diversity, reproductive success and progeny fitness (e.g. Breed *et al.* 2012a, 2012b; Jump and Peñuelas 2006; Knapp *et al.* 2001; Knight *et al.* 2005; Lowe *et al.* 2005; Ortego *et al.* 2010; Wang *et al.* 2011). The study of pollen dispersal and mating patterns has been the focus of many studies aimed to anticipate the consequences of fragmentation and low population densities in long-lived tree species for which the genetic signals of population declines are likely to require several generations to appear (e.g. Albaladejo *et al.* 2012; Bacles and Ennos 2008; Bacles *et al.* 2005; Craft and Ashley 2010; Dow and Ashley 1998; Sork *et al.* 2002). Some of these studies have found disrupted mating patterns in small or isolated tree populations (e.g. Breed *et al.* 2012a, 2012b; Rosas *et al.* 2011; Sork *et al.* 2002). However, many other studies have revealed similar or even increased pollen flow in fragmented or low tree density stands than in continuous forest or high-density populations (e.g. Bacles and Ennos 2008; Bacles *et al.* 2005; Breed *et al.* 2013; Craft and Ashley 2010; Jha and Dick 2010; Mehes *et al.* 2009; Mimura *et al.* 2009; see also Hamrick 2004; Kramer *et al.* 2008; Kremer *et al.* 2012). Thus, conclusions about the consequences of fragmentation on contemporary patterns of pollen flow are varied, probably because different pollination vectors (e.g. wind or animals), mating systems (e.g. self-compatible vs. self-incompatible), spatial distribution of remnant fragments or plants and local climates can lead to different responses of pollen flow and mating patterns to habitat fragmentation (Albaladejo *et al.* 2009; Breed *et al.* 2012a, 2012b; Knapp *et al.* 2001). For this reason, information on more species with different pollen dispersal vectors, mating systems and contrasting patterns of population fragmentation and conspecific density can help to provide a more comprehensive view of forest fragmentation genetics (Kramer *et al.* 2008).

Fragmentation and reduced conspecific density can also have important impacts on the reproductive performance of trees (Knapp *et al.* 2001). Several studies have found that low tree densities reduce the efficiency of pollination vectors, which can decrease seed set rates and production (e.g. Allison 1990; Holm 1994; Knapp *et al.* 2001; Smith *et al.* 1988) and increase the proportion of self-pollinated seeds (e.g. Breed *et al.* 2012a; Perry and Knowles 1990). However, despite the potential negative consequences of fragmentation and low population densities on reproductive performance are likely to be closely linked to the disruption of pollen flow and mating patterns, not many studies have simultaneously analysed these parameters in tree populations (e.g. Mimura *et al.* 2009; see also Breed *et al.* 2012a for progeny fitness).

Holm oak (*Quercus ilex* L.) is a monoecious wind-pollinated evergreen tree distributed across a vast area in the Western Mediterranean basin where it is the dominating and most widespread woody species (Blanco *et al.* 1997; Lumaret *et al.* 2002). Apart from its key role in Mediterranean ecosystem functioning, this species had a considerable economic importance in the past and acorns are still a valuable resource for livestock raising (Blanco *et al.* 1997; Blondel and Aronson 1999). Continuous forest are still present in many parts of the species range, but large-scale deforestation has often resulted in areas where this species is exclusively represented by a few remnant and isolated trees within extensively farmed fields (Blondel and Aronson 1999; Ortego *et al.* 2010; Vicente and Ales 2006). Previous studies on this species have analysed patterns of genetic structure at different spatiotemporal scales (Coelho *et al.* 2006; de Heredia *et al.* 2007; Lumaret *et al.* 2002; Michaud *et al.* 1992, 1995; Ortego *et al.* 2010; Soto *et al.* 2007). The patterns of seed dispersal have been also studied in holm oaks, but there is no available information on contemporary patterns of pollen flow in this species (e.g. Muñoz and Bonal 2007, 2011). Moreover, it remains unknown how mating patterns and reproductive performance are affected by chronic forest fragmentation and reduced tree densities, despite we found previous evidence that long-term forest fragmentation could be contributing to reduce genetic variability in this species (Ortego *et al.* 2010). Hence, the simultaneous study of mating patterns, pollen movement and reproductive performance could shed further light into the ecological and genetic consequences of forest fragmentation and reduced tree densities in this Mediterranean keystone species.

Here, we combine extensive paternity analyses and data on seed set and production to study the impact of reduced tree density on pollen flow, mating patterns and reproductive success in the holm oak. For this purpose, we compare a highly fragmented stand showing extremely low tree densities with a nearby stand with high conspecific density. If disrupted mating patterns and limited pollen movement is behind the reduced genetic diversity of younger cohorts previously reported in a highly fragmented and low tree density stand (Ortego *et al.* 2010), then (i) we predict that pollen movement within the low-density stand is limited by distance, (ii) we expect that pollen from a few number of local males surrounding maternal trees is involved in most paternities in the low-density stand in comparison with the high-density stand and (iii) we predict higher selfing rates and biparental inbreeding and lower progeny genetic diversity and outcrossing rates in the low-density stand than in the high-density stand. On the other hand, we (iv) expect that reproductive performance (estimated as seed set rates and seed production) is lower in the low-density stand than in the high-density stand due to limited foreign pollen availability in the former.

MATERIALS AND METHODS

Study area and plant material

The study area is located in Huecas, Toledo province, Central Spain (39°59′N, 4°13′W; see Ortego *et al.* 2010 and Bonal *et al.* 2012 for a detailed description). The low tree density holm oak stand (~1418 ha; ~0.02 trees/ha) is located in a cultivated area where some isolated remnant trees or clusters of trees grow within the agriculture matrix (Ortego *et al.* 2010). The agriculture matrix consists of extensive crops, mainly barley (*Hordeum vulgare*) and wheat (*Triticum* spp.), while vineyards

(*Vitis vinifera*) and olive groves (*Olea europaea*) are also present to a lesser extent. We collected leaves from all individuals within this area (n = 24), including two highly isolated trees located in the easternmost part of the stand that were not detected during a previous study (Fig. 1) (Ortego *et al.* 2010). Hereafter, we refer to this area as the 'low-density stand'. Along the period 2009–11, we collected 436 acorns in 15 focal trees located within this area (Table S1, see online supplementary material). Additionally, we collected 404 acorns from 11 focal trees from a nearby high tree density stand (~16 ha; ~50 trees/ha) located 900 m away from the nearest tree in the low tree density stand (Ortego *et al.* 2010) (Table S1, see online supplementary material). Hereafter, we refer to this area as the 'high-density stand'. The high-density stand was used as a local control to compare the different studied parameters with those recorded among the extremely isolated trees present in the low-density stand. Note, however, that we cannot discard that the studied high-density stand is totally exempted from some of the potential impacts of habitat fragmentation (e.g. edge effects) due to its relatively small size and isolation from the nearest continuous forest (16 km away). We did not sample and genotype all adult individuals from the high-density stand and so progeny data from these trees were mostly used for comparative analyses regarding mating patterns, pollen pool genetic structure and progeny genetic diversity (see below). We recorded the spatial location (Universal Transverse Mercator coordinates) for each sampled tree using a Global Positioning System and analyses of clonal structure allowed us to identify unique genotypes (see Ortego *et al.* 2010 for details). All collected acorns were planted to obtain seedling leaf tissues that were stored at −20°C until needed for genetic analyses.

Microsatellite genotyping

We used NucleoSpin Plant II kits (Macherey-Nagel) to extract and purify genomic DNA from adults and progeny. We amplified nine polymorphic microsatellite markers previously developed for other *Quercus* species (Table 1). Approximately 5 ng of template DNA was amplified in 10-µl reaction volumes containing 1× reaction buffer (EcoStart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 µM of each dye-labelled primer (FAM, PET, VIC or NED) and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The polymerase chain reaction programme used was 9 min denaturing at 95°C followed by 40 cycles of 30 s at 94°C, 45 s at the annealing temperature (Table 1) and 45 s at 72°C, ending with a 10-min final elongation stage at 72°C. Amplification products were electrophoresed using an ABI 310 Genetic Analyzer (Applied Biosystems) and genotypes were scored using GeneMapper 3.7 (Applied Biosystems). We used Arlequin 3.1 to test for linkage equilibrium within each pair of loci and population using a likelihood-ratio statistic, whose distribution was obtained by a permutation procedure (Excoffier *et al.* 2005). Microsatellite genotypes were tested for departure from

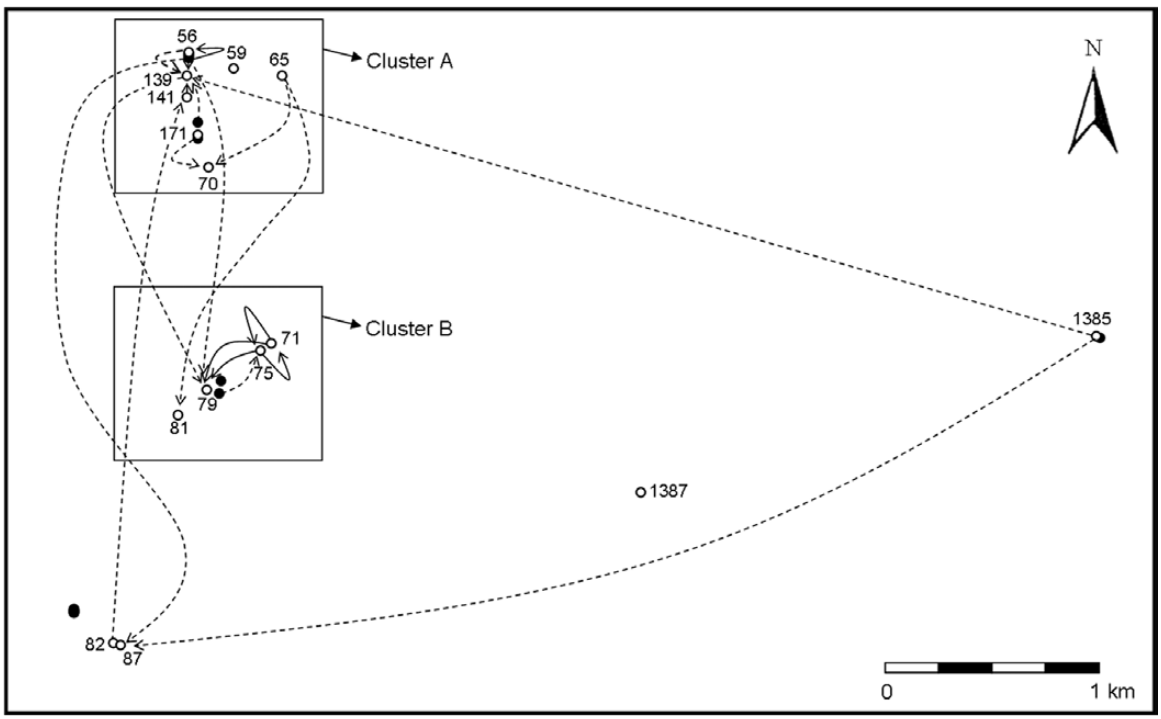

Figure 1: map of the low-density stand indicating the location of all the trees (open and filled dots) and the two main clusters of trees (cluster A and cluster B). Open dots represent the focal maternal trees analysed and arrows pollen dispersal events within the stand (from paternal to maternal trees) considering seeds assigned at the 95% CL (dashed lines: one dispersal event; solid lines: more than one dispersal event). Numbers correspond with tree codes described in Table S1, see online supplementary material.

Table 1: microsatellite loci used to genotype holm oaks (*Quercus ilex*)

Locus	*A*	H_E	H_O	T_a	Primer origin
MSQ13	11	0.85	0.86	50	Dow *et al.* (1995)
QpZAG9	10	0.79	0.92	55	Steinkellner *et al.* (1997)
QpZAG15	14	0.87	0.70	50	Steinkellner *et al.* (1997)
QpZAG36	10	0.81	0.57	50	Steinkellner *et al.* (1997)
QpZAG46	5	0.54	0.39	53	Steinkellner *et al.* (1997)
QrZAG11	13	0.85	0.95	50	Kampfer *et al.* (1998)
QrZAG20	21	0.89	0.94	55	Kampfer *et al.* (1998)
PIE020	10	0.51	0.35	50	Durand *et al.* (2010)
PIE258	12	0.82	0.95	55	Durand *et al.* (2010)

This table shows number of alleles (*A*), expected heterozygosity (H_E), observed heterozygosity (H_O) and annealing temperature (T_a, in °C) for each locus.

Hardy–Weinberg equilibrium using an exact test (Guo and Thompson 1992) based on 900 000 Markov chain iterations as implemented in the program Arlequin 3.1 (Excoffier *et al.* 2005). Microsatellite loci were also tested for the presence of null alleles, allelic dropouts or genotyping errors using Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004).

Paternity analyses

For paternity assignment of acorns collected in the low-density stand, we used the maximum likelihood method implemented by the program Cervus (Kalinowski *et al.* 2007; Marshall *et al.* 1998). The index delta (Δ) was calculated for each parent–offspring pair over all loci, and the most likely paternal tree for a particular seedling was assigned to the adult tree whose delta value was higher than a certain threshold calculated via simulations (Meagher 1986). We simulated 100 000 offspring using allele frequencies observed in our population. According to our own empirical data and preliminary maternity analyses with Cervus, we set 98.7% of loci typed and 5.8% of loci mistyped. We allowed self-fertilization and considered 48 candidate paternal trees and 50% of candidate paternal trees sampled according to pollen immigration rates (~50%) previously reported for other oaks (e.g. Abraham *et al.* 2011; Craft and Ashley 2010; Dow and Ashley 1996, 1998; Nakanishi *et al.* 2004; Pluess *et al.* 2009; Streiff *et al.* 1999). Note that this setting does not preclude estimating higher or lower levels of gene flow than 50% (Pluess *et al.* 2009). In the paternity assignment analyses, all individuals within the low-density stand were included as candidate fathers. The 15 focal maternal trees were also included as candidate fathers for their respective progeny (e.g. Abraham *et al.* 2011). Cervus allows the assignment of paternity at different confidence levels (CL) and like most studies, we present results based on paternity inferences obtained at both the 80% CL and 95% CL (e.g. Abraham *et al.* 2011; Pluess *et al.* 2009). We also used Cervus considering the same simulation parameters described above to identify offspring arising from self-fertilization in the high-density stand.

Pollen movement, mating patterns and pollen pool structure

Dispersal distances for male gametes assigned to a given analysed seed were calculated as the Euclidean distance from the paternal to the maternal tree. We used Monte Carlo simulations to evaluate the possibility that observed pollen dispersal patterns have occurred by chance and are merely constrained by the spatial distribution of the studied maternal trees (Manly 1991; e.g. Ortego *et al.* 2011a). For this purpose, we set a null model considering that male gametes identified through parentage analyses (see below) randomly disperse as an inverse function of squared distance to any studied maternal tree. We constrained pollen movement to the analysed seeds and maternal trees studied each year. We performed simulations excluding self-fertilization due to the low frequency of this phenomenon in oaks (e.g. Fernández and Sork 2005; Pluess *et al.* 2009; Sork *et al.* 2002; see also Results). For each male gamete, we calculated the distance between the paternal tree and the randomly assigned maternal tree to generate the expected frequency distribution of dispersal distances, i.e. the null model. Simulations of the null model were repeated 1000 times to obtain the expected frequency distribution of median pollen dispersal distances. The expected distribution of median pollen dispersal distances was compared with the observed median pollen dispersal distance. Tests of significance were generated by counting the number of randomized cases that resulted in an equal or larger/smaller value than the observed median pollen dispersal distance and dividing by the total number of randomizations (Manly 1991). We performed different simulations for each study year (2009 and 2010) and for male gametes assigned at both the 80% CL and 95% CL. Note that the number of collected and paternally assigned seeds was very low for 2011 (80% CL, $n = 5$; 95% CL, $n = 1$) and for this reason, we did not perform simulations for this year.

We characterized mating patterns in each stand estimating multilocus outcrossing rates (t_m), single-locus outcrossing rate (t_s), biparental inbreeding ($t_m - t_s$) and multilocus correlated paternity (r_p). These parameters were calculated for each reproductive year using the maximum likelihood procedures of Ritland and Jain (1981) as implemented in the multilocus mating system program MLTR (Ritland 2002). Standard deviations (SDs) for t_s, t_m and r_p were obtained from 1000 bootstrap replicates, with families (i.e. groups of offspring from a known mother tree) as the re-sampling unit.

We estimated pollen pool structure in each of the two studied stands conducting TwoGener analyses, a molecular analysis of variance based on male gametic genotypes (Smouse *et al.* 2001). A partition of male gametic variation into among- and within-female components yields an intraclass correlation measure, Φ_{FT}, which is informative about the degree of genetic heterogeneity among pollen clouds sampled

by maternal trees. Global estimates of Φ_{FT} for each population and pairwise Φ_{FT} estimates for each pair of maternal trees were obtained using GenAlex 6.5 (Peakall and Smouse 2006). The statistical significance of Φ_{FT} estimates was tested based on 999 permutations of pollen gametes among females. We only considered data from mothers from which at least 10 offspring had been sampled. To account for adult population structure, we calculated the fixation index F_{IS} based on multilocus genotypes of trees and then divided Φ_{FT} estimates by $(1 + F_{IS})$ following Austerlitz and Smouse (2001).

Genetic diversity of progeny and pollen pools

We used two metrics to estimate progeny genetic diversity: (i) uncorrected heterozygosity (H_O), calculated as the proportion of loci at which an individual is heterozygous and (ii) homozygosity by loci (HL), a microsatellite derived measure that improves heterozygosity estimates in natural populations by weighting the contribution of each locus to the homozygosity value depending on their allelic variability (Aparicio *et al.* 2006). H_O and HL were calculated using Cernicalin, an Excel spreadsheet available on request. For statistical analyses, we calculated average offspring heterozygosity for each maternal tree and year.

We compared progeny genetic diversity between the low-density stand and the high-density stand using generalized linear mixed models (GLMMs) with a normal error distribution and an identity link function. We included stand and year as fixed factors and maternal tree as a random effect. The precision of genetic diversity estimates may differ among trees because sample sizes (i.e. the number of genotyped seeds) varied between them. To take this into account, we used a weighted least square method, where weight equals the number of genotyped seeds (e.g. Ortego *et al.* 2011b). We also compared selfing rates between the low-density and the high-density stand. For this purpose, we used a GLMM with a binomial distribution of errors and a logit link function, including the number of selfed progeny as the response variable and the total number of genotyped seeds in a given tree as the binomial denominator. We also included stand and year as fixed factors and maternal tree as a random effect. The proportion of selfed progeny was calculated for both the 80% CL and the 95% CL data sets (Table S1, see online supplementary material). All these analyses were performed using SAS 9.2 (SAS Institute 2004).

Pollen limitation and seed set

During spring 2009, we marked with plastic labels between 8 and 15 buds in 19 and 13 trees from the low-density stand and the high-density stand, respectively. We estimated seed set rates by counting the number of successfully fertilized flowers that were starting to fructify by early June relative to the number of female flowers initially present in each bud. Buds partially or totally eaten by caterpillars were discarded from statistical analyses. We compared seed set rates between the low-density stand and the high-density stand using a GLMM with a binomial distribution of errors and a logit link function, including the number of successfully fertilized flowers as the response variable and the total number of female flowers in a given bud as the binomial denominator. We included stand as a fixed factor and tree as a random effect. A similar analysis was performed to compare seed set rates in experimental vs. control buds, in this case including treatment as a fixed factor. The experiment consisted in labelling eight additional buds in four focal trees from the low-density stand. In those buds, female flowers were experimentally supplemented *ad libitum* with foreign pollen using a paintbrush. We did so to assess any potential limiting effect of foreign pollen availability on fertilization success by further comparison with control buds.

Seed and female flower production

The number of female flowers produced was estimated in the marked buds described in the section 'Pollen limitation and seed set' and this variable was analysed using a GLMM with a normal distribution of errors and an identity link function. We included stand as a fixed factor and tree identity as a random effect. During 2009–11, we estimated seed production in 13 and 11 trees from the low-density stand and the high-density stand, respectively. Seed traps (plastic containers with a surface of 0.12 m^2 and 50 cm deep) were randomly situated under the canopies to assess acorn production. The number of traps differed between trees to cover the same proportion of canopy surface in all of them (between 1.5 and 2%). Tree surfaces were calculated on the basis of three random measures of the diameter of their canopy, considering trees to be roughly circular (see Pulido and Díaz 2005 for a similar procedure). These acorn crop estimates are reliable, as differences between the seed traps of each tree are very small compared with between-tree variability (see Bonal *et al.* 2007). We assessed the possibility of unsuspected subtractions from the traps by placing 100 marked acorns in them; none were removed by the end of the study (see also Bonal *et al.* 2007 for details). In all years, the traps were first checked on 15 September and from then on, every 15 days until 30 December, when mature acorns stopped falling. From these data, we calculated the total number of mature acorns produced by each tree per square metre (Bonal *et al.* 2012). This variable was compared between the low-density stand and the high-density stand using a GLMM with a normal distribution of errors and an identity link function, including stand and year as fixed factors and tree identity as a random effect.

Experimental estimation of selfing rates

During spring 2009, we selected between 1 and 19 branches before bud swelling in 9 trees (3 from the low-density stand and 6 from the high-density stand). These branches were isolated using paper bags to avoid natural pollination. Bags were periodically checked to determine bud swelling, strip male buds from branches and count the number of female flowers per branch. We applied two pollination treatments using paintbrushes: (i) cross-pollination with foreign pollen (33

branches from 6 trees) and (ii) self-pollination (40 branches from 9 trees). We used 10 branches as controls (i.e. bagged branches with no pollination treatment) to make sure that the sealing system and bags were adequate to prevent external pollination. None of these control branches produced acorns. Bags were removed from branches when acorns started to grow and/or all chance of natural pollination was over. Finally, we analysed seed set rates using a GLMM with a binomial distribution of errors and a logit link function, including the number of acorns produced as the response variable and the total number of female flowers in a given branch as the binomial denominator. We fitted treatment and stand as fixed factors and tree as a random effect.

RESULTS

Microsatellite data

All microsatellite markers were highly polymorphic (Table 1). We found no evidence of linkage disequilibrium among loci, indicating that the analysed markers can be treated as independent from each other. After adjusting for multiple comparisons (n = 9 loci; Bonferroni adjusted P-value = 0.030), significant departures from Hardy–Weinberg equilibrium due to homozygosity excess were observed in microsatellites QpZAG36 (only in the low-density stand) and PIE020 (in both the low-density stand and the high-density stand). MicroChecker analyses indicated that locus QpZAG36 showed evidence for null alleles in both the low-density stand and the high-density stand, with estimated frequencies of 0.14 and 0.19, respectively. Locus PIE020 only showed evidence for null alleles in the high-density stand with an estimated frequency of 0.21. Previous studies have indicated that inferred mating patterns based on microsatellite data can be sensitive to null alleles (Breed *et al.* 2012b). For this reason, we recalculated mating parameters and genetic diversity estimates excluding loci QpZAG36 and PIE020 and provide this information in Table S2, see online supplementary material. We maintained these two loci in all other analyses as their exclusion provided analogous results (data not shown).

Pollen flow, mating patterns and pollen pool structure

Paternity analyses assigned 102 seeds at the 80% CL and 43 seeds at the 95% CL. This indicates pollen immigration rates higher than 75% (80% CL: 77%; 95% CL: 90%). Pollen dispersal distance of outcrossed assigned offspring ranged from 6 to 4828 m with a median of 336 m for the 80% CL data set and 109 for the 95% CL (Table S1, see online supplementary material). Results of paternity analyses indicated overall selfing rates of 3.0% at the 80% CL and 1.4% at the 95% CL. Paternity analyses showed that 87.5% (21 out of 24) of the potential pollen donors within the low-density stand sired at least one seed for the 80% CL data set, whereas only 54.2% (13 out of 24) of them sired at least one seed for the 95% CL data set. The number of seeds sired by each parent with at least one seed assigned ranged from 1 to 12 (mean ± SE = 4.2 ± 3.1) for the 80% CL data set and from 1 to 10 (mean ± SE = 3.0 ± 3.0) for the 95% CL data set. Monte Carlo simulations revealed that median dispersal distances were much higher than expected under random dispersal in both study years and considering male gametes assigned at either the 80% CL or the 95% CL (all Ps < 0.001).

The results of MLTR analyses suggest that both stands have very high outcrossing rates (t_m) (range: 0.99–1.00) and very low estimates of biparental inbreeding ($t_m - t_s$) (range: 0.02–0.076) and multilocus correlated paternity (r_p) (range: 0.001–0.033) (Table 2). This indicates a low proportion of mating among relatives and that most seeds within families have different fathers (i.e. they are half-siblings).

Differentiation among pollen pools received by female trees was statistically significant in both the low-density stand

Table 2: genetic diversity, mating system parameters and differentiation in pollen gene pool among seed parents in each studied stand

Group	$n_{family}/n_{progeny}$	H_O	HL	t_m	t_s	$t_m - t_s$	r_p	Φ_{FT}
Low-density stand								
Adults		0.753 (0.102)	0.218 (0.106)	—	—	—	—	—
2009	9/102	0.692 (0.139)	0.280 (0.137)	0.990 (0.013)	0.937 (0.014)	0.054 (0.013)	0.009 (0.005)	0.058
2010	13/318	0.730 (0.136)	0.250 (0.134)	0.991 (0.006)	0.931 (0.012)	0.060 (0.013)	0.001 (0.001)	0.032
2011	5/16	0.680 (0.147)	0.295 (0.148)	—	—	—	—	—
High-density stand								
Adults		0.728 (0.119)	0.239 (0.119)	—	—	—	—	—
2009	8/157	0.703 (0.130)	0.269 (0.128)	0.994 (0.007)	0.954 (0.012)	0.040 (0.010)	0.033 (0.008)	0.005
2010	8/158	0.687 (0.143)	0.289 (0.141)	1.000 (0.000)	0.977 (0.004)	0.023 (0.004)	0.008 (0.003)	0.037
2011	9/89	0.681 (0.146)	0.297 (0.145)	1.000 (0.001)	0.924 (0.023)	0.076 (0.023)	0.008 (0.004)	0.043

Abbreviations: n_{family} = total number of families (i.e. mother trees) analysed, $n_{progeny}$ = total number of seeds (i.e. progeny) analysed across all families, H_O = observed heterozygosity, HL = homozygosity by loci, t_m = multilocus outcrossing rate, t_s = single-locus outcrossing rate, $t_m - t_s$ = biparental inbreeding estimate, r_p = multilocus correlated paternity, Φ_{FT} = differentiation in pollen gene pool among seed parents. SDs are indicated in parentheses.

and the high-density stand for all study years (all Ps > 0.001) (Table 2). Note that only a single tree with more than 10 genotyped seedlings was available in the low-density stand for 2011. F_{IS} values were very low in both the low-density stand (F_{IS} = 0.01) and the high-density stand (F_{IS} = 0.04). For this reason, accounting for population structure did not result in substantial changes of Φ_{FT} estimates in either stand (data not shown) (see also Slavov *et al.* 2009). The larger area of the low-density stand could have contributed to increase Φ_{FT} values relative to those obtained for the much smaller high-density stand. For this reason, we calculated Φ_{FT} values separately for two smaller clusters of trees located within the low-density stand and indicated in Fig. 1 as 'cluster A' (~12 ha) and 'cluster B' (~10 ha). Φ_{FT} values for these clusters were similar to those reported for the entire stand for 2010, the only year with three or more available trees per cluster (cluster A: Φ_{FT} = 0.035; cluster B: Φ_{FT} = 0.029; all Ps < 0.001). We also tested the hypothesis that differentiation among pollen pools is caused by excessively disproportionate pollination by a small number of males in close proximity to the sampled maternal trees (Slavov *et al.* 2009). After removing data from offspring assigned within the low-density stand at the 80% CL or 95% CL, we found that Φ_{FT} values were similar to those reported for the complete data set in both 2009 (80% CL: 0.060; 95% CL: 0.052; all Ps < 0.001) and 2011 (80% CL: 0.032; 95% CL: 0.031; all Ps < 0.001).

We analysed inter-annual differences between pollen pools received by maternal trees sampled in successive years. We did not find inter-annual differentiation in pollen pools in comparisons involving four trees from the low-density stand that were sampled during 2009 and 2010 (mean Φ_{FT} = 0.003; all Ps > 0.2). In the high-density stand, we found significant differentiation of pollen pools in comparisons involving two trees sampled in 2009 and 2010 (mean Φ_{FT} = 0.024; all Ps < 0.01) and one tree sampled in 2010 and 2011 (Φ_{FT} = 0.035; P = 0.04). However, we did not find significant differentiation of pollen pools for one tree sampled in 2009 and 2011 (Φ_{FT} = 0.008; all Ps = 0.34). Despite low sample sizes, inter-annual differences in Φ_{FT} values were significantly higher in the high-density stand than in the low-density stand either considering all the re-sampled trees (one-way analysis of variance [ANOVA], $F_{1,7}$ = 10.84, P = 0.017) or only those trees sampled in 2009 and 2010 (one-way ANOVA, $F_{1,5}$ = 29.70, P = 0.006).

Genetic diversity

Progeny heterozygosity did not differ between the low-density stand and the high-density stand (H_O: $F_{1,50}$ = 2.72, P = 0.105; HL: $F_{1,50}$ = 2.19, P = 0.145) or among years (H_O: $F_{2,49}$ = 2.84, P = 0.068; HL: $F_{2,49}$ = 2.33, P = 0.108). After controlling for maternal identity, the proportion of selfed offspring did not differ between stands (80% CL database: $F_{1,50}$ = 0.82, P = 0.369; 95% CL database: $F_{1,50}$ = 0.09, P = 0.766) or years (80% CL database: $F_{2,49}$ = 1.29, P = 0.285; 95% CL database: $F_{2,49}$ = 0.91, P = 0.409). More detailed analyses within the low-density stand indicated that these parameters are not associated with the degree of maternal tree isolation (see online Supplementary Data).

Pollen limitation and seed set

Seed set rates were significantly higher in the high-density stand than in the low-density stand ($F_{1,337}$ = 4.73, P = 0.030; tree identity: Z = 2.42, P = 0.008; Fig. 2A). Experimental cross-pollen addition in shoots from some trees in the low-density stand resulted in increased seed set rates in comparison with control shoots ($F_{1,61}$ = 8.60, P = 0.005; tree identity: Z = 0.85, P = 0.198; Fig. 2A). Seed set rates did not differ between experimental shoots with pollen addition in the low-density stand and control shoots in the high-density stand ($F_{1,172}$ = 0.48, P = 0.490; tree identity: Z = 1.11, P = 0.133; Fig. 2A). This is further supported by the pollination experiment of bagged branches (see below): the proportion of cross-fertilized female flowers producing acorns did not differ between the low-density stand (mean ± SE = 21.5 ± 4.8%) and the high-density stand (mean ± SE = 26.8 ± 16.1%) ($F_{1,31}$ = 0.36, P = 0.555; tree identity: Z = 0.73, P = 0.232). Overall, these results indicate that the lower seed set rates in the low-density stand in comparison with the high-density stand are not due to a lower fertility or poorer physiological state of trees in the former.

Seed and female flower production

The number of flowers produced per bud did not significantly differ between the low-density stand and the high-density stand ($F_{1,362}$ = 1.89, P = 0.170; tree identity: Z = 3.34, P < 0.001; Fig. 2B). The number of acorns produced by each tree per square metre neither differed between the low-density stand and the high-density stand ($F_{1,70}$ = 0.73, P = 0.397; tree identity: Z = 1.51, P = 0.065; Fig. 2C). We did not find differences in seed production among years ($F_{2,69}$ = 0.33, P = 0.722) or an interaction between year and stand ($F_{2,66}$ = 0.29, P = 0.747).

Experimental estimation of selfing rates

Seed set rates of self-fertilized female flowers were much lower than the rates in cross-fertilized female flowers ($F_{1,71}$ = 10.64, P = 0.002; tree identity: Z = 0.67, P = 0.250; Fig. 2D). We did not find differences in seed set rates between the low-density stand and the high-density stand ($F_{1,70}$ = 1.19, P = 0.279) or an interaction between the experimental treatment and stand ($F_{1,69}$ = 0.78, P = 0.381).

DISCUSSION

Our study indicates that reduced tree densities have scarce consequences on pollen flow, mating patterns, progeny genetic diversity and seed production. We have found extensive pollen immigration (>75%) into the low-density stand, indicating that long-distance pollen movement is compensating the low availability of local pollen donors. Further, pollen donors identified through paternity analyses often involved trees located far away from focal maternal trees (Fig. 1) and

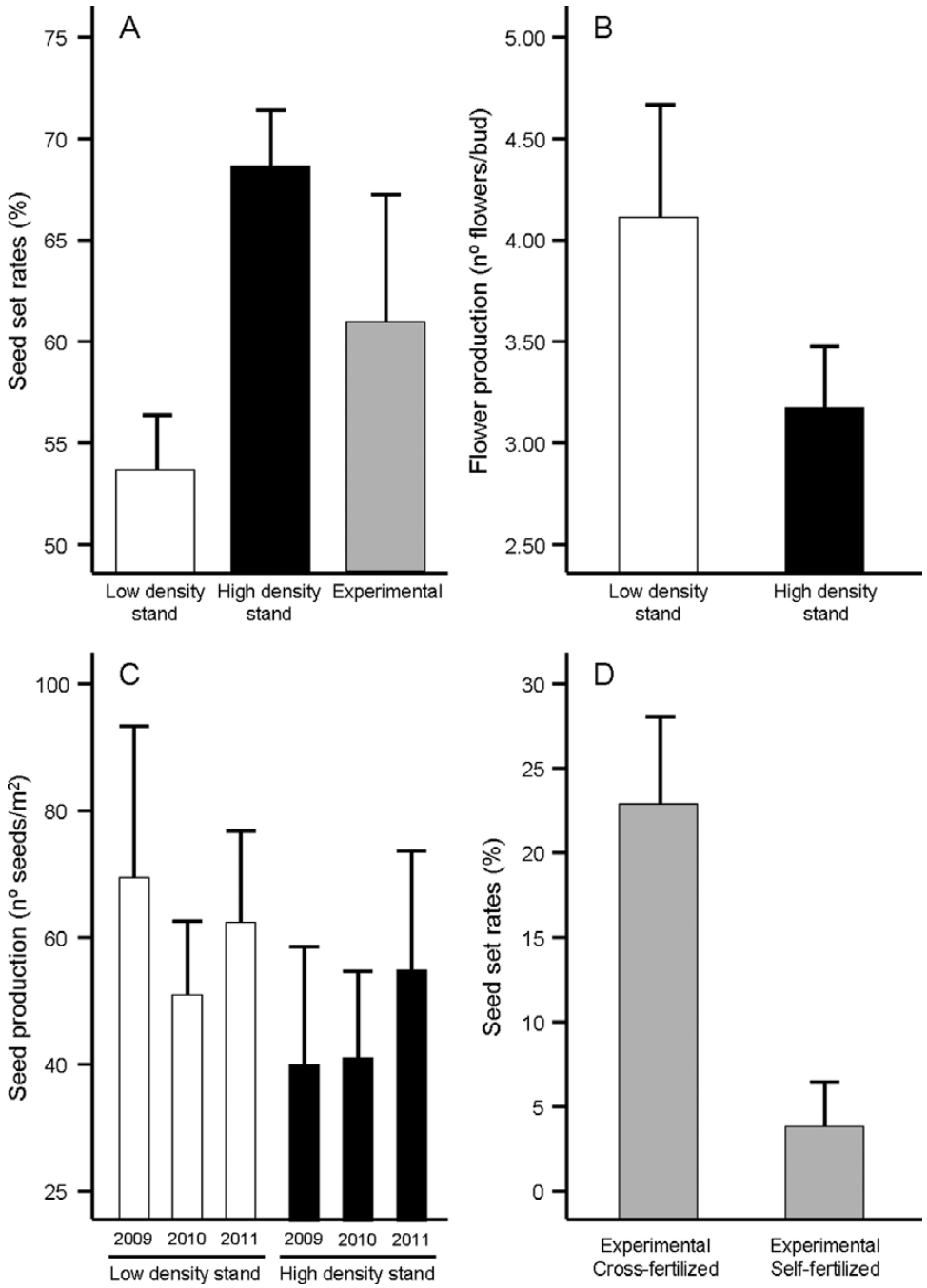

Figure 2: mean ± SE for (**A**) seed set rates in the low-density stand and the high-density stand and for trees from the low-density stand with experimental pollen supplementation; (**B**) flower production in the low-density stand and the high-density stand; (**C**) seed production in the low-density stand and the high-density stand during the three study years; (**D**) seed set rates in experimental self- and cross-fertilized buds.

Monte Carlo simulations revealed that pollen within the low-density stand moves larger distances than expected from null models of random dispersal. Thus, our results indicate widespread local pollen flow and extensive immigration of foreign pollen. The high temperatures and low precipitations characterizing the study area during the flowering period are likely to increase pollen production and favour that the pollen released into the air is not regularly washed by rainfall (see Knapp *et al.* 2001 and references therein). These local climate conditions, together with the wind-mediated pollen dispersal, can explain the long-distance pollen movement observed in holm oaks and other wind-pollinated tree species from Mediterranean environments (e.g. Albaladejo *et al.* 2012; Pluess *et al.* 2009).

We have found no evidence that the low tree density stand has disrupted mating patterns when compared with

the high-density stand, and both stands showed similar levels of outcrossing rates, biparental inbreeding and correlated paternities (Table 2). Accordingly, patterns of pollen pool differentiation were similar in the low-density stand and the high-density stand, with Φ_{FT} values (range: 0.005–0.058) comparable to those previously reported for other oaks (Fernández and Sork 2005; Fernández-Manjarres *et al.* 2006; Pakkad *et al.* 2008; Smouse *et al.* 2001; Sork *et al.* 2002). Interestingly, inter-annual differentiation between pollen pools received by maternal trees sampled in different years was significant in most trees from the high-density stand, whereas no differentiation was observed in any tree from the low-density stand. This indicates higher inter-annual heterogeneity of pollen pools received by trees in the high-density stand than in the low-density stand. One possibility to explain this pattern is that trees in the high-density stand are predominantly pollinated by a few close neighbours and inter-annual mismatches in phenology result in different groups of dominant male donors contributing to most paternities in different years (Nakanishi *et al.* 2004, 2005). Most paternities in the low-density stand are the result of long-distance pollen dispersal events (>75%) that are expected to involve small contributions of many pollen donors. As a result, it seems unlikely that inter-annual fluctuations in these small relative male contributions are detected by TwoGener analyses.

Progeny genetic diversity and selfing rates did not differ between the low-density stand and the high-density stand and were not associated with any parameter related with tree isolation (Table 2; see online Supplementary Data). The pollination experiment showed that the self-fertilization treatment resulted in a very low rate of seed set, with only 3.8% of the female flowers producing acorns when selfed. This figure is similar to the obtained for paternity-based analyses in both the low-density stand (1.4–3.0%) and the high-density stand (1.0–1.7%) and corroborates previous studies indicating that oaks are highly self-incompatible (Bacilieri *et al.* 1996; Ducousso *et al.* 1993; Yacine and Bouras 1997). Thus, a low availability of foreign pollen is not likely to be compensated with increased rates of self-fertilization in oaks. Overall, these results suggest that low conspecific densities have negligible consequences on progeny genetic diversity, a pattern expected considering the high rates of pollen immigration revealed by paternity analyses. However, this observation contrasts with a previous study performed in the same locality and showing lower heterozygosity in younger cohorts in comparison with adult trees in a low-density stand but not in the high-density stand (Ortego *et al.* 2010). The fragmented stand with low conspecific density analysed in the previous study is located close to the low-density stand here considered but it has a higher tree density and this fact could have favoured local pollinations and increased rates of biparental inbreeding (Fernández and Sork 2005). Alternatively, progeny resulted from local crosses could show higher adaptation to local environmental conditions and experience increased recruitment success despite reduced genetic diversity (Alberto *et al.* 2010; Ortego *et al.* 2012; Ramírez-Valiente *et al.* 2009). Another possible explanation for the lower heterozygosity in saplings in comparison with adults is that selection against homozygous/inbred young individuals eliminates them before becoming adults (Hufford and Hamrick 2003).

Seed set rates were lower in the low-density stand, but this did not result in reduced reproductive success estimated as seed production. The proportion of successfully fertilized flowers was significantly lower in the low-density stand than in the high-density stand and experimental cross-pollen supplementation in the low-density stand resulted in increased seed set rates in comparison with control flowers, whereas no difference was observed between the high-density stand and the low-density stand after pollen supplementation. Similar patterns of foreign pollen limitation have been previously described in other plant species in relation with population fragmentation and neighbourhood density (González-Varo *et al.* 2009; Hirayama *et al.* 2007; Knight *et al.* 2005; Severns 2003), but this phenomenon has been generally overlooked in most studies analysing the consequences of fragmentation and low conspecific densities in oaks and other wind-pollinated tree species (but see Knapp *et al.* 2001 and references therein). Our results suggest that foreign pollen availability could be limiting seed set rates in the low-density stand. Alternatively, an extremely low tree density could induce that most trees self-fertilize a high proportion of their female flowers before the arrival of foreign pollen, resulting in a high proportion of non-viable embryos and reducing seed set rates. Foreign pollen is likely to reach female flowers much earlier in the high-density stand, which could contribute to reduce the probability of selfing and increase seed set rates in this stand. We found that flower production and seed crops did not differ between the low-density stand and the high-density stand but tended to be higher in the former, which may reflect increased resource availability for isolated trees in comparison with those growing in higher-density stands (Bonal *et al.* 2012; Moreno and Cubera 2008). These results indicate that foreign pollen limitation in the low-density stand is not likely to be of great concern in terms of reduced seed production and future potential recruitment.

Overall, this study demonstrates the usefulness of combining genetic and ecological data to get a more comprehensive picture about the consequences of reduced tree densities and forest fragmentation on pollen movement, mating patterns and reproductive success. Our data suggest that low conspecific densities can reduce seed set rates and modify certain parameters related with the composition of pollen clouds, but low tree densities seem to have negligible consequences on mating patterns, progeny genetic diversity or seed production. Poor recruitment due to other ecological (e.g. reduced dispersal, edge effect) and human (e.g. ploughing of land) factors is likely to be a more important threat for the long-term persistence of fragmented forest than disrupted pollen dispersal or reduced reproductive success.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology* online.

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