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Genetic Consequences of Habitat Fragmentation in Long-Lived Tree Species: The Case of the Mediterranean Holm Oak (*Quercus ilex*, L.)

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Abstract

Large-scale forest fragmentation can increase interpopulation genetic differentiation and erode the genetic variability of remnant plant populations. In this study, we analyze the extent of clonality and the genetic variability and structure within a holm oak (*Quercus ilex*) population from Central Spain at 3 patches showing different degrees of fragmentation. For this purpose, we have typed 191 individuals (105 adults and 86 saplings) at 9 microsatellite loci. Microsatellite markers revealed an extensive clonal structure in this species, with most analyzed clumps constituting a single "genet", which in some cases extended over a considerable area (up to 318 m^2). The maximum distance between "ramets" tended to be higher in the extremely fragmented patch, suggesting that intensive management and environmental perturbation has favored clonal propagation. We have also found evidence that fragmentation has contributed to reduce genetic variability and increase genetic differentiation in holm oak saplings, indicating that the younger cohorts are suffering some negative genetic consequences of long-term population fragmentation. Finally, analyses of fine spatial genetic structure have revealed significant kinship structures up to 20–50 m that were particularly patent in the 2 less fragmented patches. Overall, our findings point to long-term genetic shifts in population structure of holm oaks in fragmented landscapes; however, further research is required on pollen dispersal and gene flow in this species.

Key words: *clonal structure, fragmentation, gene flow, genetic diversity, heterozygosity, isolation by distance*

Human activities are responsible for large-scale forest fragmentation and destruction in several ecosystems worldwide (Noss and Csuti 1994; Lindenmayer and Fischer 2006). This has often reduced continuous habitats into several smaller spatially isolated remnants, threatening the maintenance of biodiversity in such landscapes (Saunders et al. 1991). One of the consequences of fragmentation is the loss of connectivity between patches with its potential-associated genetic effects. In the case of wind-pollinated plants, reduction in fragment size and decreased pollen interchange can increase genetic differentiation between patches and erode local genetic variability (Young et al. 1996). The latter can reduce both individual fitness and the population's ability to respond to changes in selection pressures that can ultimately compromise its long-term viability (Young et al. 1996). Moreover, intensive forest management and environmental perturbations can also favor asexual over sexual reproduction strategies, which can strongly reduce population genetic diversity if only some clones dominate and colonize large areas (e.g., Kemperman and Barnes 1976; Mitton and Grant 1996).

Current empirical evidence on the genetic consequences of forest fragmentation is unclear (Lowe et al. 2005), with some studies reporting a significant loss of genetic variability and increased genetic differentiation between patches after forest fragmentation and others failing to find such effects (reviewed in Young et al. 1996; Lowe et al. 2005). These contrasting results may reflect interspecific differences in mating patterns, generation time, and longevity (Young et al. 1996). The latter may be particularly important for

long-lived organisms like trees when the temporal scale of sampling and the time elapsed since human disturbance/fragmentation strongly differ between studies (Gram and Sork 1999; Lowe et al. 2005; Craft and Ashley 2007). On the other hand, some studies have suggested that the open landscape after fragmentation facilitates airborne pollen dispersal that can compensate for the expected detrimental genetic effects of fragmentation (Young et al. 1993; Young et al. 1996; Bacles et al. 2005; Lowe et al. 2005).

The holm oak (*Quercus ilex*, L.) is widespread over the western Mediterranean basin (Blondel and Aronson 1999). In Spain, it is the most prevalent tree species and extends over a surface of approximately 5.1 million ha (Blanco et al. 1997). The holm oak plays an extremely important role for the functioning of Mediterranean forests, and it is considered a keystone species in these ecosystems (Blanco et al. 1997; Muñoz and Bonal 2007). This species is also of economic importance and has been extensively used by humans for centuries (Blanco et al. 1997). In addition, large areas of holm oak forests have been cleared for large-scale farming and former woodlands have undergone extensive fragmentation for centuries (Blondel and Aronson 1999). Stands have been traditionally felled to provide firewood, charcoal, and timber or cleared to constitute the so called "dehesas," open wooded landscapes devoted to extensive cereal cultivation and livestock raising (Vicente and Ales 2006). This long-term human management and alteration has resulted in different levels of forest fragmentation ranging from continuous forest to woodland islands of variable size, savannah-like dehesas, or even extremely isolated trees within farmland areas (Blondel and Aronson 1999; Vicente and Ales 2006).

These human-induced changes in population structure could have reduced the genetic variability of small isolated populations, although long-distance pollen dispersal and the species long lifespan (several centuries) could have counterbalanced the expected loss of genetic diversity over time (Petit et al. 2002; Lowe et al. 2005; Craft and Ashley 2007). However, in spite of the key role of the holm oak in Mediterranean ecosystems, no study has specifically analyzed the genetic consequences of fragmentation. In addition, the holm oak is known to be a vigorous resprouter after disturbance, with very effective asexual reproduction through root shooting (Espelta et al. 2003; Plieninger et al. 2004; Vicente and Ales 2006), and its clonal propagation and genetic structure has not been properly analyzed. If clones are not properly discriminated, levels of local genetic diversity and the number of different individuals present in a population may be overestimated (Mayes et al. 1998; Arnaud-Haond et al. 2007).

In this study, we analyze the extent of clonality and the genetic variability and structure within 3 patches of a holm oak population showing different degrees of fragmentation, ranging from a typical forest formation to an extremely fragmented patch where highly isolated "island trees" are the only remnant of the former continuous forest. We have used 9 microsatellite markers to type individuals and discriminate clones, obtain accurate estimates of genetic variability, and analyze genetic structure under such contrasting scenarios of population fragmentation. In particular, we tested the following predictions: 1) Due to intensive management in the highly deforested patch, we would expect larger clone sizes in this area in comparison with the less altered stand showing a forest structure; 2) due to the long generation time of this species and the time since fragmentation, we predicted reduced genetic diversity in the younger cohort (saplings) in the fragmented patch but not in the continuous forested area or in adult reproductive individuals; 3) we predicted that historical isolation and fragmentation have contributed to reduce gene flow, thus triggering genetic differentiation between the study patches in the younger cohort (saplings) but not in adult reproductive individuals; and 4) finally, we expected an isolation-by-distance pattern of fine spatial scale genetic structure that should be particularly patent in the less fragmented patches due to higher tree density and successful natural regeneration that may favor the aggregation of genetically related individuals.

Materials and Methods

Study Area and Plant Material

The study area is located in Huecas (Toledo province, Central Spain; lat 39°59′N, long 4°13′W). The climate is meso-Mediterranean with mean temperatures ranging from 24–26 °C in July to 4–6 °C in January and 350–450 mm of rainfall mainly concentrated in spring and autumn. Paleolimnologic analyses from our study area have revealed a diminishing tree cover linked to human land use since the year 3500 BC, when the abundance of holm oak pollen start to decrease at the same time that the records of first crops (mainly wheat and barley pollen) are registered (López et al. 2009). Since then, the reduction of tree cover as a consequence of farming has continued, being more severe from the Roman period onwards (López et al. 2009). Historical records of our study area indicate that, at least in some areas where we can only see isolated trees today, there were denser holm oak stands 300 years ago (Román 2006). In this study, we considered 3 patches differing in the degree of fragmentation and tree spatial isolation: 1) an extensively cultivated area with some highly isolated holm oaks growing within the agriculture matrix. The crops are mainly barley (*Hordeum vulgare*) and wheat (*Triticum* spp.), whereas vineyards (*Vitis vinifera*) and olive groves (*Olea europaea*) are also present to a lesser extent; (2) a noncultivated area covered with pastures and some scattered holm oaks; and 3) a continuous holm oak population with a forest structure (~35 ha) (Figure 1). We collected leaves from all adult reproductive individuals located in patches A ($n = 22$) and B ($n = 42$). Leaves from all holm oak saplings were also collected in patch B ($n = 38$). It should be noted that there was an absence of saplings and recruits in patch A due to recurrent ploughing and destruction of new recruits. In the holm oak forest (patch C), we randomly sampled adult

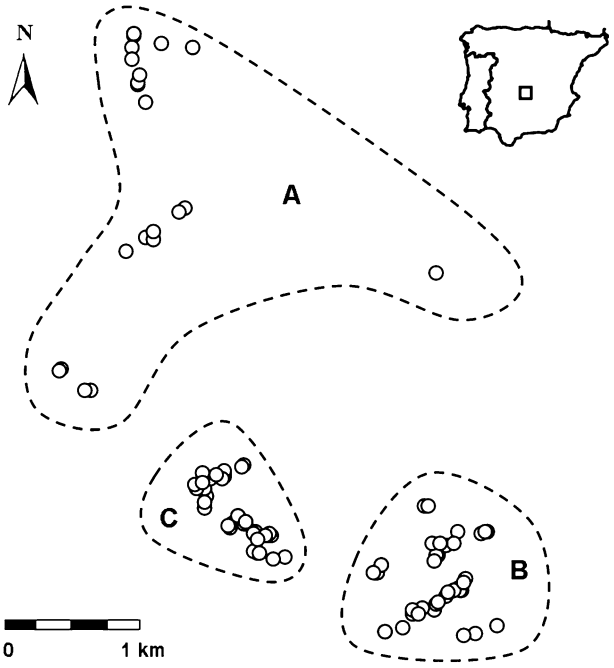

Figure 1. Map of the study area showing the spatial distribution of the studied holm oak individuals. Only adult reproductive trees are showed. Ellipses indicate study patches: (A) an extensively cultivated area with highly isolated holm oaks growing within the culture matrix; (B) a noncultivated area covered with pastures and scattered holm oaks; and (C) a continuous holm oak forest.

reproductive individuals ($n = 41$) and saplings ($n = 48$) to cover the entire patch area. Adults and saplings are evenly distributed in the studied patches, although the latter is more frequent in areas with higher density of adult reproductive trees. Spatial location (Universal Transverse Mercator coordinates) for each sampled tree was recorded using a Global Positioning System. Holm oaks usually form clusters with several stems/trees growing close to one another and to study their clonal structure we collected leaves from 2 to 7 stems from each of 27 of these aggregations ($n = 114$ stems) located in patches A ($n = 17$ aggregations) and C ($n = 10$ aggregations). It should be noted that we did not sample all the "ramets" from each cluster (in some cases more than 30). Rather, we sampled the most distant ramets from these clustered formations to cover the entire area of the suspected clone.

Genetic Analyses

We used 9 polymorphic microsatellite markers previously developed for other *Quercus* species to genotype holm oaks in the study area: MSQ4 and MSQ13, developed for *Quercus macrocarpa* (Dow et al. 1995); QpZAG9, QpZAG15, QpZAG36, and QpZAG46, developed for *Quercus petraea* (Steinkellner et al. 1997); and QrZAG7, QrZAG11, and QrZAG20 developed for *Quercus robur* (Kampfer et al. 1998). We used NucleoSpin Plant II kits (Macherey-Nagel) to extract and purify genomic DNA from leaf samples. Approximately 5 ng of template DNA was amplified in 10 μl reaction volumes containing 1X reaction buffer (EcoStart Reaction Buffer, Ecogen), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each dye-labeled primer (FAM, PET, VIC or NED), and 0.1 U of *Taq* DNA EcoStart Polymerase (Ecogen). The polymerase chain reaction program used was 9 min denaturing at 95 °C followed by 35 cycles of 30 s at 94 °C, 45 s at the annealing temperature (Ortego and Bonal 2010), and 45 s at 72 °C, ending with a 5-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).

Analysis of Clonal Structure

We studied the clonal structure of holm oaks identifying unique multilocus genotypes (e.g., Ainsworth et al. 2003; Valbuena-Carabana et al. 2008). We calculated the maximum number of unique genotypes possible at our study area using the equation:

$$N_g = \prod_{i=1}^{L} [a_i(a_i + 1)]/2$$

where L is the number of loci and a_i is the number of alleles at the locus i (Reusch et al. 1999; e.g., Ainsworth et al. 2003). The likelihood that a genotype occurs more than once by random chance was calculated as follows:

$$P_{gen} = \left(\prod_{i=1}^{l} (f_{ai} \times f_{bi})\right) 2^h$$

where f_{ai} and f_{bi} are the population allele frequencies for the alleles that constitute the genotype (a and b in this case) at the ith locus, h the number of heterozygous loci, and l the total number of analyzed loci (Parks and Werth 1993). P_{gen} indicates the probability that 2 different "genets" have the given genotype by chance, and thus, it informs on marker power for clone identification (e.g., Ainsworth et al. 2003; Valbuena-Carabana et al. 2008). Population allele frequencies were calculated using Cernicalin, an Excel spreadsheet available on request. The probability that the actual observed number of ramets with identical multilocus genotypes (n) are the result of n independent sexual mating events was estimated using P_{sex} values with the software Geneclone 2.0 (Arnaud-Haond and Belkhir 2007; Arnaud-Haond et al. 2007). We used one-way analyses of variance (ANOVAs) in SPSS 17.0 to analyze differences in clone size between patches A and C. We estimated clone size determining 1) the maximum distance between ramets and 2) the area covered by the clone.

Analyses of Genetic Variability

Microsatellite genotypes were tested for departure from 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). We also 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). Genetic diversity was evaluated calculating the total number of alleles (A), allelic richness standardized to the smallest sample size (A_R), unbiased effective number of alleles (A_e), observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}). A, A_R, and A_e were calculated with HP-Rare (Kalinowski 2005), whereas H_O, H_E, and F_{IS} were calculated using Cernicalin (Aparicio et al. 2006), an excel spreadsheet available on request. All these analyses were performed on the basis of unique genotypes, that is, multiple clonal genotypes were removed.

Each study patch and cohort was tested for heterozygosity excess in order to detect recent population bottlenecks using the program Bottleneck 1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999). We ran Bottleneck under the two-phase model (TPM) that is supposed to fit microsatellite evolution better than other methods (Di Rienzo et al. 1994). We used 1000 replications, assuming 10% of the infinite allele model and 90% of the stepwise mutation model. Statistical significance was assessed with a two-tailed Wilcoxon signed-rank test, the most powerful and robust statistics when using less than 20 polymorphic loci (Piry et al. 1999). We also investigated the distribution of the allele frequencies using the mode shift test also implemented in the program Bottleneck (Piry et al. 1999). For each locus, patch, and cohort, we calculated the M-ratio, defined as the ratio of the number of alleles to the range of allele sizes, a statistic that can detect reductions in population sizes (Garza and Williamson 2001). M-ratios were calculated as follows: $M = k/r$, the ratio of the number of alleles to the range of allele sizes, where k = the number of alleles and r = the number of possible alleles sizes between the smallest and the largest observed alleles (Garza and Williamson 2001). We then calculated mean M-ratios across all loci for each patch. This mean value was compared with $M = 0.68$, the threshold value below which a population can reasonably be assumed to have undergone a recent reduction in population size (Garza and Williamson 2001).

We used 2 metrics to estimate individual genetic diversity and inbreeding: 1) uncorrected heterozygosity (H_O), calculated as the proportion of loci at which an individual is heterozygous and 2) 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). HL is calculated as follows: $HL=(\sum E_h)/(\sum E_h + \sum E_j)$, where E_h and E_j are the expected heterozygosities of the loci that an individual bears in homozygosis (h) and in heterozygosis (j), respectively (Aparicio et al. 2006). H_O and HL were calculated using Cernicalin, an excel spreadsheet available on request. We used one-way ANOVAs in SPSS 17.0 to analyze differences in individual heterozygosity among age categories (i.e., adult reproductive trees or saplings) and study patches (A, B, or C).

Analysis of Spatial Genetic Structure

All analyses of spatial genetic structure were performed on the basis of unique genotypes, that is using a single data per genet (see also above). We first investigated population genetic structure among all sampling patches and cohorts (adult reproductive trees and saplings) calculating pairwise F_{ST} values and testing their significance with Fisher's exact tests after 10 000 permutations as implemented in Arlequin 3.1 (Excoffier et al. 2005). Second, we examined patterns of fine-scale spatial genetic structure using the software Spagedi 1.2 (Hardy and Vekemans 2002). To examine the influence of saplings on genetic structure, we analyzed the data considering all individuals (adults plus saplings) and only including adult reproductive trees (e.g., Cottrell et al. 2003; Soto et al. 2007). Spagedi computes 2 matrixes, one of pairwise relatedness and one of Euclidian distances based on tree spatial co-ordinates. Genetic relatedness between pairs of individuals was computed using the Nason's estimator of kinship coefficient (Loiselle et al. 1995). Previous research suggests that spatial autocorrelation is likely to occur only in the first distance classes where nearby trees usually have the highest pairwise relatedness values (e.g., Aldrich et al. 2005; Soto et al. 2007; Valbuena-Carabana et al. 2007). Distance classes were set at 20, 30, 50, 100, 200, 400, and 800 m. We used Spagedi to calculate the average pairwise estimates of relatedness in each distance class and obtain the 95% confidence intervals for these averages based on 20 000 permutations of genotypes among the positions of the sampled trees. To visualize the spatial genetic structure, we plotted the calculated average pairwise estimates of relatedness as a function of distance to generate spatial genetic autocorrelograms. To compare the extent of spatial genetic structure among the studied patches, we calculated the Sp statistic from the spatial autocorrelation analyses (Vekemans and Hardy 2004). The Sp statistic was determined as—$b_F/(1 - F_1)$, where b_F is the regression slope of kinship coefficient estimate (F) on distance classes and F_1 is the kinship coefficient for adjacent individuals in the first distance interval (Hardy and Vekemans 2002).

Results

After adjusting for multiple comparisons (n = 9 loci), significant departures from Hardy–Weinberg equilibrium due to homozygosity excess were observed in microsatellite QpZAG46 (Bonferroni adjusted P value = 0.030). We maintained this locus in all the analyses to make them comparable with previous research performed on holm oaks using the same panel of 9 microsatellite markers (Soto et al. 2007). We found no evidence of linkage disequilibrium among loci, indicating that the analyzed markers can be treated as independent from each other.

Clonal Structure and Genetic Variability

A total of 122 alleles were observed over the 9 analyzed loci. The maximum number of possible genotypes in the study

area (N_g) was 7.06×10^{16}, and the likelihood that individuals were erroneously assigned to the same genotype was small (all $P_{gen} < 0.0008$; Table 1). P_{sex} values were also very low for all multilocus genotypes; hence, we can assume that all resampled genotypes corresponded to true clonal lineages: P_{sex} values ranged from 1.11×10^{-8} for a single resampling to 2.42×10^{-59} for a multilocus genotype resampled 7 times. All studied holm oak aggregations (i.e., groups of multiple stems) belonged to the same genet as all sampled stems shared identical genotypes. However, in 6 cases, 2 nearby groups of stems that were a priori considered as different individuals were different ramets from the same genet (Table 1). In all cases, stems sharing the same genotype were located contiguously and no intergrowth was observed. The maximum distance between ramets ranged between 1.4 and 26.3 m (mean ± standard error [SE]; patch A = 7.5 ± 7.5 m; patch C = 3.0 ± 1.8 m), and the area covered by the studied clones ranged between 5.0 and 318.5 m^2 (mean ± SE; patch A = 73.0 ± 71.1 m^2; patch C = 57.7 ± 25.9 m^2; Table 1 and Figure 2a–b). The area covered by clones did not differ between patches (one-way ANOVA: $F_{1, 25} = 0.425$; $P = 0.520$), whereas we found a non-significant tendency for a higher maximum distance between ramets in patch A (one-way ANOVA: $F_{1, 25} = 3.432$; $P = 0.076$).

Table 1 Clonal structure in 27 holm oak clustered formations

Patch	Genet code	No. of genotyped ramets	Maximum distance between ramets (m)	Cover of genet (m^2)	P_{gen}
A	2	3	2.70	43.27	8.907×10^{-11}
A	3	6	6.30	105.31	7.862×10^{-7}
A	4	5	3.80	81.48	0.008×10^{-1}
A	6	3	3.60	72.37	9.430×10^{-10}
A	7	4	5.50	91.07	5.660×10^{-16}
A	8	2	2.30	75.11	1.080×10^{-12}
A	10	4	2.00	104.81	1.392×10^{-13}
A	11	5	4.10	88.89	6.526×10^{-14}
A	26	3	2.40	11.85	3.185×10^{-12}
A	27	7	3.20	42.90	3.299×10^{-12}
A	28–29	7	17.40	66.75	2.368×10^{-12}
A	30	5	5.90	48.31	1.387×10^{-10}
A	31	2	6.80	11.44	1.143×10^{-13}
A	33	2	6.30	5.04	6.471×10^{-11}
A	34–35	3	26.25	18.22	2.113×10^{-12}
A	49–51.1 –52–53	7	23.80	318.51	5.450×10^{-10}
A	50–51.2	2	5.00	56.15	7.526×10^{-11}
C	13–14	6	3.70	99.82	4.048×10^{-6}
C	15	5	3.50	62.28	4.542×10^{-15}
C	16–17	6	7.30	72.61	1.143×10^{-10}
C	18	6	3.90	78.81	1.029×10^{-11}
C	19	2	1.40	75.58	1.360×10^{-10}
C	20	4	2.90	26.02	3.370×10^{-12}
C	21	4	2.00	68.43	6.478×10^{-13}
C	22	4	1.40	32.62	3.678×10^{-13}
C	24	4	1.80	32.95	3.080×10^{-11}
C	25	3	2.10	27.92	5.168×10^{-10}

Each line corresponds to a single genotype, and P_{gen} indicates the likelihood that the genotype (genet) occurs more than once by random chance. Lines with multiple genet codes are aggregations that showed the same genotype and so were considered to be a single genet.

The holm oak population displayed high levels of genetic diversity (Table 2). However, diversity indexes (A_R, A_e, and H_O) showed lower values in the sapling cohort in comparison with adult reproductive trees in patch B (Table 2). We found no genetic signature of recent demographic bottleneck in any patch or cohort. Wilcoxon signed-rank tests did not reveal any significant excesses of heterozygosity under the TPM, suggesting that the studied populations (patches) have not experienced a recent and/or strong population bottleneck. Similar results were obtained using different percentage (20% and 30%) of multistep mutations in the TPM or when a global analysis including all individuals from the entire study area was performed. The mode shift test did not show any modal shift in allele frequency distribution characteristic of a population bottleneck. Similarly, the *M*-ratios were always higher than the critical value of 0.68 proposed by Garza and Williamson (2001) (Table 2).

Individual genetic diversity did not differ among the study patches (*HL*: $F_{2, 188} = 0.03$, $P = 0.968$; H_O: $F_{2, 188} = 0.09$, $P = 0.915$). However, saplings showed lower heterozygosity than adult reproductive trees in patch B (*HL*: $F_{1, 78} = 6.88$, $P = 0.010$; H_O: $F_{1, 78} = 5.89$, $P = 0.017$) but not in patch C (*HL*: $F_{1, 87} = 9.25$, $P = 0.164$; H_O: $F_{1, 87} = 1.49$, $P = 0.226$) (Figure 3b).

Analysis of Spatial Genetic Structure

F_{ST} values indicated no genetic differentiation between the studied patches when only adult individuals were considered (Table 3). However, we found a highly significant genetic differentiation between sapling cohorts and between some adult-sapling cohorts from different patches (Table 3). Interindividual genetic relatedness decreased as a function of distance class within 2 of the 3 patches of holm oak. Analyses of fine spatial genetic structure revealed significant positive kinship coefficient values within the 20–30 m distance class in patches B and C (*P*s < 0.05; Figure 4b,c). We also detected a negative kinship coefficient value at 200-m distance class in patch B ($P < 0.05$; Figure 4b). However, we found no significant kinship coefficient values in patch A (Figure 4a), although it must be noted that the long distance between trees and the absence of saplings around adult oaks may be underlying this result. When saplings from patches B and C were included into the analysis, kinship coefficient values were significantly higher than expected from a random distribution of genotypes up to 30–50 m distance (all *P*s < 0.05; Figure 5). Including saplings, we also detected negative kinship coefficient values at 200-, 400-, and 800-m distance classes in patch B (Figure 5a) and at 200 m distance in patch C (all *P*s < 0.05; Figure 5b). The *Sp* statistic revealed stronger genetic structure in the less fragmented patch when only adult individuals were considered (*Sp* values, patch A: −0.000008; patch B: 0.000008; and patch C:

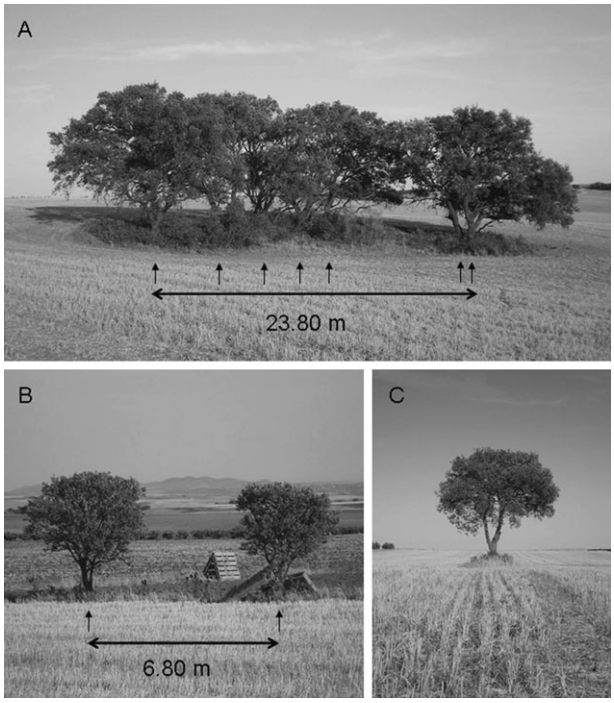

Figure 2. (**A** and **B**) The clonal structure of 2 studied genets and the maximum distance between "ramets" (small arrows indicate genotyped ramets). (**C**) a highly isolated holm oak within the matrix of cereal cultures.

0.000080). When saplings were included into the analyses, the *Sp* statistic also revealed stronger genetic structure in the less fragmented patch (*Sp* values, patch B: 0.000028 and patch C: 0.000045).

Discussion

We analyzed the extent of clonality and the genetic variability and structure in a holm oak population with patches showing different degrees of fragmentation. Microsatellite markers revealed an extensive clonal structure in this species, with most analyzed clumps/aggregations constituting a single genet. As found in other *Quercus* species, all genets comprised numerous closely spaced ramets in absence of intergrowth with only some genets showing a certain tendency to fragmentation in patch A when the area between different ramets has been ploughed (sensu Mayes et al. 1998; e.g., Figure 2B). Clonal structure is likely to be favored by a recurrent clear cutting and a coppice-like management that can stimulate a profuse sprout and stump emergence (Espelta et al. 2003; Valbuena-Carabana et al. 2008). This type of management has been widespread in patch A, where farming is more intensive, and this may explain why the maximum distance between ramets tended to be higher there. It should be noted that maximum distance between ramets could be a better index of clone size in the study system in comparison with clone area because most oaks in the highly fragmented patch are located in field margins and only linear clonal propagation is possible due to the recurrent ploughing of fields (e.g., Figure 2B). The case of genet 49 (in patch A) is especially astonishing, as it constitutes by itself a small wood with 7 trunks with diameters at breast height (dbh) more than 30 cm and a maximum distance between ramets of 23.80 m (Figure 2A). Thus, certain clones can dominate a very large area reducing effective population sizes and local genetic variability (Ainsworth et al. 2003; Valbuena-Carabana et al. 2008). On the other hand, clonal reproduction may also have a positive buffering effect by prolonging the life of genets and, thus, maintaining original population genetic variability over long time periods (Valbuena-Carabana et al. 2008). The clonal structure of holm oaks has also important implications for long-term ecological research on this species because several trees could be considered independent units despite being the ramets of a single "cryptic" genet with the potential problem of pseudoreplication (see Ainsworth et al. 2003).

Similar to previous studies on other oak species, data on genetic diversity only considering a single genotype data per genet revealed high levels of variability in all patches within the study area (e.g., Aldrich et al. 2005; Fernandez and Sork 2005; Valbuena-Carabana et al. 2008). Accordingly, heterozygote excess and shift in allele frequency distribution were not significant, and *M*-ratio values were relatively high for all study patches. As found in several other study organisms, the long generation time of many tree species can contribute to minimize genetic drift by buffering against the rapid loss of genetic diversity (Petit et al. 2002; Aldrich et al. 2005; Fernandez and Sork 2005; Craft and Ashley 2007; see also Hailer et al. 2006). Thus, holm oaks, with a lifespan of several centuries, are likely to retain high levels of genetic variability over time, and our results suggest that the current genetic composition of adult trees in the studied population

Table 2 Mean values of genetic diversity in the study patches

Sample	*n*	*A*	A_R	A_e	H_E	H_O	F_{IS}	*M*-ratio
Adult reproductive trees (patch A)	22	78	8.67	5.16	0.69	0.64	0.07	0.77
Adult reproductive trees (patch B)	42	104	9.34	5.60	0.69	0.68	0.01	0.85
Adult reproductive trees (patch C)	41	93	8.55	5.33	0.69	0.66	0.04	0.83
Saplings (patch B)	38	90	8.36	5.08	0.69	0.59	0.14	0.75
Saplings (patch C)	48	97	8.77	5.84	0.69	0.61	0.12	0.91

The table shows sample size (*n*), total number of alleles observed (*A*), standardized allelic richness (A_R), unbiased effective number of alleles (A_e), expected heterozygosity (H_E), observed heterozygosity (H_O), inbreeding coefficient (F_{IS}), and Garza-Williamson index (*M*-ratio).

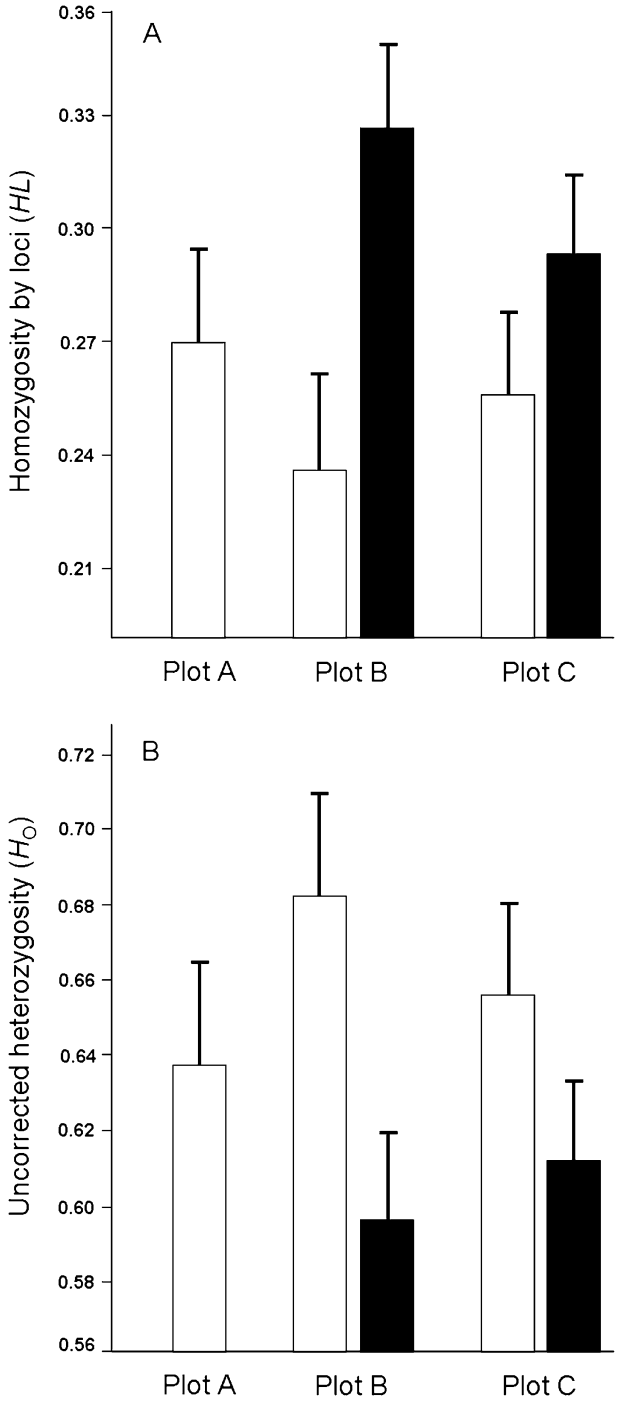

Figure 3. Mean ± SE for (**A**) homozygosity by loci (*HL*) and (**B**) uncorrected heterozygosity (H_O) for sapling (filled bars) and adult reproductive (open bars) holm oaks in the 3 study patches.

has probably been primarily determined by the variability present prior to fragmentation (Craft and Ashley 2007). This could explain both the high allelic diversity observed and the similar values reported in patches with contrasting levels of fragmentation (Craft and Ashley 2007).

Table 3 Pairwise F_{ST} values between adult and sapling cohorts from the different studied patches (below the diagonal) and corresponding *P* values (above the diagonal)

	Adults-A	Adults-B	Adults-C	Saplings-B	Saplings-C
Adults-A	—	0.661	0.958	**<0.001**	0.319
Adults-B	−0.003	—	0.284	**<0.001**	0.352
Adults-C	−0.008	0.002	—	**<0.001**	0.156
Saplings-B	**0.031**	**0.027**	**0.028**	—	**<0.001**
Saplings-C	0.002	0.001	0.003	**0.023**	—

Capital letters refer to patch code (A, B, or C). Values in bold are statistically significant after Bonferroni corrections ($P < 0.05$).

By contrast, reduced genetic variability would be expected to arise in younger holm oak cohorts that have emerged after fragmentation (i.e., saplings) if the isolation of their parents has reduced the levels of gene flow and increased levels of self-fertilization or inbreeding (Young et al. 1996; Fernandez and Sork 2005). Saplings showed lower levels of genetic diversity (allelic richness and number of effective alleles) and lower heterozygosity (*HL* and H_O) than adult reproductive trees in the fragmented patch (B). If reduced levels of genetic diversity increase expression of deleterious recessive alleles or reduce the heterozygote advantage for traits controlled by genes experiencing some form of balancing selection, overall population fitness could be reduced (Charlesworth and Charlesworth 1987; Falconer and Mackay 1996). Another possible explanation for the differences in heterozygosity between adults and saplings is that selection against relatively homozygous young individuals eliminates them before becoming adults, thus increasing the proportion of more heterozygous individuals among adult reproductive trees (Fernandez and Sork 2005).

We have found a complete absence of genetic differentiation among the study patches when only adult reproductive individuals were considered, probably because the current distribution of genetic variability in this cohort is likely to represent that existing before fragmentation, as has been previously suggested for other long-live tree species (Petit et al. 2002; Aldrich et al. 2005; Fernandez and Sork 2005; Craft and Ashley 2007). However, we have found strong genetic differentiation between saplings from patches B and C and between saplings and adult reproductive trees in some pairwise comparisons, suggesting that long-term fragmentation has reduced gene flow and increased genetic drift particularly in younger cohorts (Lowe et al. 2005). Analyses of fine spatial-scale genetic structure have revealed a clear pattern of isolation by distance in the studied holm oak population, with a typical positive spatial autocorrelation at short distances that subsequently declines through zero and becomes negative at larger distance classes (e.g., Peakall et al. 2003). When only adult reproductive trees were considered, we detected kinship structures up to 30 m distance which suggests restricted dispersal within this distance. As expected, the inclusion of saplings in analyses of patches B and C revealed a stronger pattern (up to the 50 m distance class), an effect probably mediated by most saplings being related to

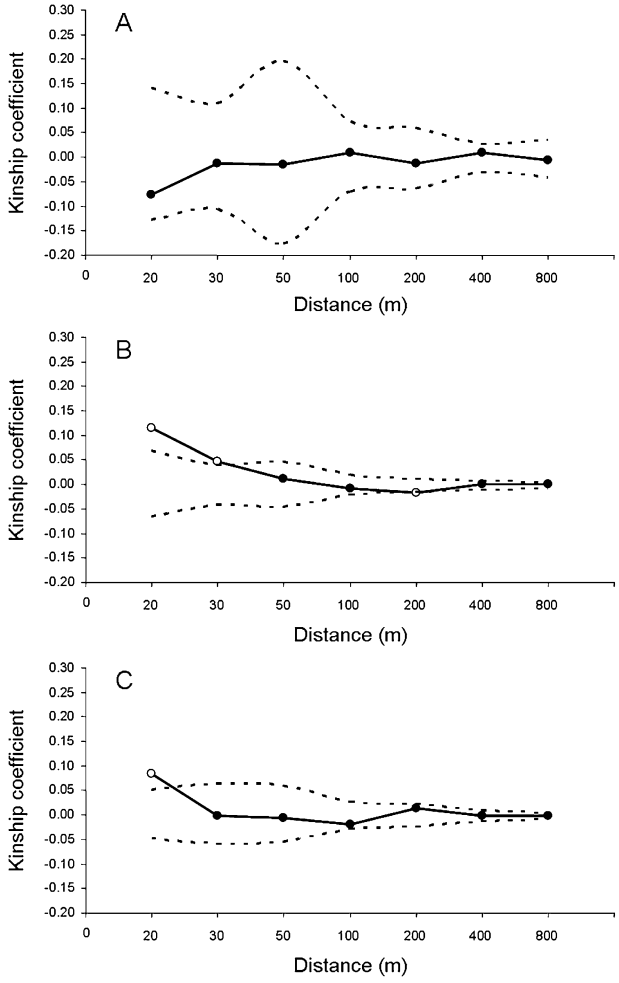

Figure 4. Correlogram plots of the Nason's kinship coefficient as a function of distance considering reproductive trees from the 3 study patches (patch A: panel A; patch B: panel B; and patch C: panel C). Open symbols indicate kinship values that deviate significantly from expected of a random distribution of genotypes. Dotted lines indicate 95% confidence limits under the null hypothesis (based on 20 000 permutations of spatial locations among individuals).

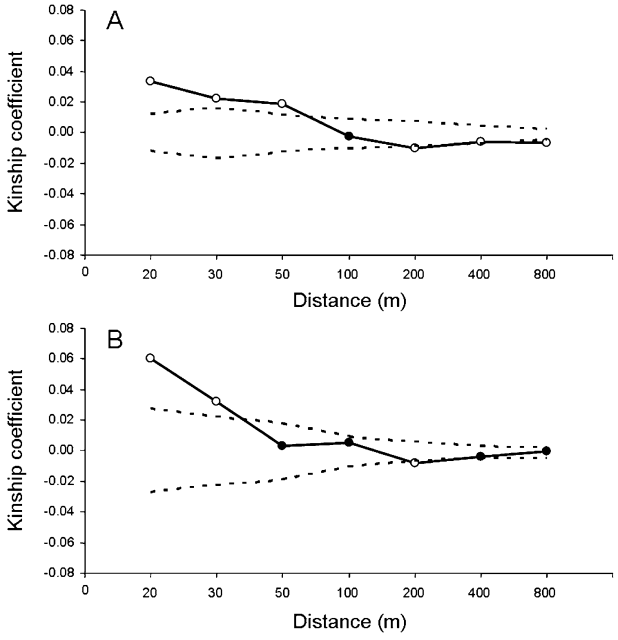

Figure 5. Correlogram plots of the Nanson's kinship coefficient as a function of distance for considering both saplings and reproductive trees from patch B (panel A) and patch C (panel B). Open symbols indicate kinship values that deviate significantly from expected of a random distribution of genotypes. Dotted lines indicate 95% confidence limits under the null hypothesis (based on 20 000 permutations of spatial locations among individuals).

close adult trees (e.g., Soto et al. 2007). The *Sp* statistic suggested stronger genetic structure in the less fragmented patches, probably because higher tree density and successful natural regeneration in that patches have favored the spatial aggregation of genetically related individuals. These results support previous research on both this and other oak species, which have generally reported significant kinship structures within 50 m distances (Soto et al. 2007; reviewed in Aldrich et al. 2005). This pattern of fine spatial genetic structure may have resulted from restricted seed dispersal, given that (rodents) the main acorn dispersers in the study area disperse the acorns at distances below 50 m (Muñoz and Bonal 2007; Muñoz A, unpublished data). On the other hand, pollen dispersal has never been directly studied in the holm oak, but the patterns reported in other oak species have revealed larger dispersal distances which suggest that restricted pollen movement is not the main factor contributing to the observed kinship structures (Dow and Ashley 1996; Dow and Ashley 1998a, b).

Overall, we have found that the fragmentation of our holm oak population has probably reduced both the genetic diversity and the levels of heterozygosity of individuals that have emerged after fragmentation. The most probable explanation is that isolation of trees caused by fragmentation has reduced levels of gene flow and increased self-fertilization or the chance of crosses between individuals sharing a common ancestry. In addition, we have found some remarkable cases of clonal structure among these adult trees, which may have been favored by a recurrent clear cutting and a coppice-like management stimulating a profuse sprout and stump emergence. Conservation managers should be aware that the number of stems present in a patch may not always equal the number of individuals present, and thus, effective population sizes can potentially be significantly underestimated, particularly in patches adjacent to cultivated land. Our results also suggest that holm oak forest fragmentation may lead to the loss of genetic diversity that might compromise their long-term conservation. On the other hand, revegetation of cleared areas with genetically diverse individuals would help to maintain population genetic variability (Burgarella et al.

2007). Besides implementing techniques for the maintenance of keystone scattered trees, such as planting seeds, managing grazing regimes etc. (Manning et al. 2006, 2009), further research is also needed on the consequences of reduced genetic diversity on individual fitness (seed and pollen production, seed dispersal, seedling survival, etc.) and on understanding the patterns of gene flow estimated by direct analyses of mating behavior (paternity analyses, identification of pollen donors) between trees/patches with different degree of fragmentation and spatial isolation. This knowledge will help to ensure the long-term sustainability of species occupying such fragmented landscapes that are nowadays threatened worldwide (Gibbons et al. 2008; Manning et al. 2009).

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