

RESEARCH PAPER

Population genetic structure in *Myrtus communis* L. in a chronically fragmented landscape in the Mediterranean: can gene flow counteract habitat perturbation?

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ABSTRACT

Ancient managed landscapes provide ideal opportunities to assess the consequences of habitat fragmentation on the patterns of genetic diversity and gene flow in long-lived plant species. Using amplified fragment length polymorphism (AFLP) and allozyme markers, we quantified seed-mediated gene flow and population genetic diversity and structure in 14 populations of *Myrtus communis* (myrtle), a common endozoochorous shrub species of forest patches in lowland agricultural Mediterranean areas. Overall, allozyme diversity for myrtle was low ($P_{95} = 25\%$; $A = 1.411$; $H_e = 0.085$) compared to other known populations, and a significant portion of populations (57%) had lower levels of allelic diversity and/or heterozygosity than expected at random, as shown by simulated resampling of the whole diversity of the landscape. We found significant correlations between allozyme variability and population size and patch isolation, but no significant inbreeding in any population. Genetic differentiation among populations for both allozyme and AFLP markers was significant ($\Phi_{ST} = 0.144$ and $\Phi_{ST} = 0.142$, respectively) but an isolation-by-distance pattern was not detected. Assignment tests on AFLP data indicated a high immigration rate in the populations (ca. 20–22%), likely through effective seed dispersal across the landscape by birds and mammals. Our results suggest that genetic isolation is not the automatic outcome of habitat destruction since substantial levels of seed-mediated gene flow are currently detectable. However, even moderate rates of gene flow seem insufficient in this long-lived species to counteract the genetic erosion and differentiation imposed by chronic habitat destruction.

INTRODUCTION

Habitat fragmentation is the process by which an original habitat is reduced and broken into a constellation of habitat remnants that differ in size, shape and connectedness (Fahrig 2003). As a consequence, natural areas and effective population sizes of the species within them are reduced, while the population extinction risk through demographic, environmental and genetic stochasticity is increased (Lande 1988; Hanski 1998). For instance, genetic erosion of remnant populations is expected through the action of drift, increased inbreeding and

reducing incoming gene flow (Ellstrand & Elam 1993; Young *et al.* 1996), which might first result in ill-adapted offspring (Reed & Frankham 2003; Leimu *et al.* 2006) and finally in population extinction (Saccheri *et al.* 1998). Furthermore, the potential for adaptation might also be compromised in the long term as a result of the reduction in variability upon which natural selection can act (Young & Clarke 2000). However, it is now known that the genetic consequences of habitat fragmentation for plant species are more diverse than predicted by simple theoretical models, and that they are related to the differential ability of species to spread their genes across the

landscape (Young *et al.* 1996; Cruzan 2001). Remarkably, some studies have revealed high levels of among population gene flow (via pollen and/or seeds) even in highly fragmented landscapes (e.g. Sork *et al.* 2005; Bacles *et al.* 2006; Jordano *et al.* 2007).

Nowadays, the destruction of tropical ecosystems is considered one of the main threats to biodiversity, and many researchers are devoted to quantifying the ecological, biological and genetic impacts of habitat loss in tropical trees, which are additionally considered to be especially vulnerable (Ward *et al.* 2005). According to theoretical predictions, it has been empirically supported that while the deleterious effects of inbreeding are observed immediately after the fragmentation process, genetic impoverishment is only evident after many generations, which in long-lived species may take centuries. Thus, current studies in these ecosystems are showing the pre-fragmentation population genetic diversity (Lowe *et al.* 2005).

Only recently, have researchers shifted their attention to temperate areas, such as Europe (e.g. Bacles *et al.* 2004; Honnay *et al.* 2006; Jump & Peñuelas 2006), with a much longer history of deforestation dating back to 6000 BP (Williams 2000). In the Mediterranean Basin, for example, forest management has been so intense and severe in past millennia (Grove & Rackham 2001) that forests ecosystems have become 'relictual' (*sensu* McIntyre & Hobbs 1999), being <10% of the potential forest canopy (Primack 1995). Thus, these Mediterranean landscapes provide ideal 'laboratories' to empirically assess the consequences of chronic fragmentation on the patterns of genetic diversity and gene flow in forest plant species. However, in the Mediterranean most studies have been conducted on rare and threatened species (Torres *et al.* 2003; Juan *et al.* 2004), in which the levels of diversity might be compromised because of their own rarity (Gitzendanner & Soltis 2000). Notably, it has recently been confirmed that common plant species are at least as vulnerable as rare species to habitat fragmentation (Honnay & Jacquemyn 2007).

Patch turnover dynamics, in addition to genetic effects like selection on inbred or outbred progeny, can largely determine the maintenance or loss of genetic diversity in fragmented habitats. Indeed, even for long-lived species extinction-colonisation dynamics can have long-term consequences on the present genetic structure. For example, Wade & McCauley (1988) have shown how genetic sub-structuring can occur among a set of populations despite the fact that gene flow is sufficient but not of a random nature. Empirically, in *Sorbus torminalis* Angelone *et al.* (2007) found significant genetic differentiation without any geographical structuring caused by non-directional colonisation events in metapopulation-like dynamics. Thus, the quality and position of particular sources left in a fragmented landscape may have more impact on the genetic outcome of a subdivided population than gene flow levels by themselves.

Highly polymorphic markers, such as amplified fragment length polymorphisms (AFLPs), coupled to the recent development of assignment tests have proved to be an effective tool for detecting immigration events on an ecological time scale (e.g. Tero *et al.* 2003; He *et al.* 2004). In fact, although assignment methods have mainly relied on the use of microsatellite markers (Manel *et al.* 2005), comparative studies have highlighted the higher discriminatory power of AFLPs over microsatellites with a similar analytical effort (Campbell *et al.* 2003; Gaudeul *et al.* 2004). This is supported by the fact that increasing the number of loci is more critical than increasing the allelic diversity per locus (Bernatchez & Duchesne 2000). However, the dominant nature of AFLPs does not allow the assessment of levels of inbreeding, which is a key parameter in fragmentation studies. Allozymes, despite their inherent lower level of polymorphism, have provided useful insights into the historical influence of gene flow and genetic drift in shaping population genetic diversity and structure (e.g. Tomimatsu & Ohara 2003; Bacles *et al.* 2004; van Rossum *et al.* 2004). Further, because of their codominant nature, they can be used to assess inbreeding at both the species and population level (Lowe *et al.* 2004).

In this study, we combined the use of AFLP and allozyme variation and geostatistical data in the common long-lived, animal-dispersed shrub *Myrtus communis* L. (myrtle) living in an extremely and chronically fragmented area, the Guadalquivir River Valley (southern Spain) (Aparicio 2008). Specifically we asked the following questions: (i) are levels of genetic diversity higher in large and well-connected forest patches compared to small and isolated patches; (ii) are levels of inbreeding higher in small and more isolated populations; and (iii) how frequent are among-population seed-mediated gene flow events?

MATERIALS AND METHODS

Study species

Myrtus communis (myrtle; Myrtaceae) is a common circummediterranean shrub up to 4-m high that inhabits low and warm land in holm oak (*Quercus ilex* subsp. *ballota*), cork oak (*Quercus suber*) and pine (mainly *Pinus pinea* and *Pinus halepensis*) forest stands. Myrtle is self-compatible, with polliniferous flowers pollinated by bees and flies (J. P. González-Varo, unpublished results). The fleshy fruits ripen quickly in autumn and are profusely removed by a wide array of small and medium-sized bird species and mammals (Herrera 1984; Aronne & Russo 1997; Traveset *et al.* 2001). Secondary dispersal of seeds by ants has also been reported (Aronne & Wilcock 1994), but seeds germinate in the next season and no permanent soil seed-bank is formed (Traveset *et al.* 2001).

Study site, selected populations and landscape metrics

The study site was in the countryside around the Guadalquivir River valley in western Andalusia (southern Spain),

an agricultural landscape of *ca.* 21,000 km² that is an extreme example of ancient habitat management and deforestation (Grove & Rackham 2001). More recently, the spread of mechanisation in agriculture and reforestation (mostly conifers) have generated the current landscape, where scattered forest patches covering about 1% of the potential area are embedded within the highly agricultural matrix (Aparicio 2008).

In the present study, we focused on 14 patches (*i.e.* populations) of the 162 patches where myrtle is known to occur in the Guadalquivir River valley, representing a wide range of population sizes and degrees of isolation (Fig. 1, Table 1). Inter-patch distance for the selected patches ranged from 4 to 222 km. We estimated myrtle population size by multiplying the average density of individuals in 30 quadrats of 100 m² regularly placed in each patch by the patch area. We measured patch isolation by (i) the Euclidean distance to the nearest neighbour patch (edge-to-edge) where myrtle was detected (ENN), and (ii) the proximity index (PROXI). The first metric is a distance-based method commonly used in fragmentation studies, whereas the second is a weighted distance area-based metric defined as:

$$\text{PROXI} = \sum_{k=1}^n \frac{A_{ik}}{d_{ik}^2}$$

where A_{ik} is the area (in ha) of patch i within a specified neighbourhood, and d_{ik} is the distance (in m) between the focal patch and patch i . This index is dimensionless and it increases as the neighbourhood (defined by a

specified search radius) is increasingly occupied by patches containing myrtle populations. To optimise this variable, we tested four search radii of 1, 2, 5 and 10 km. Given that all the resulting values were highly correlated (Pearson's correlation coefficient ranged from 0.73 to 0.99), we used the 10-km radius as the most integrative measure of the landscape configuration around each focal patch.

Geostatistical metrics were computed with the spatial pattern analysis software FRAGSTATS 3.3 (McGarigal *et al.* 2002), based on a 30-m cell grid generated with ARCGIS 9.0 of the 162 patches where myrtle is found.

Allozyme electrophoresis

In spring 2005, 30 adult plants of myrtle were sampled in the field from the abovementioned 14 populations. To avoid the collection of genets, we collected young leaves of individual plants at least 10 m apart; these were immediately refrigerated. Small pieces of leaves were crushed in three drops of extraction buffer (Werth 1975), and the extracts were stored at -80 °C until electrophoresis.

Allozyme electrophoresis was carried out in 9% starch gels following the general protocols of Weeden & Wendel (1989), and with the staining solutions of Soltis *et al.* (1983). Thirty enzyme systems were assayed in three different buffer systems, eight of which provided consistent patterns of activity and reproducibility throughout the study. The 6-phosphogluconate dehydrogenase (6-PGD, EC 1.1.1.44), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37), menadiene

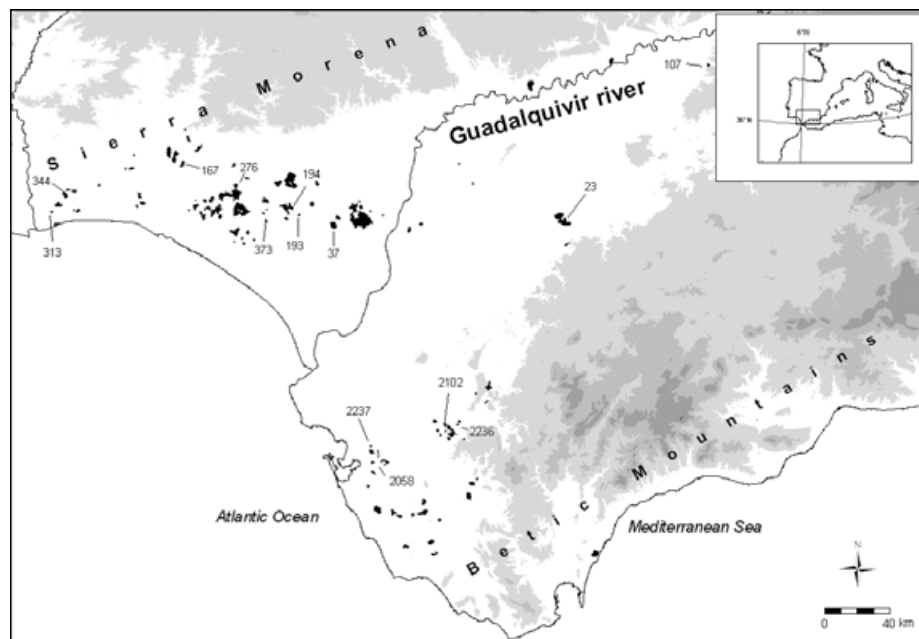


Fig. 1. Map of the Guadalquivir River valley (southern Spain) with the 162 forest patches where *Myrtus communis* (myrtle) is known to occur (black polygons). Labels correspond to the 14 patches selected for this study.

Table 1. Population code, patch area, census of population size, patch isolation and geographical location in UTM coordinates for 14 studied myrtle populations in a fragmented landscape in southern Spain.

population code	patch area (ha)	census size ^a	isolation ^b		UTM geographical coordinates		N _{allozy}	N _{AFLP}
			ENN (km)	PROXI (10 km)	x	y		
23	201.6	3360	0.360	23.3	283248	4125617	30	12
37	244.0	29821	1.333	83	207286	4124616	30	13
107	10.45	69	16.151	0	330626	4177460	28	15
167	32.3	215	0.095	63.3	157819	4145382	30	8
193	14.9	693	0.920	207.4	195908	4128150	30	15
194	93.3	933	0.547	20.5	193081	4130731	30	13
276	61.6	205	0.457	36.4	175107	4137700	30	15
313	8.2	247	2.222	0.3	114440	4129063	28	13
344	30.9	515	0.150	17.4	118838	4135093	30	15
373	9.7	130	1.315	0.7	184187	4128706	24	15
2058	21.4	3499	2.058	0.4	220139	4046577	30	15
2102	5.0	605	0.765	3.3	244201	4056919	30	14
2236	13.0	950	0.690	26.6	248179	4059035	30	13
2237	5.9	238	0.949	1.1	219512	4051899	30	14

AFLP = amplified fragment length polymorphism; UTM = universe transverse mercator; N_{allozy} and N_{AFLP} = sample size for the allozyme and AFLP surveys, respectively.

^a Census size was estimated in 30 quadrats of 100 m² in each patch and then multiplying the average quadrat density per patch area.

^b Patch isolation was measured as the Euclidean distance to the nearest neighbour patch (ENN) and with the Proximity index (PROXI), calculated at a search radius of 10 km around each focal patch (see text for details).

reductase (MNR, EC 1.6.99) and phosphoglucosmutase (PGM, EC 2.7.5.1) enzyme systems were resolved in morpholine–citrate buffer 2 (Weeden & Wendel 1989). The phosphoglucosmutase (PGI, EC 5.3.1.9) and triosephosphate isomerase (TPI, EC 5.3.1.1) systems were resolved in lithium–borate/tris–citrate buffer 6, and the shikimate dehydrogenase (SKDH, EC 1.1.1.25) system was resolved in histidine–citrate buffer 4. Overall, multilocus allozyme profiles were accessible for 410 individual plants.

AFLP fingerprinting

Silica gel-dried young leaves from a random subset of 15 plants from each population were ground with a mixer mill (Retsch MM200, Haan, Germany), and 20 mg of the resulting fine powder were used for DNA isolation with the DNeasy 96 Plant Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol.

After a preliminary screening of 12 primer pairs with the AFLP Plant Mapping Kit (PE Applied Biosystems, Foster City, CA, USA), we selected the three primer pairs that provided the highest number of polymorphic fragments evenly distributed in the range of 75–500 bp (*EcoRI*–*ACA/MseI*–*CTT*, *EcoRI*–*AGC/MseI*–*CTG*, and *EcoRI*–*AGC/MseI*–*CTC*). Amplified labelled fragments were analysed on an ABI 3730 DNA Analyzer (PE Applied Biosystems) and fingerprint profiles were scored with GENEMAPPER 3.7 (PE Applied Biosystems). Unambiguous well-shaped peaks were scored as present (1) or absent (0) to construct a binary character matrix. To confirm AFLP reproducibility and reliability, 15 randomly

chosen samples were duplicated (starting with new DNA isolations) and the scored profiles of 1s and 0s compared side-by-side with their replicates, yielding an average of 94% for scoring repeatability. Overall, scoreable AFLP profiles were generated for 190 individuals.

Data analysis

Within-population genetic diversity

The independence of pairs of allozyme loci in each population was assessed through linkage disequilibrium tests, implemented with FSTAT 2.9.3 (Goudet 2002). The neutrality of the resolved allozyme loci was checked with the Ewens–Watterson neutrality test, implemented with POPGENE 1.32 (Yeh *et al.* 1999). The following standard measures of genetic diversity were computed directly from the allozyme allele frequencies: percentage of polymorphic loci at the 95% level (P₉₅), number of alleles per locus (A) and Nei's within-population gene diversity or expected heterozygosity (H_e). All measures were computed with GDA 1.0 (Lewis & Zaykin 2001). The inbreeding coefficient (F_{IS}) within populations was estimated with FSTAT 2.9.3 (Goudet 2002), and significant departures from Hardy–Weinberg proportions were assessed by randomising alleles among individuals within populations (3360 randomisations).

Genetic diversity parameters are sensitive to sample size, and resampling methods can be used to distinguish between insufficient sampling and a true reduction in genetic diversity (see Banks *et al.* 2000). Furthermore, in the absence of a control (*i.e.* an untouched habitat) at the scale of the study, it is difficult to ascertain whether genetic

diversity has been effectively modified. Therefore, we considered the landscape diversity ($n = 410$ individuals) as the control. First, resampling with replacement (bootstrapping) was applied to the allozyme dataset for sample sizes of $n = 1, 2, 3, \dots, 410$, and the procedure was repeated 1000 times for each sample size. Next, we calculated the means, and recorded from the sorted results the 25th and 976th estimates to obtain a 95% confidence interval (CI) to estimate the expected diversity from a random sample within the sampling range. Finally, we contrasted the observed diversity for each fragment with the expected diversity at a given sample size. We focused on the genetic diversity parameters A and H_e (restricted to polymorphic loci only). Simulations were performed using `MATLAB R12` and functions can be requested directly from J. F. Fernández-Manjarrés. Unlike the allozymes (see Results), a resampling analysis on the levels of H_e based on our AFLP dataset (not shown) showed that at least 35 individuals per population would have been necessary to obtain accurate estimates of within-population genetic diversity. Therefore, we decided to concentrate our site analysis of genetic diversity on allozyme data and use AFLP data at the global population level through the analysis of population structure and assignment tests.

Correlations between allozyme population genetic diversity and both census size and degree of isolation were investigated through Pearson correlations. We also used partial correlations to ensure that the effects of isolation on genetic diversity parameters were independent of the effects of population size. Census size and isolation metric variables were \log_{10} transformed to meet the assumption of linearity (Tabachnick & Fidell 1996). The spatial location of each patch centroid was also characterised by x and y Universe Transverse Mercator (UTM) geographical coordinates and correlated with the genetic parameters to check for the existence of geographical trends in the distribution of genetic diversity.

Among populations genetic structure

For both allozymes and AFLPs, total genetic diversity was partitioned among and within populations with an analysis of molecular variance (AMOVA) based on individual pairwise genetic distances, following Huff *et al.* (1993) and Peakall *et al.* (1995). This method provides a common pathway for the analysis of both dominant and codominant data, allowing direct comparisons. AMOVA was performed with the software `GENALEX 6.0` (Peakall & Smouse 2006).

We examined the spatial distribution of the genetic structure through isolation by distance analysis in the studied populations for both markers using the Mantel test for correlating geographic distance with pairwise population Φ_{ST} values derived with `GENALEX 6.0` (Peakall & Smouse 2006). Congruence in the genetic structure was also detected for both markers with the Mantel test by correlating allozyme and AFLP population pairwise Φ_{ST} matrices. Mantel tests were performed with the software `PASSAGE 1.1` (Rosenberg 2004) and statistical significance was determined by setting 1000 permutations for each test.

Assignment test

To estimate the immigration rate in the set of analysed populations, AFLP data were subjected to individual-based assignment tests by applying the maximum likelihood method for dominant data implemented in `AFLPOP 1.1` (Duchesne & Bernatchez 2002). Because we are aware that we did not sample all the potential source populations, our aim was not to allocate individuals to some of the sampled populations, but to detect genetic outliers (*i.e.* migrant genotypes) in the sampled populations. This program allocates individuals on the basis of log-likelihoods using a 'leave-one-out' procedure in which each individual is withdrawn from its putative population and the allelic frequencies for this population are computed anew. Given that pollen flow might result in ambiguous assignments when low levels of stringency are used (He *et al.* 2004), allocation tests were conducted setting the minimum log-likelihood difference (MLD) to 1 or 2. At the levels of 1 and 2, the program assigns a multilocus genotype to a certain population only if it is 10 or 100 times more likely, respectively, than another one. Before the assignment test, we removed monomorphic loci and used the 'locus filtering' tool of `AFLPOP 1.1` to detect clusters of loci that may be linked (*i.e.* redundant in information content), and removed 23 loci from the analysis. Other settings in the program were: replace zero frequencies by $(1/[\text{sample size} + 1])$ and calculate a P-value for each individual's log-likelihood by creating empirical distributions from 1000 randomly generated genotypes based on the presence frequencies of each population. When the P values for an individual were below a certain warning threshold (<0.001 in our case) for all candidate populations, it was concluded that the individual did not originate from any of the sampled populations (Duchesne & Bernatchez 2002).

Prior to the allocation test, we assessed the power of our dataset for accurate assignment of the real genotypes with the population assignment simulator of `AFLPOP 1.1`. The simulator generated 1000 random genotypes based on the observed allele frequencies in each sampled population. After that, the simulated genotypes were reassigned to the most probable population under $\text{MLD} = 1$. The simulation process was repeated 10 times to check for the consistency of the results. Because of non-significant Φ_{ST} pairwise values (results not shown), we pooled the pairs of populations 37 and 193, 2058 and 2102, and 167 and 373 in the allocation analysis, thus increasing the sample size of some populations and diminishing the risk of misassignments due to similar allele frequencies between population pairs.

RESULTS

Within-population genetic diversity

The eight satisfactorily resolved enzyme systems provided 12 allozyme loci in the 14 populations, seven of which were polymorphic at the 95% level. Two loci were scored

Table 2. Inbreeding coefficient (F_{IS}) and Ewens–Watterson test for neutrality for seven polymorphic allozyme loci in the myrtle.

locus	F_{IS}	Ewens–Watterson neutrality test		
		observed value	U95%	L95%
<i>Pgi-2</i>	0.011	0.658	0.978	0.336
<i>Tpi-1</i>	−0.008	0.867	0.998	0.503
<i>Pgm-1</i>	−0.044	0.752	0.993	0.422
<i>Idh-1</i>	−0.054	0.859	0.998	0.506
<i>Idh-2</i>	−0.039	0.869	0.998	0.504
<i>Mdh-1</i>	−0.074	0.868	0.998	0.503
<i>6pgd-2</i>	0.298	0.863	0.997	0.506
Mean (95% CI)	−0.015 (0.019 to −0.044)			

95% CI = 95% confidence interval, U95% and L95% = upper and lower confidence limits.

A locus is considered neutral when the observed homozygosity falls within the 95% CI obtained under the null hypothesis of neutral evolution.

for the enzyme systems PGI, IDH, 6-PGD and SKDH, and one locus for each of PGM, TPI, MDH and MNR. None of the 79 tests used to detect linkage disequilibrium between pairs of loci in each population were significant after adjustments were made for the significance level ($\alpha = 0.05$) with the sequential Bonferroni correction, suggesting that the scored allozyme loci were independent. According to the Ewens–Watterson test, all seven polymorphic loci could be considered neutral (see Table 2).

At the population level, allozyme diversity was low in all populations, although no population was monomorphic at all loci (Table 3). The percentage of polymorphic loci (P_{95}) ranged from 16.7 (five populations) to 41.7 (two populations). The number of alleles per locus (A) ranged from 1.167 in population 313 to 1.667 in population 23. Gene diversity (H_e) values were consistently low among populations, with an average value (\pm SD) of 0.085 (\pm 0.019). No population departed significantly from Hardy–Weinberg equilibrium (Table 3). The estimate of F_{IS} per locus varied between −0.074 (MDH-1) and 0.298 (6-PGD-2), and was −0.015 (not significantly different from zero) over all loci and populations (see Table 2).

It is apparent from the resampling simulations conducted on gene diversity values that the mean sample size selected to conduct the allozyme study was sufficient to encompass the variability present in the whole landscape (Fig. 2). The resampling analysis showed that eight out of 14 populations (57%) harboured an allelic diversity (A) that was on the edge or below the levels that would be expected for a random sample in this landscape, and further, four populations also displayed lower levels of heterozygosity (H_e) than expected.

All allozyme genetic diversity parameters, except F_{IS} , showed positive and significant correlations with census size (Table 4), indicating that small populations were less genetically diverse. Moreover, H_e also correlated significantly with the degree of isolation, as measured by PROXI, even after controlling for census size, suggesting

Table 3. Genetic diversity assessed by 12 allozyme loci in 14 populations of myrtle occurring in a highly fragmented landscape in southern Spain.

population	Allozymes			
	P_{95}	A	H_e	F_{IS}
23	41.7	1.667	0.130	−0.096 ^a
37	33.3	1.583	0.109	−0.017 ^a
107	16.7	1.333	0.062	−0.055 ^a
167	16.7	1.417	0.082	−0.085 ^a
193	25.0	1.500	0.087	−0.085 ^a
194	25.0	1.500	0.100	−0.030 ^a
276	16.7	1.333	0.087	−0.020 ^a
313	16.7	1.167	0.066	−0.038 ^a
344	16.7	1.250	0.080	0.102 ^a
373	25.0	1.417	0.064	−0.062 ^a
2058	25.0	1.333	0.071	0.059 ^a
2102	33.3	1.417	0.079	0.120 ^a
2236	41.7	1.500	0.092	−0.089 ^a
2237	16.7	1.333	0.079	0.127 ^a
mean	25.0	1.411	0.085	−0.012 ^a
SD	9.24	0.133	0.019	0.080

^a Non-significant ($P > 0.05$) after permutation procedures.

that the larger and closer the neighbouring patches, the higher the gene diversity of the focal patch. However, no genetic diversity measure from allozymes displayed a significant relationship when ENN was used as the metric of isolation. Correlations between genetic and geographical variables were not significant, except that F_{IS} correlated with the geographic coordinate y (see Table 4), indicating that the previous significant correlations were not affected by geographic trends in the genetic diversity at the study site.

Among populations genetic structure

AMOVA of both the allozyme and AFLP datasets were concordant and gave virtually the same result ($\Phi_{ST} = 0.144$ and $\Phi_{ST} = 0.142$, respectively), revealing that a moderate proportion of the variance occurred among populations (Table 5). However, the extant genetic subdivision of the myrtle populations was not linked to spatial distance because the Mantel test results did not indicate a pattern of isolation by distance, either for allozymes ($r = 0.297$; $P = 0.058$) or for AFLPs ($r = 0.205$; $P = 0.105$; Fig. 3). Congruence between markers in explaining the patterns of population differentiation was further supported by the significant correlation detected between the population pairwise Φ_{ST} values for both datasets ($r = 0.307$; $P = 0.032$).

Assignment test

Based on the AFLPOP simulation analysis, the assignment success was above 94% in all the 11 analysed populations, which means that the probability of misassignments in

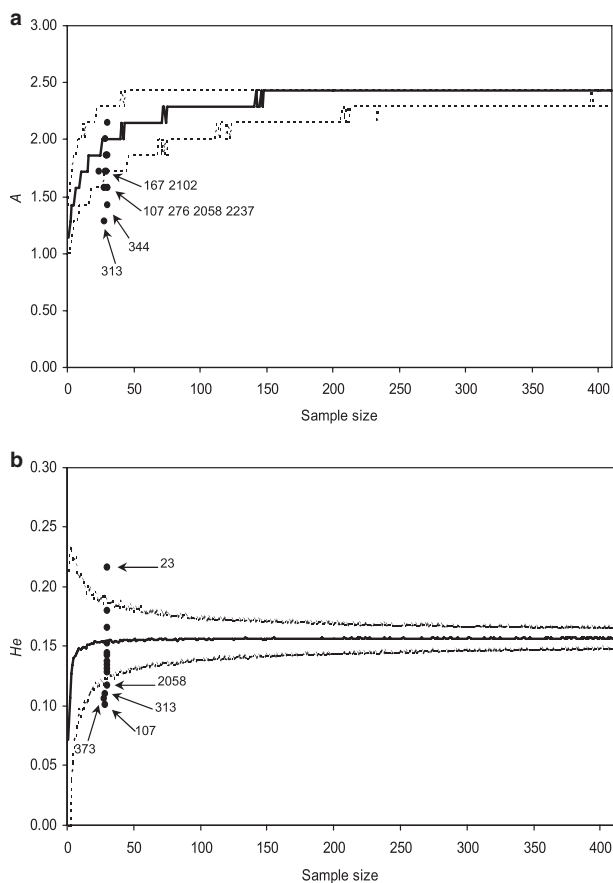


Fig. 2. Results of bootstrap simulations illustrating (a) the number of alleles (A), and (b) the expected heterozygosity (H_e) as a function of sample size. Resampling was performed using 1000 iterations for each sample size. Only populations departing from random expectations are labelled. Note that the values of the sampled populations are higher than those reported in Table 2 because only polymorphic loci were considered for resampling analyses.

our dataset was very low (<6%). A high assignment success is rapidly achieved when markedly different allelic frequencies occur among populations. Thus, while *ca.* 15 individuals per population are generally not enough for obtaining good estimates of within-population diversity, our sampling effort accurately detected immigration events (*e.g.* Albert *et al.* 2006).

As expected, increasing the level of stringency in the assignment test conducted over the real sampled individuals resulted in a greater number of individuals that could not be confidently allocated. Fifty-four (28%) and 84 (44%) individuals could not be confidently allocated when the MLD was set to 1 and 2, respectively. Most individuals that could be confidently allocated were assigned to their putative population (78% and 80% for MLDs set to 1 and 2, respectively) (Fig. 4). Nevertheless, a considerable proportion of individuals genetically resembled another population much more closely than that from which they were collected, indicating the pres-

Table 4. Pearson's product moment correlation coefficients between allozyme-derived genetic variability parameters and population size, degree of isolation and geographical location for 14 populations of myrtle.

population size	isolation metrics		UTM coordinates			
	ENN	PROXI	PROXI (\times census size) ^a	x	y	
Allozymes						
P_{95}	0.634*	-0.071 ^b	0.281 ^b	-	0.469 ^b	-0.382 ^b
A	0.593*	-0.195 ^b	0.577*	0.455 ^b	0.477 ^b	-0.017 ^b
H_e	0.685**	-0.433 ^b	0.655*	0.563*	0.204 ^b	0.011 ^b
F_{IS}	0.023 ^b	-0.042 ^b	-0.377 ^b	-	-0.145 ^b	-0.537*

^a Pearson's partial correlation coefficients for PROXI after controlling for the effect of census size (\times census size).

^b Non-significant, $P > 0.05$.

* $P < 0.05$.

** $P < 0.01$.

Table 5. Allozymes and AFLPs AMOVA results for 14 populations of myrtle in a chronic fragmented landscape in southern Spain.

source of variation	df	SS	Φ_{ST}	P
Allozymes				
among populations	13	77.856	0.144	<0.001
within populations	396	399.728		
AFLPs				
among populations	13	729.213	0.142	<0.001
within populations	176	3040.635		

Df = degree of freedom; SS = sum of squares.

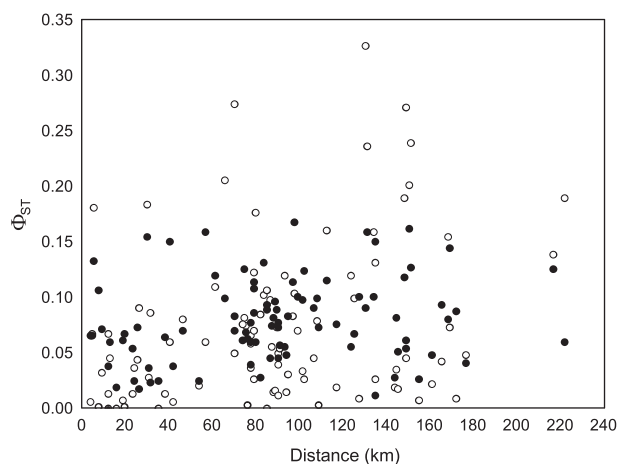


Fig. 3. Scatter plot of Φ_{ST} population pairwise values for allozymes (empty circles) and AFLPs (filled circles) against geographical distance for 14 populations of myrtle in a fragmented landscape in southern Spain.

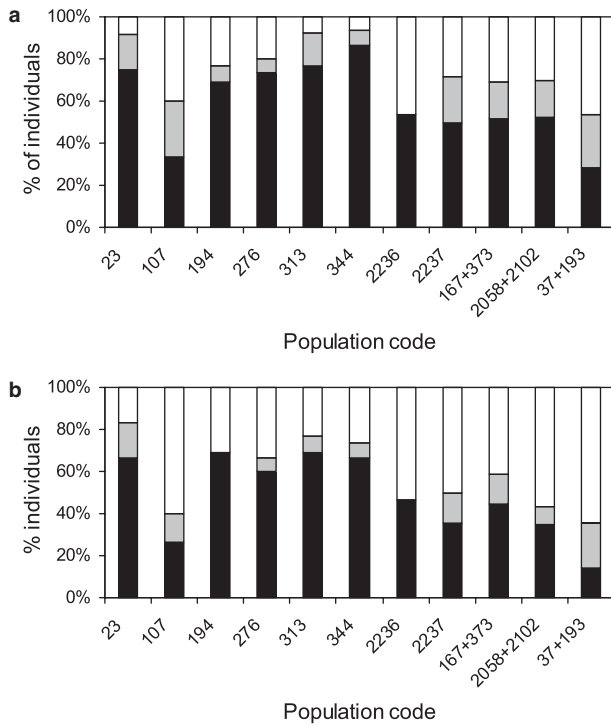


Fig. 4. Results of the assignment test conducted in populations of myrtle in a fragmented landscape with AFLPOP 1.1. (a and b): The results under minimal log-likelihood difference (MLD) set to 1 and 2, respectively. Black bars represent individuals assigned to their putative population; grey bars represent individuals assigned to a population different from the putative population (*i.e.* migrants); and white bars represent individuals not confidently assigned to any population.

ence of migrant genotypes in the set of analysed populations (Fig. 4). Thus, 30 myrtle plants out of 136 (22%) from ten populations were at least 10 times more likely to belong to a population other than their putative populations, whereas 21 out of 106 plants (20%) from nine populations displayed a probability at least 100 times higher.

DISCUSSION

Within-population genetic diversity

In general, our allozyme-based estimates of genetic diversity were lower than those reported for plants in highly fragmented landscapes (*e.g.* Tomimatsu & Ohara 2003; Rossum *et al.* 2004), and also contrasted with the allozymic diversity found by Messaoud *et al.* (2006) for North African (Tunisia) populations of myrtle, where more than twice the allozymic diversity ($P_{95} = 60.3$ versus 25.0; $H_e = 0.215$ versus 0.085) has been reported.

More importantly, the bootstrap analysis over the allozyme data showed that eight out of 14 studied populations had a number of alleles (A) that was below or on the lower boundary of what would be expected at

random in this landscape. Additionally, four populations also displayed lower levels of gene diversity (H_e) than expected. Given that these populations are not spatially concentrated in the landscape (see Fig. 1), the genetic impoverishment is not a consequence of geographic trends at the scale of the study, but is more likely the result of local demographic events (*i.e.* genetic drift after population bottlenecks or founder events) associated with the fragmentation process. In fact, five of those eight impoverished populations (107, 167, 276, 313 and 2237) are small populations that currently harbour <250 individuals (see Table 1). Moreover, genetic drift has an immediate effect on the number of alleles through the stochastic loss of low-frequency alleles, whereas detectable impacts on heterozygosity may take several generations to become evident (Barrett & Kohn 1991; Jump & Peñuelas 2006).

We have found an overall significant correlation between allozyme genetic diversity and population size because most genetically impoverished populations (low number of alleles and gene diversity) were also small populations. However, contrary to other studies (*e.g.* Tomimatsu & Ohara 2003; Llorens *et al.* 2004; Honnay *et al.* 2006; Jump & Peñuelas 2006), the significant correlation between H_e and population size in our study suggests that habitat loss has had a negative and detectable impact on genetic diversity despite the fact that myrtle is a long-lived woody species. Only one population (23) showed higher variability (H_e) than expected, as assessed by the bootstrap analysis, but at present it is difficult to conclude whether this population is representative of the pre-fragmentation genetic structure of the populations in the studied area. Nevertheless, considering that population 23 is located in one of the largest forest patches, the local population size appears to be an important variable in the maintenance of gene diversity.

Unexpectedly, the inbreeding coefficient (F_{IS}) within populations was not correlated with population size. However, as shown by two recent meta-analyses (Leimu *et al.* 2006; Honnay & Jacquemyn 2007), empirical support to the expected outcome of increased inbreeding in small populations is not general. For example, Honnay & Jacquemyn (2007) argued that selection acting against homozygote progeny at the earliest stages of development might be responsible for the lack of correlation between inbreeding (estimated from adult individuals) and local population size. However, to clarify the observed pattern, further studies focused on sampling among different age cohorts will be needed.

It is generally expected that genetic variability and isolation metrics should be correlated (Young *et al.* 1996); however, relatively few empirical works have detected such significant relationships. Historical landscape configurations (*e.g.* Llorens *et al.* 2004), life history traits of species (mainly pollen and seed dispersal) (*e.g.* Honnay *et al.* 2006), or the way in which isolation has been measured (Storfer *et al.* 2007) are not mutually exclusive factors that might explain this fact. In our study, the use of two

isolation metrics gave contrasting results. We detected significant relationships between gene diversity parameters and the proximity index (PROXI), whereas correlations were not significant with the distance to the nearest neighbour (ENN). These results indicate that myrtle populations immersed in a framework of large neighbouring patches were more diverse than less connected populations, which might be the direct consequence of historically high incoming gene flow (via both pollen and seeds) from the vicinity. The lack of relationship between diversity measures and ENN probably reflects the often unrealistic picture that distance-based metrics provide of patch isolation, because they (i) do not consider the proximity of other neighbouring patches beyond the closest one; and (ii) do not take into account the amount of suitable habitat around the focal patch (Bender *et al.* 2003). Distance-weighted area-based metrics, such as proximity indices, seem more biologically realistic because they reflect the number of sources of dispersers that are proximate to a patch as a function of their sizes and distances (Bender *et al.* 2003).

Among populations genetic structure

Unlike the results at the within-population level (results not shown), allozymes and AFLPs gave concordant patterns at the population level. First, AMOVA for both allozymes and AFLPs provided virtually the same results ($\Phi_{ST} = 0.144$ and $\Phi_{ST} = 0.142$, respectively), indicating that myrtle populations in the study area display a significant moderate degree of subdivision, and that they do not constitute a single large panmictic unit. Second, the pattern of differentiation among populations between markers was significantly correlated, as assessed by the Mantel test. This result seems to reflect that, whereas concordance is not detected at the lowest level, each marker type is similarly responsive to the forces of gene flow and drift (Maguire *et al.* 2002; Gaudeul *et al.* 2004).

The population genetic structure derived both from the analysis of the allozyme and AFLP data was not consistent with a pattern of isolation by distance, as expected in a system with a patchy distribution of genetic variability (McCaughey 1993). Two candidate alternative scenarios can explain the disruption of the historical equilibrium between gene flow and genetic drift. On the one hand, the more influential effects of genetic drift on gene flow in a situation of extreme isolation would cause population allele frequencies to evolve independently, resulting in a lack of relationship between genetic and spatial distances (Hutchison & Templeton 1999). On the other hand, a moderate frequency of long-distance dispersal events might result in some spatial structure and ultimately distort the isolation-by-distance pattern (McCaughey 1993; Hutchison & Templeton 1999). We have found support for a scenario involving high levels of seed-mediated gene flow from the results of the assignment test, as will be explained below.

Assignment test

As revealed by the assignment test conducted on the AFLP dataset, at least one individual in ten of the 11 analysed populations showed a genetic resemblance 10 times higher to a population other than the putative one and, furthermore, in nine of the populations some individual plants displayed a resemblance to other population that was at least 100 times higher. The estimated immigration rate in the myrtle populations was similar regardless of the stringency imposed in the assignments (20–22%). It is unlikely that effective pollen flow could generate such a high genetic resemblance to another population because the male gamete contains only half the genetic load of an individual. Thus, effective pollen flow in the last few generations would have generated individuals of mixed ancestry that could not have been allocated to any population with the high stringency criteria used in this study. Therefore, the outlier genotypes detected by the assignment test can be considered the products of successful seed dispersal events, *i.e.* migrants (*cf.* He *et al.* 2004).

Several reasons made us confident of the reliability of the immigration rate estimated in this study. First, the analysis of the simulated data prior to the assignment of the real genotypes revealed a low risk of misassignment (<6%) with our AFLP dataset. Second, most individuals that could be confidently allocated were assigned to their putative populations, which highlights the good performance of the method identifying the sampled populations. Finally, a high number of loci (167 unlinked polymorphic loci in our AFLP dataset) and a relatively high genetic differentiation across populations ($\Phi_{ST} = 0.142$ among the sampled myrtle populations) are two parameters known to increase the efficiency and accuracy of the assignments (Duchesne & Bernatchez 2002; Campbell *et al.* 2003; Gaudeul *et al.* 2004; Manel *et al.* 2005).

The immigration rate estimated in populations of myrtle (20–22%) is much lower than that reported from assignment tests in a metapopulation of the wind-dispersed *Hypochaeris radicata* (68.7%; Mix *et al.* 2006), but higher than those reported for metapopulations of *Silene tatarica* (8.6%; Tero *et al.* 2003) and *Banksia hookeriana* (6.8%; He *et al.* 2004), whose seeds are primarily dispersed by gravity. More importantly, our estimate much resembled that obtained for the frugivore-dispersed tree *Prunus mahaleb* in a southern Spain population, in which data were obtained by means of maternity analysis (19–20%; García *et al.* 2007). In southern Spain, myrtle has a wide array of seed dispersal vectors, which can drive the relatively high immigration rate calculated. This includes small birds (European robins, blackcaps and Sardinian warblers), medium-sized birds (blackbirds, song thrushes and azure-winged magpies) and mammals (mainly foxes) (Herrera 1984; J. P. González-Varo, personal observation). We cannot assess the distance covered by the dispersal events that we detected because logistic

limitations meant that we did not sample all the possible source populations. However, it is known that these frugivores act as 'legitimate seed dispersers', because they release the seeds intact after defecation or regurgitation. Some of them are very efficient in carrying seeds over long distances across the landscape because of their migratory nature (Herrera 1984), facilitating the connection between patches. In fact, in a recent study to determine the differential contribution of dispersers on the seed dispersion curve of *P. mahaleb*, Jordano *et al.* (2007) demonstrated that mammals and medium-sized birds (some of them common to myrtle) were the major vectors for seed dispersal beyond 300 m, involving dispersal events between 1.5 and 17 km. Thus, the Mediterranean landscape, despite its dry and dissected features, may have some resilience to habitat subdivision, and gene flow may not be totally blocked.

An effective method of seed dispersal should also make possible the colonisation of suitable new habitats. Because small populations are more susceptible to extinction, through either stochastic (demographic, environmental and genetic) or extrinsic events (anthropogenic habitat loss and deterioration, *etc.*; Lande 1988; Hanski 1998), a population extinction–recolonisation dynamic is likely to be enhanced in highly fragmented landscapes, even in long-lived species (Bacles *et al.* 2004; Angelone *et al.* 2007). Theoretical studies have stressed that this population turnover might lead to a decrease in the overall genetic diversity relative to the situation in which extinction is absent (McCauley 1991; Hanski 1998), especially if seed sources are scarce, which might explain the overall low levels of genetic diversity in the myrtle populations in this fragmented landscape compared to other regions, as discussed above (Tunisia; Messaoud *et al.* 2006). However, the precise patterns of extinction–recolonisation dynamics in this particular Mediterranean landscape need to be further explored, as we have obtained evidence of seed-mediated gene flow but not of the precise spatial scale and rates at which it occurs.

CONCLUSIONS

Our results join a growing wealth of evidence clearly suggesting that genetic isolation is not the automatic outcome of habitat destruction. The overall low levels of genetic diversity, together with the significant genetic structuring, the lack of isolation by distance produced by the patchy distribution of genetic diversity, and the relatively high levels of gene flow seem to point to an extinction–colonisation population dynamic in the studied populations of myrtle. Our study detected low levels of genetic diversity in small populations but also higher levels of diversity in less isolated and larger patches. This heterogeneity in genetic diversity distribution implies that the long-term maintenance and enhancement of the habitat relies largely on the connectivity among patches to preventing smaller local populations from genetic erosion. Overall, in addition to the demographic events, selection

also appears to be playing a major role, as evidenced by the mostly unchanged values of inbreeding across the landscape. Finally, our findings highlight that substantial levels of gene flow seem insufficient to avoid allelic losses in portions of the landscape and counteract significant genetic differentiation of populations even in this long-lived species.

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