consense packages of Phylip 3.7 (Felsenstein, 2007). Branch reliability was assessed by bootstrapping with 1000 replicates.

To assess genetic structure among samples, we conducted a principal coordinates analysis (PCoA) on a genetic distance matrix generated from the binary presence–absence matrix, using GenAlEx 6.4. An analysis of molecular variance (AMOVA) was performed with two hierarchical levels (among and within populations) for the whole data set. To examine the pattern of isolation-by-distance, we tested the correlation between genetic distances (pairwise Nei distances, Hedrick, 2005, and pairwise F_{ST} values) and Euclidean geographic distances among localities using a Mantel test (implemented in GenAlEx 6.4 with 1000 permutations).

cpDNA analyses

A pilot study of six plastid regions (Shaw *et al.*, 2007) revealed the sequences of the *rpl*32 and *psb*D to be the most variable. A total of 78 individuals from the five populations (as for AFLPs) were included in the sequencing study. The *rpl*32 and *psb*D regions were amplified and sequenced as described by Shaw *et al.* (2007).

The 78 accessions were manually aligned in a matrix. The resulting plastid haplotype matrix was analysed under statistical

parsimony using TCS 1.20 (Templeton *et al.*, 1992; Clement *et al.*, 2000). One gap was found in *D. rodriguezii* (which characterizes all individuals from Porter); it was analysed as missing data, but was coded as present/absent for the statistical parsimony analysis. Genetic differentiation among populations was estimated with pairwise F_{ST} values and overall F_{ST} as implemented in ARLEQUIN 3.5 (Excoffier & Lischer, 2010).

We estimated the ratio of pollen migration (m_p) to seed migration (m_s) following the protocol of Petit (1992) and Petit *et al.* (1992), using the formula

$$m_p/m_s = [2(1/F_{STc} - 1) - (1/F_{STn} - 1)]/(1 - 1/F_{STc}),$$

where F_{STc} is cytoplasmic variation among sites and F_{STn} is nuclear variation among sites.

RESULTS

Genetic diversity and relatedness patterns

A total of 187 polymorphic AFLPs were scored for the 78 sampled individuals. The proportion of loci that were polymorphic (Table 1) varied from 28.0% (Mesquida) to 54.3% (Colom). The total expected heterozygosity (H_T) was 0.116 ± 0.009, and the average within-population expected

heterozygosity was 0.095 ± 0.004. Favàritx and Colom showed the highest H_E, whereas Mesquida and Porter had the lowest (Table 1). Both Br and PLs followed the same genetic trend after standardization to the minimum sample size ($n = 12$), showing the highest genetic diversity values in Colom and Favàritx (Table 1). We found high levels of pairwise relatedness for Porter ($r = 0.5$, the expected value for full sibs), Mesquida and Pudent, and low values for Colom and Favàritx (Table 1).

The number of private alleles was nine in Colom (4.62%, $n = 195$) and five in the other populations (2.89–3.55%, $n = 141$–173; Table 1). Genetic diversity was correlated with census population size when considering the proportion of polymorphic loci ($r^2 = 0.78$, $P = 0.045$, $n = 5$), but not when using H_E ($r^2 = 0.18$, $P = 0.477$, $n = 5$).

Genetic structure and differentiation among populations

AFLP-derived pairwise F_{ST} values ranged from 0.007 (Colom–Pudent) to 0.082 (Favàritx–Porter; Table 2). The population NJ tree (Fig. 2) showed a moderately supported split between Mesquida + Porter and Colom + Favàritx + Pudent (68% bootstrap support), while further divisions had low support. A PCoA of AFLP variation revealed very little genetic structure among populations. A plot of the two principal coordinates (accounting for 56% of the variance) showed a main cluster formed by some individuals from all populations, whereas some individuals from Colom, Favàritx and Pudent departed from the main group (Fig. 3).

The AMOVA for the whole data set, with two hierarchical levels, assigned most of the total genetic variance to variation within populations (92%), whereas variation among the five populations was low (Table 3). The Mantel test did not detect any isolation-by-distance when Nei distances or F_{ST} were used as inputs (Nei: $r = 0.107$, $P = 0.25$, $n = 10$; F_{ST}: $r = 0.030$, $P = 0.35$, $n = 10$).

Five distinct cpDNA haplotypes were identified (GenBank numbers: JQ755966–JQ756121). Each haplotype was fixed and characteristic of one population, with two exceptions: (1) all individuals but one from Colom and all individuals from
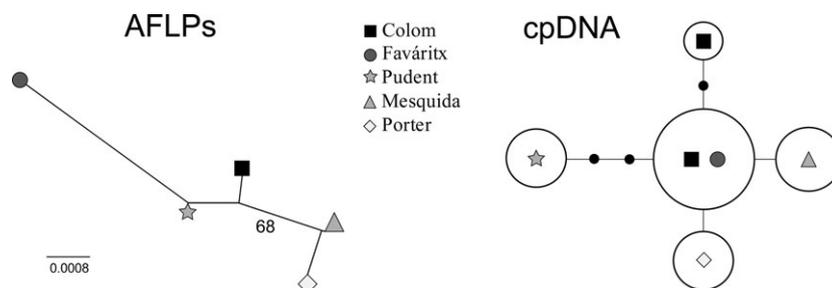
Table 2 Pairwise F_{ST} values of *Daphne rodriguezii* for the AFLP fingerprint (values below the diagonal) and cpDNA sequences (values above the diagonal). Populations were sampled from the islet of Colom and from four localities in Menorca (Balearic Islands).

	cpDNA				
	Porter	Mesquida	Favàritx	Colom	Pudent
AFLPs					
Porter		1.000	1.000	0.941	1.000
Mesquida	0.045		1.000	0.923	1.000
Favàritx	0.082	0.053		0.004	1.000
Colom	0.046	0.037	0.030		0.936
Pudent	0.025	0.040	0.030	0.007	

AFLPs, amplified fragment length polymorphisms.

Figure 2 On the left, unrooted neighbour-joining tree of *Daphne rodriguezii* populations from the islet of Colom and from four localities in Menorca based on pairwise Nei's genetic distances from applied fragment length polymorphisms. Support for branches is indicated by bootstrap values based on 1000 replicates. On the right, TCS haplotype network of genetic relationships among *D. rodriguezii* populations based on cpDNA. The size of each haplotype symbol is proportional to its frequency, and black circles represent mutational steps separating observed haplotypes.
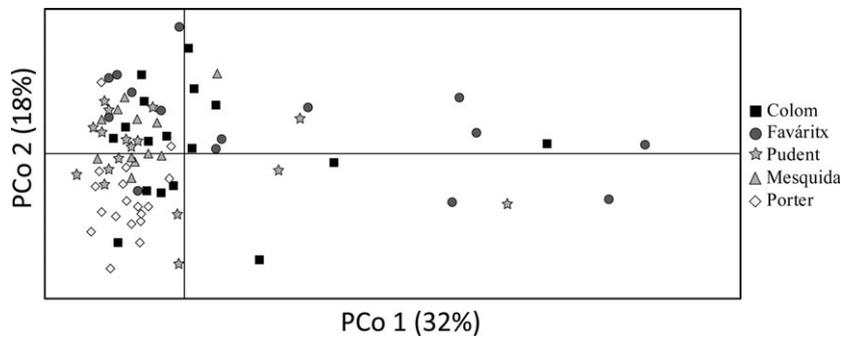
Figure 3 Principal coordinates analysis (PCoA) of applied fragment length polymorphism genetic variation in *Daphne rodriguezii* populations from the islet of Colom and from four localities in Menorca. Populations do not show a clear pattern of spatial structure (the first two coordinates explain 56% of the variance), but some individuals from Colom, Favàritx and Pudent depart from the main cluster, which groups all extant populations.

Table 3 Analysis of molecular variance (AMOVA) of *Daphne rodriguezii* for the AFLP fingerprint considering the whole data set with two hierarchical levels.

Source of variation	d.f.	SS	MS	Est. var.	Percentage of variation
Among populations	4	134.202	33.550	1.266	8*
Within populations	73	1016.196	13.920	13.920	92
Total	77	1150.397		15.186	

AFLPs, amplified fragment length polymorphisms.
Populations were sampled from the islet of Colom and from four localities in Menorca (Balearic Islands). SS, MS and Est. var. refer to the sum of squares, mean squares, and estimated variance, respectively.
*$P = 0.001$.

Favàritx shared the same haplotype; and (2) one individual from Colom displayed a haplotype that was different from that of all other individuals of all populations.

Statistical parsimony analysis of the *rpl*32 and *psb*D loci resulted in a network of the five plastid haplotypes of *D. rodriguezii* (Fig. 2) with the central, most frequent and probably ancestral haplotype shared between Favàritx and Colom. Haplotypes from Porter and Mesquida were connected to this haplotype by a single mutation, the additional haplotype from Colom was connected by two mutations, and the haplotype from Pudent by three mutations.

Pairwise F_{ST} values for cpDNA ranged from 0.004 (Colom–Favàritx) to 1.000 (six population pairs; Table 2). Overall F_{ST} for cpDNA was 0.971. Estimated pollen migration (m_p) was much higher than seed migration (m_s), resulting in a pollen-to-seed migration ratio of 820.

DISCUSSION

Seed versus pollen gene-flow patterns in *D. rodriguezii*

The plastid and nuclear markers gave us different and complementary information with which to disentangle the

patterns of gene flow in *D. rodriguezii*. The high pollen-to-seed migration ratio obtained reflects the clear predominance of pollen-mediated gene flow (Petit *et al.*, 2005). Maternally inherited cpDNA showed a clear differentiation among populations (except between Favàritx and Colom), which suggests an old isolation by seeds before lizards disappeared, whereas the low genetic differentiation shown by the biparentally inherited AFLP markers points to the important role of pollen gene flow in counteracting the genetic isolation of populations by seed.

Comparisons of the genetic differentiation in cpDNA and nDNA in angiosperms have usually shown a stronger spatial structure in cpDNA than in nDNA (Ennos, 1994; El Mousadik & Petit, 1996; McCauley *et al.*, 1996). This is related to the double effective population size of biparentally inherited nDNA as compared with maternally inherited markers (Birky *et al.*, 1983), to dispersal by both pollen and seeds of the nuclear genome but only by seeds of the plastid genome (Petit *et al.*, 1993; Ennos, 1994), and to a general predominance of pollen-mediated over seed-mediated gene flow (reviewed in Petit *et al.*, 2005). This predominance might be partially attributable to the relatively limited seed dispersal ranges compared with pollen dispersal ranges in most of the species studied so far (e.g. in *Quercus* and *Pinus*; Petit *et al.*, 2005). In fleshy fruited plants, however, low levels of differentiation in cpDNA are common (e.g. Oddou-Muratorio *et al.*, 2001; García-Verdugo *et al.*, 2010). Most of the fleshy fruited plants that have been studied are dispersed by birds and mammals, whose wide-range movements provide good connections between populations, especially in the range of a few kilometres (e.g. García *et al.*, 2007; Jordano *et al.*, 2007). The comparatively limited range of lizards is consistent with the strong differentiation in cpDNA found in our study.

Pollen-mediated gene flow has provided connectivity among populations despite the limitations in seed dispersal. The floral morphology of *D. rodriguezii* points to pollination by long-tongued insects, such as butterflies and moths. Diurnal insect visitors to the flowers are extremely rare (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2011), but nocturnal moths

might be important pollinators (Sam Pons & A. Traveset, pers. obs.). Moths are strong fliers, with local movements in the range of a few kilometres (Del Socorro & Gregg, 2001), and records of extreme long-distance movements reaching hundreds of kilometres (Hendrix *et al.*, 1987; Gregg, 1993). Minimum distances between current Menorcan populations range from *c.* 5 to 20 km, well within the flying range of moths, which would explain the low molecular differentiation among populations in nuclear markers.

Differentiation in nuclear markers was particularly low between Colom, Pudent and Favàritx (northern Menorcan coast), as shown by the PCoA and the NJ tree. This suggests that neither the sea strip between Colom and Menorca nor the present distance between these Menorcan populations have been efficient barriers for pollen gene flow. The large number of individuals in Colom might have favoured higher frequencies of pollen-mediated gene flow in this area (Cruzan, 2001). Moreover, the largest remaining populations in Menorca are on the northern coast (Pudent and Favàritx), and the presence of sparse individuals and historical records along this coast suggest that this area might have harboured larger populations for longer, compared with the southern and eastern parts of the island. This could have contributed to the maintenance of higher levels of gene flow via pollen between northern populations.

Colom and Favàritx are the only populations sharing a haplotype (Fig. 2), which points to a good connection by seeds between them until relatively recent times. The star-shaped pattern observed in the cpDNA network (Fig. 2) also points to these being the ancestral populations. AFLP analyses showed that these two populations have higher heterozygosity and lower relatedness than the others, and that Colom has the highest number of private alleles and polymorphic loci (Table 1). The vicariant event caused by the separation of Colom and Menorca did not lead to genetic differentiation between Colom and Favàritx populations in cpDNA markers (Fig. 2). In contrast, the clear differentiation in cpDNA haplotypes among Menorcan populations suggests that they were isolated from each other before Colom and Menorca became definitely separated, after the LGM (see Introduction). We may thus conclude that the isolation between Menorcan populations for seed movement occurred long before the lizard population became extinct (*c.* 2000 years ago).

The seemingly old isolation by seeds among Menorcan populations does not seem to be related to fragmentation in the lizard populations. On the contrary, the genetic patterns observed across the extant populations of *P. lilfordi* inhabiting the islets surrounding Menorca suggest that Menorcan populations were connected before the lizard became extinct on the island (Terrasa *et al.*, 2009). In addition, there is no apparent past or present physical barrier preventing the movement of lizards between *D. rodriguezii* populations in Menorca, and the coastal shrubland is well preserved along most of the coast, especially in the north, which would have ensured a good connectivity (Fig. 1). Given the limited

movement range of lizards (Santamaría *et al.*, 2007), however, they can connect only continuous populations, the connectivity failing as soon as populations are separated by a few hundreds of metres. Thus, even a slight degree of fragmentation in the plant population might preclude connectivity by seeds, even with continuous and abundant lizard populations. Many factors could lead to such minor fragmentation. For instance, the onset of the Mediterranean xericity and the climatic oscillations of the Quaternary might well have resulted in range contractions, and the species might not have fully recovered to regain the near continuous distribution required for lizards to ensure good connectivity.

Consequences of genetic erosion and restoration of the disrupted seed dispersal mutualism

Daphne rodriguezii shows low levels of within-population genetic variability compared with average values for other long-lived perennial ($H_E = 0.25$, $n = 37$), endemic ($H_E = 0.20$, $n = 7$) and outcrossing ($H_E = 0.27$, $n = 38$) plants (as reviewed in Nybom, 2004; for studies using RAPD markers, which yielded similar results to studies based on AFLPs, as shown in a subset of studies using both markers). As expected according to the population sizes, Colom had high levels of genetic diversity and low levels of relatedness between individuals. The lowest levels of diversity and the highest relatedness values were found in the smallest populations (Porter and Mesquida). In addition, Porter is the most isolated population, and this seems to have promoted the lower heterozygosity and higher relatedness found in this population as compared with Mesquida, which has a similar population size but is less isolated. However, any effects of inbreeding depression are not yet apparent in fruit set or in germinability in these populations (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010, 2011). The high values of genetic diversity and low relatedness found in Favàritx are remarkable, and seem inconsistent with its rather reduced population size. Favàritx is the closest population to Colom, and relatively frequent gene flow by pollen with this large and genetically diverse population could have counteracted genetic erosion resulting from genetic drift in Favàritx, despite its small size (Cruzan, 2001). In addition, the presumed longer persistence of larger populations in northern Menorca, as noted previously, could also have favoured pollen-mediated gene flow.

The loss of the seed dispersal mutualism hinders regeneration in Menorcan populations, which has a direct impact on the genetic diversity and relatedness associated with small population sizes (see above). It may also have indirect effects on genetic patterns, through negative effects on pollen-mediated gene flow. Recruitment is very dependent on nurse plants, and these suitable sites become difficult to reach when the seed disperser is missing (Traveset & Riera, 2005; Rodríguez-Pérez & Traveset, 2010). Plant persistence can be maintained for some time within suitable patches, but whenever plants disappear from one patch, the lack of seed

dispersal precludes the re-colonization of these vacated sites as well as the colonization of unoccupied suitable sites. This marks an inexorable long-term trend towards population decline, leading to larger distances between individuals within populations and increasing isolation of the remaining nuclei, as a consequence of the disappearance of individuals in between. The ability of pollinators to provide connectivity among plant patches depends on their movement range and on the spatial arrangement of those patches, which is ultimately dependent on seed dispersal. Seed dispersal disruptions could thus indirectly lead to disruptions in pollen-mediated gene flow through increasing fragmentation. Severe pollen limitation has, in fact, been detected in Menorcan populations (Rodríguez-Pérez & Traveset, 2011), which might be a result of this phenomenon. This suggests that current gene flow via pollen might be scarce, but this state of affairs might be too recent to have resulted in significant genetic differentiation in nuclear markers.

The small sizes of the extant populations in Menorca together with difficulties in regeneration in the absence of dispersers are a cause of concern. In this situation, further declines and increasing isolation are expected. This trend can hardly be reversed without restoring the lost dispersal mutualism, which seems the only way to attain self-sustaining populations in the long term. Restoration of the mutualism in Menorca is difficult, as the causes that led to the extinction of *P. lilfordi* (i.e. the introduction of carnivorous mammals) persist, which hinders the success of a reintroduction on the island (IUCN, 1998). A feasible alternative could be the introduction of the plant on any of the islets offshore Menorca where the lizard still occurs (16 islets; Terrasa *et al.*, 2009) and where the habitat is favourable for the plant. According to our results, Colom is the most suitable donor population for an introduction programme. It has the largest size (which reduces the impact of seed collection on recruitment), as well as high levels of genetic diversity and low relatedness (implying the good quality of the seed source and favouring the adaptive potential of the introduced individuals).

Concluding remarks

Our results suggest that the loss of seed dispersal mutualism in Menorcan populations had a direct impact on the genetic diversity and relatedness associated with small population sizes and increasing isolation. Isolation by seed dispersal among populations seems to have been prior to lizard extinction, but gene flow has been maintained by pollination. However, the limited regeneration in the absence of seed dispersers may ultimately hinder pollinator-mediated gene flow as a result of reduced probabilities of effective pollination among increasingly distant and scarce individuals. Difficulties in recruitment point to further declines and increasing isolation. Restoration of the lost dispersal mutualism seems the only way to attain self-sustaining populations in the long term and to prevent the species falling into an extinction vortex (Gilpin & Soulé, 1986).

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BIOSKETCH

The research team is a multidisciplinary group with interests in animal–plant mutualistic interactions, island ecology, and phylogeography.

Author contributions: J.R.-P. and A.T. conceived the ideas; J.R.-P. and A.T. collected the samples; E.C. and J.R.-P. performed the genetic analyses in the laboratory under the supervision of P.V.; M.E., M.C.-C. and G.V.-A. analysed the data; and M.C.-C. led the writing, with contributions from most authors.

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