

# Linking genetic diversity, mating patterns and progeny performance in fragmented populations of a Mediterranean shrub

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## Summary

1. The long-term persistence of fragmented plant populations is predicted to be threatened by a loss of genetic variability and increasing inbreeding, which might lower offspring fitness through inbreeding depression. Assessing plant progeny performance together with measurements of genetic diversity and mating patterns is therefore essential in the understanding of the role of the historical (i.e. genetic diversity in adults) and contemporary (i.e. current mating patterns) genetic effects of fragmentation on inbreeding depression, thus, on recruitment potential.

2. We evaluated genetic diversity, mating patterns and progeny performance at different life stages in seven populations of a Mediterranean shrub (myrtle *Myrtus communis*) that differed in size and degree of isolation (Large, Small-connected and Small-isolated populations). The study was conducted in the Guadalquivir Valley (SW Spain), a chronically and severely fragmented landscape characterized by *c.* 1% of woodland cover.

3. Parameters of genetic diversity ( $A_r$ ,  $H_o$  and  $H_e$ ) of adult plants were in general higher in the Large populations than in the two types of Small populations, which were similar. Outcrossing rates were higher in Small-connected populations (mean:  $t_m = 0.62$ ), intermediate in Large ( $t_m = 0.35$ ) and lower in Small-isolated populations ( $t_m = 0.13$ ), and were positively correlated with the genetic diversity of progenies.

4. Several measurements of progeny performance were higher in Small-connected populations, intermediate in Large and lower in Small-isolated populations, in particular those related with the quantity of viable seedlings produced (germination and survival). Outcrossing rates rather than the genetic diversity of adult plants were positively correlated with these measurements of progeny performance.

5. We thus conclude that contemporary mating patterns (outcrossing rates) have a more critical influence on progeny performance than either population fragmentation or the historical levels of genetic diversity.

6. *Synthesis and applications.* It may be possible to enhance either the fitness or certain levels of genetic diversity in progenies by promoting outcrossed matings in fragmented populations of self-compatible plant species. In our study species, this would be feasible either by controlling honeybee *Apis mellifera* hives or maintaining and/or enhancing landscape connectivity around small patches.

**Key-words:** genetic drift, germination, habitat fragmentation, honeybees, inbreeding depression, Mediterranean woodland, *Myrtus communis*, offspring fitness, outcrossing rates, seedling growth

## Introduction

Habitat fragmentation is considered to be one of the major threats to the global biodiversity of terrestrial ecosystems (Sala

*et al.* 2000), and the reduction in size and increase in isolation of populations may place the long-term persistence of even common and naturally abundant species in jeopardy (Hobbs & Yates 2003; Honnay & Jacquemyn 2007; Aguilar *et al.* 2008). The genetic consequences of habitat fragmentation on plant populations (i.e. loss of local genetic variability through

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increased genetic drift and/or inbreeding) have received growing attention over the last two decades (Ellstrand & Elam 1993; Young, Boyle & Brown 1996; Leimu *et al.* 2006; Honnay & Jacquemyn 2007; Aguilar *et al.* 2008). The sessile nature of plants makes small and isolated populations more prone to suffer the deleterious effects of genetic drift (reduced genetic diversity) unless sufficient immigrant gene flow via pollen and/or seed occurs (Young, Boyle & Brown 1996). Moreover, inbreeding in plants may occur either through selfing (in self-compatible species) or outcrossing with close relatives (i.e. biparental inbreeding), and habitat fragmentation may alter the levels of both components of mating patterns by lowering mate availability (Franceschinelli & Bawa 2000; González-Varo, Albaladejo & Aparicio 2009) and/or shifting the composition, abundance and behaviour of pollinators (Aizen & Feinsinger 1994; England *et al.* 2001). Indeed, it has been reported that the proportion of progeny generated by cross-pollination (outcrossing rate) is generally lower in fragmented populations than in large or continuous populations (Aguilar *et al.* 2008; Eckert *et al.* 2010; but see, e.g. Mathiasen, Rovere & Premoli 2007), although most studies to date have been conducted on tropical trees.

The long-term persistence of fragmented plant populations is predicted to be threatened by these negative genetic consequences (Hobbs & Yates 2003). Increased inbreeding, and thus homozygosity, may lead to the expression of lethal or harmful recessive alleles that could reduce offspring fitness (inbreeding depression) at different life stages (Husband & Schemske 1996; Dudash & Fenster 2000). Therefore, progeny performance in fragmented plant populations should be influenced by (i) the history of the genetic drift and inbreeding that have shaped the genetic pool of adult plants and (ii) the ecological factors affecting contemporary mating patterns (Ellstrand & Elam 1993; Young, Boyle & Brown 1996; Dudash & Fenster 2000; Leimu *et al.* 2006). However, compared with the abundant empirical literature on the effects of fragmentation on genetic diversity in plants, studies assessing progeny performance in fragmented plant populations are still scarce (but see Menges 1991; Kéry, Matthies & Spillmann 2000; Kolb 2005; Yates *et al.* 2007), whereas works assessing patterns of progeny performance in conjunction with measures of genetic diversity and mating patterns are even rarer (but see Cascante *et al.* 2002; Mathiasen, Rovere & Premoli 2007; Broadhurst, Young & Forrester 2008). This is shown by the fact that none of the fitness measurements included in a recent meta-analysis (Leimu *et al.* 2006) evaluating the relationships between plant population size, genetic diversity and fitness were based on progeny analyses, but on flower, fruit and seed production as plant fitness surrogates. These parameters of adult plants are fitness components that may not be related to offspring fitness; for example, the effects of inbreeding depression on seed production are usually weak in self-compatible species (Husband & Schemske 1996). Hence, there is a clear need for more empirical information, not only on the effects of habitat fragmentation on plant progeny performance but also on the underlying mechanisms. Such studies will help us to understand the influence of historical (i.e. the genetic diversity of adults) and

contemporary (i.e. the contemporary mating patterns) genetic effects on the inbreeding depression of fragmented plant populations and thus on their demographical performance (recruitment potential). From an applied perspective, these studies will be useful because management practices may differ depending on whether the main driver of inbreeding depression is the loss of genetic diversity (e.g. favouring immigration, natural or assisted) or the contemporary disruption of mating patterns through an increase of selfing and/or biparental inbreeding (e.g. increasing plant density and pollinator abundance and/or diversity).

In this study, we examined genetic diversity, mating patterns and progeny performance in Mediterranean myrtle (*Myrtus communis* L., Myrtaceae) populations occurring in remnant woodland patches in an extremely fragmented landscape. Myrtle is an insect-pollinated shrub and one of the main components of woodland understories and late successional shrublands throughout the Mediterranean Basin. The study was conducted in the Guadalquivir Valley (SW Spain), an intensively cultivated area in which woodland vegetation – covering just *c.* 1% of the valley surface area at present (Aparicio 2008) – was virtually eliminated by human intervention several centuries ago. We selected myrtle populations differing in size and degree of isolation and used allozyme markers to estimate genetic diversity and mating pattern parameters. To link the genetic parameters with progeny performance at different life stages, we also conducted a greenhouse experiment. Specifically, we addressed two main questions: (i) Does the performance of myrtle progenies decrease with increasing habitat fragmentation? and (ii) Is variation in progeny performance between myrtle populations mainly linked to the genetic diversity of adult plants or to their contemporary mating patterns?

## Materials and methods

### STUDY SPECIES

The Mediterranean myrtle *Myrtus communis* L. is a sclerophyllous shrub and the sole representative of the Myrtaceae in the Mediterranean Basin. It grows up to 4 m high and inhabits warm fertile lowlands. Flowers are hermaphroditic, with a white open dish-shaped corolla of 2–3 cm in diameter. Insects, mostly hymenoptera ( $\geq 75\%$  of visits) and diptera, visit myrtle flowers in search of pollen (González-Varo, Arroyo & Aparicio 2009). The species is self-compatible and shows a density-dependent mixed-mating system (mean outcrossing rate:  $t_m = 0.35$ ) within large populations; the greater the plant density, the higher the outcrossing rates (González-Varo, Albaladejo & Aparicio 2009). Its fruits are dark-blue berries that contain a mean  $\pm$  SD of  $5.2 \pm 2.7$  seeds weighing  $10.8 \pm 4.1$  mg each, which are mainly dispersed by birds (González-Varo 2010).

### STUDY AREA AND POPULATIONS

The Guadalquivir Valley (western Andalusia, southern Spain) is a large (21 000 km<sup>2</sup>) lowland area that has been intensively cultivated for centuries, and in which the transformation of natural habitats has resulted in just *c.* 1% of Mediterranean woodland cover being left in over 530 natural or semi-natural (i.e. native pine plantations) forest patches (cork oak *Quercus suber*, holm oak *Q. ilex* subsp. *ballota*

and/or stone pine *Pinus pinea*) scattered throughout the Valley (for further details, see Aparicio 2008; see map in Fig. S1, Supporting information).

Of the c. 160 forest patches in which myrtle is known to occur in this area (Albaladejo *et al.* 2009), seven were selected with myrtle populations of different sizes and degrees of isolation, which were assigned to three ordinal fragmentation categories: 'Large' (two populations), 'Small-connected' (three populations) and 'Small-isolated' (two populations) (Table 1). All patches were surrounded by continuous agricultural habitats with sharp edges. The Large populations (> 2000 individuals) were located in the surroundings of the Doñana National Park, an area characterized by large woodland patches supporting abundant myrtle populations, whereas Small populations ( $\leq 70$  individuals) were scattered throughout the whole Guadalquivir Valley (see Fig. S1, Supporting information). The number of adult myrtle shrubs in each population was estimated by extrapolating myrtle density to the patch area in Large populations and by direct counting in both types of Small populations (see González-Varo 2010, for details). We quantified isolation as the distance to the nearest population (with at least 30 plants more or less clumped together) within a 10-km radius from the focal population based on high-resolution digital cartography of the study area (Aparicio 2008) and detailed on-ground searches (see also R2 cover in Table 1). The distance to the nearest population for the Small-connected populations was 0.8–1.6 km, and > 7 km for the Small-isolated populations. In a previous study, González-Varo, Arroyo & Aparicio (2009) found that the myrtle pollinator assemblage was generally more diverse in large patches than in small-isolated patches, where it was dominated by domestic honeybees *Apis mellifera* because of the prevalence of bee-keeping activities and their strong influence on isolated populations.

#### GENETIC DIVERSITY IN THE ADULT POPULATION

Between 2005 and 2007, 23–30 adult myrtle shrubs were sampled in the field from each of the seven studied populations. Young leaves were collected from individual plants (at least 10 m apart whenever possible) and were immediately refrigerated. Small pieces of leaves were crushed in three drops of extraction buffer and subjected to horizontal starch gel electrophoresis as described by Albaladejo *et al.* (2009) for the same species. In that study, 12 allozyme loci were resolved in 14 populations, although only a mean of 25% were

polymorphic at the 95% ( $P_{95}$ ). We thus focused our study on the four enzyme systems described by Albaladejo *et al.* (2009) as having consistently the most polymorphic loci across the studied populations: the isocitrate dehydrogenase (IDH, EC 1.1.1.42), phosphoglucosomutase (PGM, EC 2.7.5.1), phosphoglucosomerase (PGI, EC 5.3.1.9) and triosephosphate isomerase (TPI, EC 5.3.1.1). Each enzyme system resolved one polymorphic locus and a total of 11 alleles were found: two in each of the *Idh-2* and *Tpi-2* loci, three in the *Pgm-1* locus and four in the *Pgi-2* locus. The four loci were polymorphic in all populations apart from the CRB population, where *Pgm-1* was monomorphic. Overall, multilocus allozyme profiles were generated for 195 adult plants.

For each population, genetic diversity statistics including allelic richness ( $A_r$ ), observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) under Hardy–Weinberg equilibrium were calculated using FSTAT v 2.9.3 (Goudet 2002). Inbreeding coefficient ( $F_{IS}$ ) was computed for each population and its statistical significance was examined through exact tests using GENEPOP 4.0 (Rousset 2008).

#### PROGENY SAMPLING DESIGN

Twenty ripe fruits were collected from throughout the canopy of each of 12 mother plants per population during November 2006 (totalling 84 mother plants and 1680 fruits). During 2006, rainfall in the region was very close to the average (550 mm; <http://www.aemet.es>) and in each population most myrtle plants (> 90%) produced fruits. Fruits were dissected and two seeds from each fruit were randomly separated: one for mating system analyses and the other for the greenhouse experiment (only one seed per fruit for each experiment was sampled to avoid the effects of correlated mating in single pollination events). For each mother plant, the mean seed mass was obtained by weighing 20 seeds from the remaining seed pool ( $n = 1680$  seeds).

#### MATING PATTERNS AND PROGENY GENETIC DIVERSITY

From each of five populations (DBL, CHP, CCL, PTR and CRB) spanning the studied range of population size and isolation (see Table 1), allozyme analysis of progeny arrays was performed to examine mating patterns. Owing to time constraints, we conducted the extractions of seed material from the remaining two populations

**Table 1.** Characteristics of the seven myrtle *Myrtus communis* populations examined in this study

| Type/population | Coordinates<br>(latitude<br>N–longitude W) | Approximate<br>population<br>size | Patch<br>size (ha) | Nearest<br>population<br>(km) | R2 cover<br>(%) | NND<br>(m) | Conspecific<br>density |
|-----------------|--|-----------------------------------|--------------------|-------------------------------|-----------------|------------|------------------------|
| Large           |  |                                   |                    |                               |                 |            |                        |
| DBL             | 37°13.3'–6°18.0'                           | 8900                              | 240                | 1.5                           | 22.5            | 5.1        | 8.8                    |
| CHP             | 37°14.5'–6°17.0'                           | 2100                              | 90                 | 1.5                           | 13.7            | 7.4        | 7.2                    |
| Small-connected |  |                                   |                    |                               |                 |            |                        |
| CCL             | 37°14.0'–5°58.6'                           | 70                                | 44                 | 1.2                           | 14.3            | 11.0       | 1.3                    |
| ALM             | 37°14.9'–6°33.7'                           | 40                                | 10                 | 1.6                           | 10.9            | 9.3        | 5.4                    |
| PTR             | 36°34.2'–6°07.5'                           | 30                                | 2                  | 0.8                           | 15.4            | 7.9        | 5.6                    |
| Small-isolated  |  |                                   |                    |                               |                 |            |                        |
| GBL             | 37°23.3'–6°51.9'                           | 50                                | 32                 | > 7.0                         | 6.1             | 11.7       | 2.1                    |
| CRB             | 37°43.6'–4°55.4'                           | 70                                | 10                 | > 10.0                        | 1.9             | 13.9       | 2.1                    |

'R2 cover' is the woodland cover (%) within a 2-km radius around the patch centroid (obtained using GIS package ArcMap, ESRI Inc., Redlands, California, USA), which can be considered as an inverse estimate of forest fragmentation at a landscape scale (see González-Varo 2010). 'NND' and 'Conspecific density' denote, respectively, the average nearest neighbour distance between individuals in the population and the number of adult myrtles within a 15-m radius (both measured using 16–34 focal individuals per population).

6 months later (autumn 2007) than the other five populations, which were performed in spring 2007. Unexpectedly, germinability (see next) was then negligible and thus, we considered this missed material was due to loss of seed viability after 1 year.

The 20 myrtle seeds per mother plant sampled for this objective were germinated in Petri dishes and germination percentages of well over 90% were obtained. Emergent seedlings were crushed in four drops of extraction buffer and subjected to horizontal starch gel electrophoresis as described by González-Varo, Albaladejo & Aparicio (2009). The same four polymorphic loci used for genotyping the adult plants were also tested, although one enzyme system (TPI) did not resolve any loci with the seedling extracts. Fortunately, an alternative enzymatic system (uridine diphosphoglucose pyrophosphorilase: UGPP, EC 2.7.7.9) not tested by Albaladejo *et al.* (2009) was found to resolve one polymorphic locus (see González-Varo, Albaladejo & Aparicio 2009). Thus, four loci were also resolved in the progenies with a total of 11 alleles: two in each of the *Idh-2* and *Pgm-1* loci, three in the *Ugpp-2* locus, and four in the *Pgi-2* locus. Overall, 710 seedlings were sampled (8–19 per mother plant; mean  $\pm$  SD = 13.7  $\pm$  2.4) from 9 to 12 mother plants per population and multilocus allozyme profiles were successfully generated for 701; some families (i.e. seeds belonging to the same mother plant) and seedlings were lost by fungal infection.

Mating patterns in the five populations were characterized by multilocus outcrossing rates ( $t_m$ ), biparental inbreeding ( $t_m - t_s$ ; being  $t_s$  the average of single locus outcrossing rates) and correlated paternity ( $r_p$ ), defined as the proportion of progeny sired by the same father. Mating variables ( $t_m$ ,  $t_m - t_s$  and  $r_p$ ) were estimated at population level using MLTR v 3.3 (Ritland 2002). The Newton–Raphson algorithm was used as very little data was missing from our data set (<1.3%). The errors for the estimates were obtained by bootstrap methods (setting 1000 replicates), with families as the resampling unit.

Genetic diversity parameters in progenies were estimated with FSTAT v 2.9.3 (Goudet 2002) by averaging values obtained for each group of seedlings from the same maternal plant (i.e. family). These estimates are particularly useful for comparisons between populations of progenies; however, they are not directly comparable with those from adult populations as all seedlings from each mother plant are at least half-sibs, and also because only three common loci (of four) were analysed in both generations (adults and progeny).

#### PROGENY PERFORMANCE: GREENHOUSE EXPERIMENT

Progeny performance, measured as germination, growth and survival, was evaluated in a greenhouse experiment by sowing seeds from the seven study populations. In December 2006, a total of 1680 seeds (7 populations  $\times$  12 mother plants per population  $\times$  20 seeds per mother) were individually planted in trays of 60 pots (5  $\times$  5 cm and 17 cm depth). Each pot was filled with horticultural mixture (peat and perlite *c.* 5 : 1) and the seeds sowed at a depth of *c.* 0.5 cm. Trays were watered twice a week and their position randomly changed throughout the greenhouse every 2–3 weeks until the end of the experiment. Seed germination and seedling mortality were monitored weekly until day 105 after sowing and fortnightly thereafter. For each seedling, the number of days between sowing and emergence was noted (hereafter ‘emergence time’) as an inverse estimate of emergence speed. Seedling heights were measured on days 75, 110, 185, 280 and 406 after the start of the experiment and were used as seedling size estimates throughout the experiment. At the end of the experiment (February 2008), the seedlings were harvested and dried at 60 °C for 72 h; root and shoot dry biomass

were then measured separately, providing accurate measurements of seedling size.

Some seedlings grew anomalously (hereafter, ‘anomalous seedlings’), and were shorter and bore smaller leaves than normal seedlings (see also Traveset, Riera & Mas 2001). At the end of the experiment, these seedlings were 3.7-fold smaller (mean dry wt = 0.66 g,  $n = 40$ ) than normal seedlings (mean = 2.42 g,  $n = 998$ ) (Student’s  $t = 9.8$ ,  $P < 0.001$ , d.f. = 1036). These seedlings were included in the seed germination and seedling survival analyses, but excluded from all growth analyses because it is reasonable to expect that anomalous seedlings have much lower chances of survival in the wild. The ‘final seedling production’ was calculated as the percentage of seeds that generated normal seedlings that survived until the end of the experiment, i.e. final seedling production = cumulative germination – (mortality + anomalous growth); thus, this variable combines germination, survival and viability (i.e. normal growth).

#### STATISTICAL ANALYSES

To test for differences in progeny performance between and within population types (Large, Small-connected and Small-isolated), general and GLMM were fitted with SAS 9.2 (procedures MIXED and GLIMMIX, respectively; Littell *et al.* 2006). Population type (main factor) and population identity (nested factor) were included in the models as fixed factors. Mother plant identity was included in the models as a random factor to account for the effect of testing seeds and seedlings from the same mother plant (Bolker *et al.* 2009). Satterthwaite’s approximation method was used to estimate the degrees of freedom of the models and thus to identify the denominator of the  $F$ -tests (Littell *et al.* 2006). Binomial distribution and logit link function (GLIMMIX models) were used for modelling percentages (germination, anomalous growth, seedling mortality and final seedling production) and normal distribution and identity link function (MIXED models) for modelling emergence time, height and dry biomass of seedlings. The effects of ‘seed mass’ on the measured variables (germination, mortality and growth) and the effects ‘emergence time’ on the growth variables (height and dry biomass) were checked for by including these parameters as continuous covariates in the models. No collinearity was observed between the covariates (Pearson’s  $r = -0.027$ ,  $P = 0.38$ ,  $n = 1081$  seedlings). For the MIXED models, a covariate was included if, according to the Akaike’s information criterion (AIC), the covariate improved the model fit (i.e.  $AIC_{\text{with covariate}} < AIC_{\text{without covariate}}$ ). Before fitting GLIMMIX models, significant effects between the covariates and the response variables were tested for by performing simple logistic regressions, and the covariates were included in the final models if a significant ( $P < 0.05$ ) effect was found.

Given the low number of populations studied, the nonparametric Kendall’s rank correlation coefficient ( $\tau$ ) was used to test for associations between genetic diversity, mating patterns and progeny performance.

## Results

#### POPULATION GENETIC DIVERSITY AND MATING PATTERNS

Although genetic diversity parameters varied within each population type (see Table 2), they were in general higher in Large populations (mean values:  $A_r = 2.4$ ,  $H_o = 0.39$ ,  $H_e = 0.38$ )

**Table 2.** Genetic diversity of adult plants for the seven myrtle *Myrtus communis* populations examined in this study, and mating system estimates and the genetic diversity of progenies in five out of these seven populations

| Type/population | Genetic diversity of adults |           |             |             |                 | Genetic diversity of progeny* |                        |           |             |             | Mating system |             |              |
|-----------------|-----------------------------|-----------|-------------|-------------|-----------------|-------------------------------|------------------------|-----------|-------------|-------------|---------------|-------------|--------------|
|                 | $n_{\text{plants}}$         | $A_r$     | $H_o$       | $H_e$       | $F_{\text{IS}}$ | $n_{\text{families}}$         | $n_{\text{seedlings}}$ | $A_r$     | $H_o$       | $H_e$       | $t_m$         | $t_m - t_s$ | $r_p$        |
| Large           |                             |           |             |             |                 |                               |                        |           |             |             |               |             |              |
| DBL             | 30                          | 2.6 (0.9) | 0.36 (0.14) | 0.37 (0.14) | 0.032           | 12                            | 178                    | 1.7 (0.4) | 0.28 (0.22) | 0.23 (0.17) | 0.23 (0.07)   | 0.03 (0.01) | 0.17 (0.16)  |
| CHP             | 30                          | 2.3 (0.5) | 0.41 (0.15) | 0.39 (0.12) | -0.052          | 10                            | 163                    | 2.0 (0.5) | 0.30 (0.19) | 0.28 (0.19) | 0.46 (0.11)   | 0.01 (0.02) | 0.05 (0.10)  |
| Small-connected |                             |           |             |             |                 |                               |                        |           |             |             |               |             |              |
| CCL             | 30                          | 2.2 (0.5) | 0.30 (0.18) | 0.28 (0.16) | -0.069          | 9                             | 108                    | 1.9 (0.3) | 0.25 (0.15) | 0.27 (0.16) | 0.51 (0.12)   | 0.04 (0.02) | -0.09 (0.08) |
| ALM             | 24                          | 2.1 (1.0) | 0.26 (0.20) | 0.28 (0.23) | 0.061           | -                             | -                      | -         | -           | -           | -             | -           | -            |
| PTR             | 23                          | 2.3 (0.5) | 0.36 (0.18) | 0.40 (0.11) | 0.092           | 11                            | 134                    | 2.0 (0.5) | 0.35 (0.20) | 0.34 (0.17) | 0.72 (0.12)   | 0.06 (0.02) | 0.06 (0.08)  |
| Small-isolated  |                             |           |             |             |                 |                               |                        |           |             |             |               |             |              |
| GBL             | 30                          | 2.4 (1.0) | 0.41 (0.26) | 0.37 (0.25) | -0.103          | -                             | -                      | -         | -           | -           | -             | -           | -            |
| CRB             | 28                          | 1.8 (0.5) | 0.29 (0.19) | 0.30 (0.22) | 0.029           | 10                            | 118                    | 1.5 (0.4) | 0.20 (0.15) | 0.19 (0.14) | 0.13 (0.10)   | 0.02 (0.05) | 0.19 (0.42)  |

Mean values ( $\pm$ SD) are reported.  $A_r$ , allelic richness;  $H_o$ , observed heterozygosity;  $H_e$ , gene diversity;  $F_{\text{IS}}$ , inbreeding coefficient;  $t_m$ , multilocus outcrossing rates;  $t_m - t_s$ , biparental inbreeding;  $r_p$ , correlated paternity.

\*Estimated by averaging values obtained for families, i.e. seedlings from the same mother plant (see 'Materials and methods').

than in both types of Small populations (Small-connected:  $A_r = 2.2$ ,  $H_o = 0.31$ ,  $H_e = 0.32$ ; Small-isolated:  $A_r = 2.1$ ,  $H_o = 0.35$ ,  $H_e = 0.33$ ), which had similar average values; however, mean  $H_o$  was higher in Small-isolated than in Small-connected populations. Inbreeding coefficients ( $F_{\text{IS}}$ ) were low, ranging from -0.103 (GBL) to 0.092 (PTR), and not significantly different from zero either in any particular population or overall ( $P > 0.25$  for all populations).

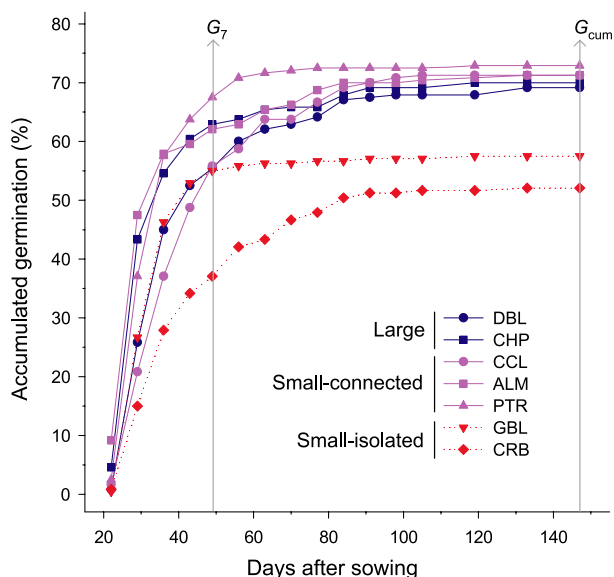
Outcrossing rates ( $t_m$ ) varied enormously across the five populations examined, ranging from predominantly selfed ( $t_m = 0.13$  in CRB) to predominantly outcrossed ( $t_m = 0.72$  in PTR; Table 2). Between population types, outcrossing rates were higher for the Small-connected populations (mean:  $t_m = 0.62$ ), intermediate for the Large populations (mean:  $t_m = 0.35$ ), and lower for the Small-isolated population CRB ( $t_m = 0.13$ ). All differences in the  $t_m$  between population pairs were significant (i.e. non-overlapping 95% CI). Biparental inbreeding ( $t_m - t_s$ ) was in general low across all the populations (range = 0.01–0.06). Correlated paternity ( $r_p$ ) ranged between -0.09 and 0.19 and was higher for the populations showing the lowest  $t_m$  (Table 2).

Parameters of genetic diversity ( $A_r$ ,  $H_o$  and  $H_e$ ) in progenies were slightly lower than in the adult population (Table 2). It is worth noting that the population outcrossing rate was positively correlated with  $A_r$  and  $H_e$  in progenies (Kendall's  $\tau = 0.80$ ,  $P = 0.05$ ,  $n = 5$  in both cases) and so the ranking of genetic diversity of progenies between population types was Small-connected ( $A_r = 2.0$ ,  $H_o = 0.30$ ,  $H_e = 0.31$ )  $\geq$  Large ( $A_r = 1.9$ ,  $H_o = 0.29$ ,  $H_e = 0.26$ )  $>$  Small-isolated ( $A_r = 1.5$ ,  $H_o = 0.20$ ,  $H_e = 0.19$ ). The same patterns were found when we estimated genetic parameters in adults and progeny using only the three common loci analysed in both generations (results not shown).

## PROGENY PERFORMANCE

### Germination, anomalous growth and survival

Seed germination varied between populations either in rate or in final percentage (Fig. 1). Emergence time ranged from 34.3 (GBL) to 44.4 (CRB) days after sowing, and differed significantly between populations but not between population types (Table 3, Table S1, Supporting information). Besides the cumulative germination (hereafter,  $G_{\text{cum}}$ ), the variation in the percentage of germination was also tested at 7 weeks after sowing (hereafter,  $G_7$ ), which was half the amount of time before  $> 95\%$  of the final percentage of germination was reached in all populations (Fig. 1).  $G_7$  ranged from 37.1% to 67.5% and  $G_{\text{cum}}$  from 52.5% to 73.3% (both at CRB and PTR, respectively) and only differed significantly between population types (Table 3, Fig. 2a). The germination percentage was higher in Small-connected populations (mean values:  $G_7 = 61.8\%$ ,  $G_{\text{cum}} = 72.2\%$ ), intermediate in Large ( $G_7 = 59.2\%$ ,  $G_{\text{cum}} = 69.6\%$ ) and lower in Small-isolated populations ( $G_7 = 46.0\%$ ,  $G_{\text{cum}} = 55.0\%$ ; Fig. 2a). The percentage of anomalous seedlings ranged from 1.0% (PTR) to 6.9% (CRB) and seedling mortality ranged from 2.4% (ALM) to 10.0% (CRB)



**Fig. 1.** Patterns in the accumulative percentage of myrtle *Myrtus communis* seeds germinating in the greenhouse of the seven populations studied (two Large, three Small-connected and two Small-isolated populations). The grey arrows indicate population values of 'germination after 7 weeks' ( $G_7$ ) and 'cumulative germination' ( $G_{cum}$ ).

(Fig. 2b). The mean percentages of anomalous seedlings and seedling mortality were in general lower in Small-connected populations (5.6% and 2.9%, respectively) than in both Large (7.7% and 4.6%) and Small-isolated populations (8.0% and

4.3%; Fig. 2b). However, these two variables did not differ either between or within population types (Table 3), probably because of the high levels of variability within each population (Fig. 2b). Final seedling production ranged between 43.3% (CRB) and 68.3% (PTR), and varied significantly only between population types, with Small-connected populations having the highest (66.9%), Large populations intermediate (61.9%) and Small-isolated populations the lowest (48.3%) success in seedling production (Fig. 2a). Seed mass had positive but non-significant effects on the germination percentage ( $G_7$  and  $G_{cum}$ ) and final seedling production. Seed mass (Table S1, Supporting information) differed significantly among populations (MIXED model:  $F_{4,56} = 2.91$ ,  $P = 0.03$ ), but not between population types ( $F_{2,56} = 1.59$ ,  $P = 0.21$ ).

### Seedling growth

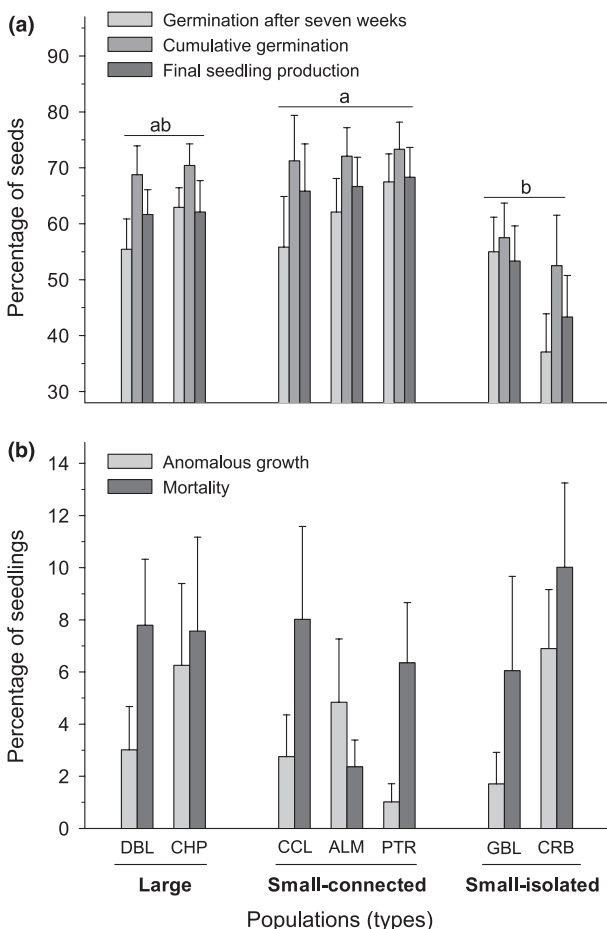
Seedling height varied significantly only between populations types the first time measurement were taken (i.e. 'after 75 days') (Table 3); mean height was higher for Small-connected (26.4 mm), intermediate for Large (24.2 mm) and lower for Small-isolated populations (23.0 mm). Variation in seedling height among populations (within types) was significant after 75 and 110 days, marginally significant ( $P < 0.10$ ) after 185 days and non-significant ( $P > 0.10$ ) thereafter (Table 3, Fig. 3a), indicating that variation in seedling height was higher during the earliest growth stages (Table 3). Whereas seed mass had positive effects (significant only 75 days after sowing) on seedling height, emergence time had negative effects

**Table 3.** Results of the general and GLMM examining the effect of population type and population nested within types on several fitness parameters measured in the progenies of seven fragmented myrtle *Myrtus communis* populations ( $P$ -values  $< 0.05$  are shown in bold type and  $< 0.1$  in italics)

| Fitness measurements                              | Population type |      |              | Population (within type) |      |              | Covariates in the models |
|---|-----------------|------|--------------|--------------------------|------|--------------|--------------------------|
|   | d.f.            | $F$  | $P$          | d.f.                     | $F$  | $P$          |                          |
| <b>Germination, anomalous growth and survival</b> |                 |      |              |                          |      |              |                          |
| Germination after 7 weeks                         | 2, 63           | 3.97 | <b>0.024</b> | 4, 63                    | 1.45 | 0.227        | Sm                       |
| Cumulative germination                            | 2, 66           | 4.17 | <b>0.020</b> | 4, 67                    | 0.06 | 0.993        | Sm                       |
| Emergence time                                    | 2, 72           | 0.22 | 0.801        | 4, 70                    | 5.27 | <b>0.001</b> | Sm                       |
| Anomalous growth                                  | 2, 106          | 0.71 | 0.494        | 4, 104                   | 1.47 | 0.215        |                          |
| Mortality   | 2, 88           | 1.09 | 0.341        | 4, 93                    | 0.75 | 0.562        |                          |
| Final seedling production*                        | 2, 66           | 4.70 | <b>0.012</b> | 4, 67                    | 0.24 | 0.914        | Sm                       |
| <b>Seedling height</b>                            |                 |      |              |                          |      |              |                          |
| After 75 days                                     | 2, 71           | 6.21 | <b>0.003</b> | 4, 71                    | 3.03 | <b>0.023</b> | <b>Sm, Et</b>            |
| After 110 days                                    | 2, 75           | 0.14 | 0.868        | 4, 74                    | 3.12 | <b>0.020</b> | <b>Sm, Et</b>            |
| After 185 days                                    | 2, 74           | 0.00 | 0.997        | 4, 73                    | 2.30 | <i>0.067</i> | <b>Sm, Et</b>            |
| After 280 days                                    | 2, 72           | 0.91 | 0.409        | 4, 72                    | 1.77 | 0.145        | <b>Sm, Et</b>            |
| After 406 days                                    | 2, 73           | 1.41 | 0.251        | 4, 72                    | 1.54 | 0.201        | <b>Sm, Et</b>            |
| <b>Seedling dry biomass</b>                       |                 |      |              |                          |      |              |                          |
| Shoot dry wt                                      | 2, 75           | 0.51 | 0.600        | 4, 75                    | 1.97 | 0.108        | <b>Et</b>                |
| Root dry wt                                       | 2, 72           | 0.51 | 0.606        | 4, 72                    | 0.49 | 0.744        | <b>Et</b>                |
| Total seedling dry wt                             | 2, 74           | 0.02 | 0.981        | 4, 74                    | 1.13 | 0.349        | <b>Et</b>                |

All models included the mother plant identity as a categorical random factor. Seed mass and/or emergence time were included as covariates in some models (see text for further details), and their acronyms ('Sm' and 'Et', respectively) are shown in bold when they produced significant ( $P < 0.05$ ) effects on the response variable in the model.

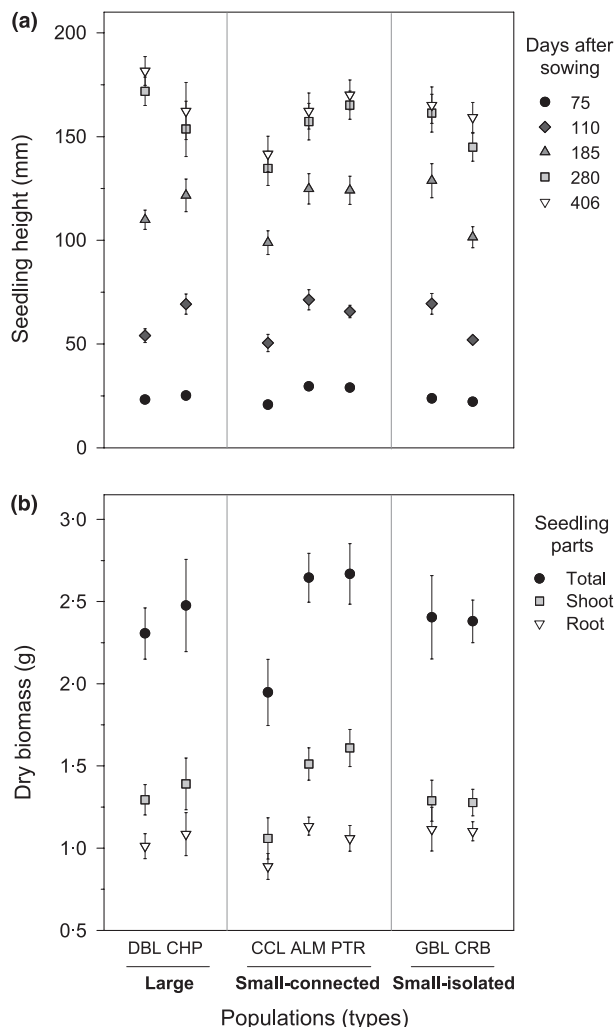
\*Final seedling production = cumulative germination - (anomalous seedlings + mortality).



**Fig. 2.** (a) Percentage (mean  $\pm$  SE) of seed germination (after 7 weeks and cumulative) and percentage of seeds that produced normal seedlings that survived until the end of the experiment (i.e. final seedling production = cumulative germination – [anomalous growth – mortality]); and, (b) percentage of anomalous growth and mortality among the germinated seedlings in the seven myrtle *Myrtus communis* populations studied. Contrasts (differences of least squares means) among types of Large, Small-connected and Small-isolated populations for seed germination percentages (after 7 weeks and cumulative) and final seedling production are shown above the bars; different letters indicate the types that differed ( $P > 0.05$ ).

(significant in all models), indicating that early emerged seedlings were taller (see Table 3). Indeed, at the population level, emergence time was significantly correlated with seedling height after 110 and 185 days (Kendall's  $\tau = -0.71$ ,  $P = 0.02$ ,  $n = 7$ ), but not thereafter ( $P > 0.25$ ).

Differences in seedling dry biomass (total, shoot and root) were not significant either between or within population types (Table 3; see Fig. 3b). It was, however, remarkable that total seedling dry biomass in the Small-connected population CCL was much lower (20–38% less) than in the other populations, as was the shoot biomass (21–52% less). Mean total seedling biomass ranged from 1.95 g (CCL) to 2.67 g (PTR), mean shoot biomass from 1.06 g (CCL) to 1.61 g (PTR) and mean root biomass from 0.89 g (CCL) to 1.13 g (ALM). Unexpectedly, seedling dry biomass was not correlated with the final seedling height (after 406 days) at population level (Kendall's



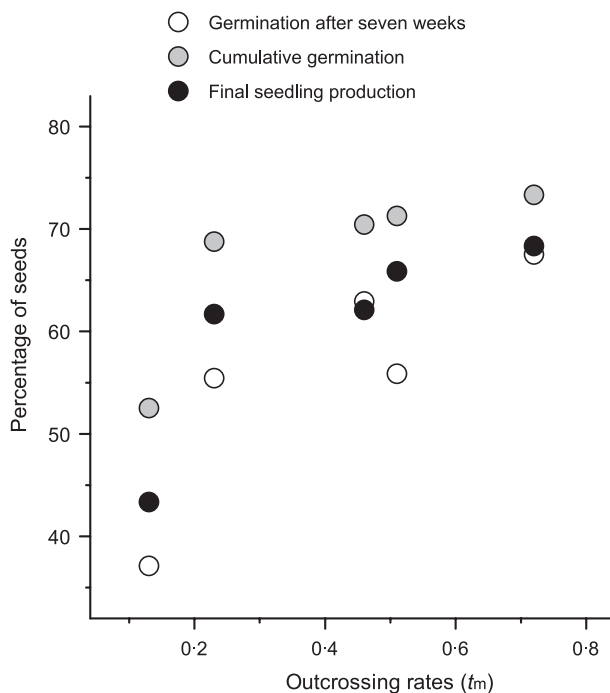
**Fig. 3.** Seedling growth measurements (mean  $\pm$  SE) of the seven populations (two Large, three Small-connected and two Small-Isolated) of myrtle *Myrtus communis* tested in the greenhouse. (a) Seedling height at days 75, 110, 185, 280 and 406 after sowing; and (b) dry biomass (total, shoot and root) at the end of the experiment.

$\tau = 0.33$ ,  $P = 0.3$ ,  $n = 7$ ), probably because of the high heterogeneity in the degree of branching in seedlings. Emergence time had significant negative effects on seedling biomass (total, shoot and root; Table 3), reflecting the fact that early emerged seedlings were larger.

The results for seedling growth (height and biomass) were the same after correcting for emergence time (i.e. growth rates instead of absolute values), and emergence time also had significant effects in all models (see Table S2 and Fig. S2, Supporting information).

#### INFLUENCES OF POPULATION GENETIC DIVERSITY AND MATING PATTERNS ON PROGENY PERFORMANCE

We found significant effects of mating patterns and adult genetic diversity on some progeny performance measurements. Population outcrossing rates ( $t_m$ ) were significantly positively associated with germination after 7 weeks ( $G_7$ ; Kendall's



**Fig. 4.** Relationships between the outcrossing rate and the percentage of seed germination after 7 weeks, cumulative germination and final seedling production (i.e. cumulative germination minus anomalous and dead seedlings) in five fragmented myrtle *Myrtus communis* populations.

$\tau = 0.80$ ,  $P = 0.05$ ,  $n = 5$ ), cumulative germination ( $G_{cum}$ :  $\tau = 1.0$ ,  $P = 0.014$ ,  $n = 5$ ) and final seedling production ( $\tau = 1.0$ ,  $P = 0.014$ ,  $n = 5$ ; see Fig. 4), and negatively associated with the percentage of anomalous seedlings ( $\tau = -0.80$ ,  $P = 0.05$ ,  $n = 5$ ). Among the genetic diversity parameters in the adult populations, only expected heterozygosity ( $H_e$ ) was positively associated with seedlings' shoot biomass ( $\tau = 0.62$ ,  $P = 0.05$ ,  $n = 7$ ).

## Discussion

Habitat fragmentation can have negative effects on genetic diversity and mating patterns of plant populations (Aguilar *et al.* 2008) and thus on offspring fitness as a result of inbreeding depression (Cascante *et al.* 2002). One of the most notable results of this study is that the performance and genetic diversity of progenies in severely fragmented *Myrtus communis* populations depends much more on contemporary mating patterns (outcrossing rates) than on genetic diversity in adult plants.

### GENETIC DIVERSITY AND MATING PATTERNS

In general, we found that Large populations exhibited higher levels of genetic diversity than both types of Small populations. However, inbreeding coefficients ( $F_{IS}$ ) were very similar between populations and non-significantly different from zero. These results are consistent with those reported by Albaladejo

*et al.* (2009), who found positive significant associations between population size and genetic diversity ( $A$  and  $H_e$ ) in 14 myrtle populations in the same study area, but non-significant correlations with  $F_{IS}$ .

We found huge mating pattern variation between the myrtle populations. In particular, the outcrossing rate varied among populations from predominantly selfed ( $t_m = 0.13$ ) to mostly outcrossed ( $t_m = 0.72$ ), a range as broad as the variation between species typically considered as having mixed-mating systems ( $0.2 < t < 0.8$ ; see Goodwillie, Kalisz & Eckert 2005). Unexpectedly, the highest outcrossing rates were not found in Large populations, but in Small-connected populations ( $t_m$  in Small-connected > Large > Small-isolated populations). Notably, high-outcrossing rates translated into enhanced genetic diversity in the progeny, which was ranked between population types in the same order as population outcrossing rates.

The spatial arrangement of relatives (conspecific plant density and nearest neighbour distances) and the pollinator assemblage (composition, abundance and behaviour) are considered as the main ecological factors influencing mating patterns in animal-pollinated plants (Franceschinelli & Bawa 2000; England *et al.* 2001; González-Varo, Albaladejo & Aparicio 2009). Since the highest outcrossing rates did not occur in Large populations, where mate abundance was the highest (Table 1), it could be argued that differences in outcrossing rates among the studied populations were influenced by dissimilarities in pollinator assemblages. Indeed, in another study that included five of the myrtle populations used in this study, we found that the myrtle pollinator assemblage is nearly monopolized (i.e. high-visitation rates and near absence of other species) by domestic honeybees *A. mellifera* L. in the Small-isolated populations GBL and CRB because of beekeeping in the immediate vicinity of the patches (see González-Varo, Arroyo & Aparicio 2009). Honeybee hives can also be found in Large populations (DBL and CHP), although the higher flower abundance in these woodlands seems to strongly dilute their effects (González-Varo, Arroyo & Aparicio 2009), a process that could also occur in Small-connected patches at a landscape scale (see R2 cover in Table 1; see also Steffan-Dewenter *et al.* 2002). Introduced honeybees can reduce pollen limitation and improve fruit production in myrtle (González-Varo, Arroyo & Aparicio 2009) and other self-compatible plants through high flower visitation rates (reviewed in Goulson 2003). However, they are regarded as selfing promoters as they tend to forage on many flowers on the same plant (England *et al.* 2001), and this could explain the high level of selfing (87%) found in CRB, a honeybee-dominated population. González-Varo, Arroyo & Aparicio (2009) also found substantial variation between populations in the frequency of visits by medium- and large-sized wild bees (genera *Amegilla*, *Bombus*, *Megachile* and *Pseudapis*) as opposed to small-sized wild bees (genera *Ceratina*, *Lasioglossum* and *Nomioides*). For example, large bees (excluding honeybees) accounted for 45% of visits in PTR ( $t_m = 0.72$ ), but only 13% in DBL ( $t_m = 0.23$ ), where both small bees and flies accounted for 51% of visits. Such

between-population differences in pollinator assemblages imply the existence of variation in spatial foraging behaviour and pollen load capacity (Herrera 1987), two factors of major importance in determining mating patterns in plant populations (England *et al.* 2001).

#### PROGENY PERFORMANCE: PAST INHERITANCE OR CONTEMPORARY ACQUISITION?

Myrtle population types differed in germination success (early and cumulative) and thus in final seedling production, with higher values being found in Small-connected, intermediate values in Large and lower values in Small-isolated populations (Fig. 2a). Moreover, seedling loss because of both anomalous growth and seedling mortality (from 7% in ALM and PTR to 17% in CRB) accounted for important differences between germination and final seedling production in each population. Interestingly, such quantitative variation either in production or the loss of seedlings did not depend on the genetic diversity of the adult plants but on the outcrossing rates, that is, on the contemporary mating patterns of the populations: the greater the outcrossing rate, the higher the germination rate and the lower the percentage of anomalous seedlings (Fig. 4). This finding is congruent with the differences found in the percentage of germination between myrtle progenies obtained from controlled self- and cross-pollinations (52% and 79%, respectively; J. P. González-Varo and A. Traveset, unpubl. data), which may be caused by the expression of lethal deleterious recessive alleles occurring in homozygosis (Husband & Schemske 1996; Charlesworth & Charlesworth 1999). Furthermore, this result agrees with other studies that have reported positive relationships between germination percentages and outcrossing rates in fragmented populations of other plant species (Cascante *et al.* 2002), regardless of population sizes (Mathiasen, Rovere & Premoli 2007).

Along with the final germination percentage, the timing of seed germination may also have important consequences on the real opportunities for seed germination in the wild (Verdú & Traveset 2005), especially in light of the unpredictable rainfall that characterizes the Mediterranean climate. A clear example is the Small-isolated population CRB, which showed not only the lowest, but also the slowest germination rate (see Fig. 1, Table S1, Supporting information). However, emergence time (inverse of early emergence) was related neither to the adult genetic diversity nor to the outcrossing rates. Early emergence can also favour fitness in other ways and, for instance, allows myrtle seedlings to achieve a proper size before having to cope with drought stress in the summer season (Verdú & Traveset 2005). This idea is supported by (i) the negative significant effects of 'emergence time' on all seedling growth measurements (height and biomass) and by the fact that (ii) seedling height varied significantly only between and/or within population types in the earliest measurements, but not thereafter. The trend towards more similar-sized seedlings in populations over time (but see dry wt in CCL) was probably favoured by space limitation in the pots and/or the optimal growth conditions in the greenhouse (see Traveset, Riera & Mas 2001).

Finally, we found a positive correlation between gene diversity ( $H_e$ ) in the adult populations and seedling shoot biomass. This relationship might be explained by the accumulation of mildly deleterious alleles, which can be expressed at life stages, such as seedling growth (Husband & Schemske 1996; Charlesworth & Charlesworth 1999), in populations historically suffering the influence of genetic drift and inbreeding (i.e. low  $H_e$ ). Probably, complex interactive effects between historical and contemporary genetic processes are shaping progeny performance in long-term fragmented myrtle populations. However, taken as a whole, our results clearly show that outcrossing rates were much more decisive for progeny performance than the genetic diversity of adult plants, particularly in terms of the production of viable seedlings. Our study correspond to a single progeny generation and its early stages (and thus should be regarded with caution); nevertheless, it reveals the mechanism (mating between plants, which is indirectly dependent on fragmentation) that may greatly affect the future of the populations.

#### IMPLICATIONS FOR MANAGEMENT

This is one of the few studies evaluating the genetic effects of fragmentation on plant progeny performance. We found that genetic diversity and several fitness measurements of myrtle progenies were positively correlated with outcrossing rates, independently of either population size or adult genetic diversity. We thus conclude that contemporary mating patterns are more critical for progeny performance than population fragmentation or initial levels of genetic diversity. The implication for management is that it may be possible to enhance either fitness or certain levels of genetic diversity in progenies by promoting outcrossed matings in fragmented populations of self-compatible plants. Evidence from this and a companion study (González-Varo, Arroyo & Aparicio 2009) suggest that, at least in our study species, this might be possible by controlling beekeeping in the immediate surroundings of small-isolated patches and favouring the abundance of certain wild pollinator species (i.e. increasing species-specific resource requirements; see Potts *et al.* 2005). Our results also suggest that a diverse pollinator assemblage can be preserved, whereas the effects of beekeeping can be diluted, by maintaining and/or enhancing landscape connectivity, particularly around small patches (see Steffan-Dewenter *et al.* 2002). It is expected that such actions would benefit other Mediterranean shrubland species besides myrtle.

The study area is an important source of honey, pollen and beeswax production within the region (Andalusia) and the country (Spain), which ranks first among European producers; it is noteworthy that more than 500 000 hives were legally settled in 2006 in Andalusia, more than 90% of which were transient (Anonymous 2006). Our studies indicate that the presence of hives nearby small-isolated patches may have detrimental effects on the local pollinator assemblage and, through honeybee pollination, on the genetic structure and vigour of plant progenies. These findings highlight the need, on the basis of landscape connectivity, for managers to restrict beekeeping

locations to sites that minimize their impact on remnant patches.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Map showing the study area and the location of the study populations.

**Fig. S2.** Seedling growth rate measurements (height and biomass) in the seven myrtle *Myrtus communis* populations tested in the greenhouse.

**Table S1.** Seed mass and emergence time for each of the seven myrtle *Myrtus communis* populations studied.

**Table S2.** Results of the general linear mixed models examining growth rate (height and biomass) parameters.

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