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Exploring the impact of neutral evolution on intrapopulation genetic differentiation in functional traits in a long-lived plant

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Abstract Most plant species, particularly long-lived plants, harbor a large amount of genetic variation within populations. A central issue in evolutionary ecology is to explore levels of genetic variation and understand the mechanisms that influence them. In this study, our goals were to examine the impact of neutral evolutionary processes on the genetic variance and functional diversity within three populations of a long-lived plant (*Quercus suber* L.). For this purpose, we genotyped the progeny of 45 open-pollinated mother trees from three populations originating from Spain, Portugal, and Morocco using six microsatellite markers. Seedlings were planted in a common garden trial and were phenotypically characterized by seven leaf functional traits. Molecular analyses revealed weak genetic differences between Iberian and Moroccan populations. Nevertheless, high genetic differentiation was observed among maternal families within populations. Differentiation between particular maternal families from the same population reached values of 29.2 %, which far exceeds the values reported between the most genetically distant populations for this species (11.7 %). Maternal families differed also in phenology, leaf size, and shape traits. In the Moroccan population, there were correlations among matrices of distances for molecular markers, leaf shape traits (e.g., leaf circularity index), and phenology, indicating that maternal families with contrasting phenologies were genetically and functionally distinct. This, together with the moderate heritability for phenology in Moroccan population, suggests that besides selective forces, neutral evolutionary processes have promoted intrapopulation genetic divergence and contribute to maintain high levels of genetic variation within this population. Overall, our results reinforce the importance of intrapopulation studies in long-lived plants under an evolutionary context.

Keywords Population divergence · Genetic diversity · Phenotypic traits · Leaf shape · Assortative mating · *Quercus*

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Introduction

A central issue in evolutionary ecology is to explore levels of genetic variation and understand the mechanisms that influence on them. The existence of genetic variation within populations is considered essential to effectively respond to biotic and abiotic changes (Hughes et al. 2008; Jump et al. 2009). Most plant species, particularly long-lived plants, harbor an important amount of genetic variation within populations (Hamrick 2004; Petit and Hampe 2006; White et al. 2007). Several evolutionary forces can contribute to maintain such

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levels of intrapopulation genetic variation. In particular, selective processes such as divergent selection in space and time can lead to genetic differentiation and, thus, favor genetic variation within populations. For example, when habitat is heterogeneous, then differences in micro-environmental conditions such as soil moisture and fertility, shading, slope, altitude, orientation, and solar radiation can exert different selective pressures originating spatially divergent selection (White et al. 2007). Genetic differentiation associated with short-distance environmental variations has been documented by an increasing number of studies in many plants (Owuor et al. 1997; Li et al. 1999; Huang et al. 2002; Matesanz et al. 2011) including forest tree species (Mitton et al. 1989; Cobb et al. 1994; Mitton and Duran 2004). Annual fluctuations in weather within a stand may also contribute to create and maintain genetic variation within populations (Bürger and Gimelfard 2002). This happens when different genotypes are favored by selection at different times due to variations in precipitations and temperature (i.e., temporal divergent selection; Ellner and Hairston 1994). Many studies show associations between annual fluctuations in weather and variations in allele frequency of loci across cohorts, which has been usually interpreted as evidence of temporal divergent selection (Epperson 1992; Kelly et al. 2003; Jump and Peñuelas 2005).

Restrictions to gene flow among individuals can be another cause of genetic differentiation within populations. For instance, topography and population characteristics such as tree density and population size can facilitate, modify, interrupt, and restrict pollen movement among individuals within populations (i.e., physical barriers to gene flow). Environmental heterogeneity can also cause phenological disparities because genetic exchange decreases between individuals growing in different microenvironments (i.e., temporal barriers to gene flow; Hirao and Kudo 2008; Cavender-Bares and Pahlich 2009). Phenology can be affected by a high number of environmental cues such as light, temperature, nutrient content, and water availability (Stanton et al. 2000). Thus, the relevance of phenology as a barrier to gene exchange will depend on the degree of environmental heterogeneity and the sensitivity of the species to the environmental variation. Most importantly, when phenology is also heritable, then this kind of assortative mating will contribute to generate genetic differentiation within populations (Fox 2003; Gustafsson and Lönn 2003; Soularue and Kremer 2012). Indeed, recent studies demonstrate that restrictions in gene flow caused by flowering time divergence are a reproductive isolating mechanism increasing genetic variation, promoting adaptive divergence, and, potentially, leading to adaptive radiation and sympatric speciation (Petit et al. 1997; Weis and Kossler 2004; Cavender-Bares and Pahlich 2009).

Cork oak (*Quercus suber* L.) is a wind-pollinated, evergreen species widely distributed in western Mediterranean Basin. Like other sympatric Mediterranean oak species, cork oak defines the ecosystem and plays a keystone resource role for wildlife (Bussotti et al. 2003; Aronson et al. 2009). Previous studies have shown the existence of high levels of genetic variation in functional traits within populations (Ramírez-Valiente et al. 2011). Balancing selection, caused by year-to-year fluctuations in climate, has been suggested as one of the potential mechanisms promoting genetic differentiation and increasing genetic variance within populations (Ramírez-Valiente et al. unpublished).

Here, our goals were to determine the extent to which neutral evolutionary processes have influenced on levels of genetic variation and genetic differentiation within populations and explore the potential underlying mechanisms for the observed pattern. For this purpose, we studied the progeny of 45 mother trees originating from three distant cork oak populations from Morocco, Spain, and Portugal. Specifically, first, we compared the genetic differentiation among populations and among maternal families within populations in neutral molecular markers in order to understand how genetic variation is structured in hierarchical groups. Second, we investigated whether differentiation at neutral markers was correlated with phenology and a set of morphofunctional traits across maternal families. Significant positive correlations would be evidence that factors affecting neutral evolution such as restrictions to gene flow among individuals also shape levels of genetic variation in functional traits within populations.

Materials and methods

Populations and common garden

The study was conducted with the progeny of 45 mother trees originating from the three populations of the Western distribution range of the species (Ramirez-Valiente et al. 2014). A more detailed description of the three plots is given in Catalan (2003) and a summary of their geographic and climatic characteristics is reported in Table 1.

Within each population, 15 mother trees were randomly selected. They were separated at least 150 m from each other to avoid familial structures (Soto et al. 2007). Seeds were collected from the mother trees during winter of 1996. They were sown in the beginning of 1997 and grown in a nursery for a year under optimal conditions of water and nutrient availability for the species. Then, they were planted in a common garden experiment during the spring of 1998.

The common garden experiment of cork oak (*Q. suber* L.) was established in the "Sierra de Andújar" Natural Park (38° 21′ 54″ N, 3° 51′ 40″ W, 560 m a.s.l.), which is located in Southern Spain, within the core of the cork oak natural range. The trial follows a design of randomized complete blocks. Climate in the trial site is Mediterranean, characterized by dry

Table 1 Location and climatic characterization of the studied populations

Population	Country	Latitude (N)	Longitude (W)	Altitude (m)	Pa (mm)	Ps (mm)	T (°C)
Alcácer do Sal (P)	Portugal	38° 22′	8° 32′	30	709	31	16.3
La Almoraima (S)	Spain	36° 16′	5° 22′	118	813	20	17.4
Aïn Rami (M)	Morocco	35° 04′	5° 12′	425	865[a]	10[a]	17.4

[a] Obtained from Worldclim (Hijmans et al. 2005)

Pa annual precipitation, *Ps* summer precipitation, *T* mean annual temperature

warm summers and moderately cold winters. The average annual precipitation is 617 mm and the average annual temperature is 14.7 °C. The average temperatures for the most contrasting months are 5.9 °C in January and 25.3 °C in July. Most precipitation falls between October and January (329.3 mm), and very little during the summer months (34.3 mm on average from June to September) (data for the interval 1987–2005, taken from the closest climatic station to the plot, located about 4 km from the common garden).

Microsatellite genotyping

From five to eight plants from each maternal family (264 individuals in all) were genotyped using six nuclear microsatellites transferred to cork oak from other *Quercus* species. DNA was extracted from leaves following the method described by Doyle and Doyle (1990). A total of six $(GA)_n$ nuclear microsatellites were used, transferred from other *Quercus* species—*Qp*ZAG9, *Qp*ZAG15, and *Qp*ZAG46 developed in *Quercus petraea* (Matts.) Liebl. (Steinkellner et al. 1997); *Qr*ZAG7, *Qr*ZAG11, and *Qr*ZAG20 developed in *Quercus robur* L. (Kampfer et al. 1998). Amplification and scoring were performed following Soto et al. (2003). Only four individuals amplified with all microsatellites in the maternal family "P40" from Portugal. As a consequence, this maternal family was excluded from further analyses.

Leaf morphology

Leaf morphology was determined for eight plants per each maternal family in 2006. Climatic conditions during this year were similar to average values. Annual rainfall was 600.6 mm and annual temperature 15.6 °C. Four sun spring leaves from three orientations (N, SE, and SW) were collected for each sampled plant (12 leaves per plant in total) to characterize leaf morphology (N=360 plants × 12 leaves/plant =4,320 leaves in all). Then, leaves were digitalized and analyzed using the software WINFOLIA v. 2002 and Image J. Average leaf size of the plant was estimated by means of several morphological leaf traits—individual leaf area, maximum length, maximum width, and perimeter. All these parameters were strongly correlated among each other (all r>0.80, P<0.0001) so we finally used only leaf area (leaf size from now on) for further analyses. Leaf shape was measured by (1) circularity index ($4\pi A/P^2$), where A is the leaf area and P is the perimeter. This index grades the leaf shape between circular and filiform. (2) Aspect ratio or elongation factor, which is the ratio of maximum horizontal width to vertical length (W/L) and (3) perimeter square/area ratio (PA), which is usually called shape factor. High PA indicates more complex leaf shape (e.g., lobing and dissection). Leaves were oven dried at 65 °C to a constant moisture after scanning. Specific leaf area (SLA) was estimated in one leaf per orientation (three leaves per plant) using the ratio of dry weight to leaf area of one leaf.

Biochemical traits

Two biochemical leaf traits that are highly related to control of water losses and water use efficiency were also measured: 13-carbon isotope discrimination ($\Delta^{13}C$) and nitrogen leaf content per unit mass (N_{mass}). For this purpose, after drying the leaf material sampled for leaf morphology, it was ground in a ball mill following Ramírez-Valiente et al. (2011). This material was used to determine the isotopic composition of C^{13} ($\delta^{13}C$) with a MicroMass Isochrom mass spectrometer and leaf nitrogen content by the Kjeldahl method (Vapodest 50, Gerhardt) for each plant. The $\delta^{13}C$ values were used to estimate the isotopic discrimination ($\Delta^{13}C$) as:

$$\Delta^{13}C(‰) = \delta_a - \delta_p / \left(1 + \delta_p / 1,000\right)$$

where δ_p (‰) refers to $\delta^{13}C$ of bulk leaf material and δ_a is the $\delta^{13}C$ of the air acting as carbon source (it was assumed to be −7.9‰).

Phenology

Since cork oak plants in the common garden were still in a juvenile stage in the sampled year, we could not record their reproductive phenology. Nevertheless, vegetative and reproductive phenologies have been suggested to be under common genetic control and regulation (Rohde and Bhalerao 2007; Horvath 2009). In fact, as observed in other forest tree species from temperate climates, Díaz-Fernández (2000) showed that reproductive and vegetative phenologies are highly correlated in

cork oak. In this study, we evaluated vegetative phenology in the progeny of the 45 maternal families in spring 2006 since it is associated to flowering phenology in adults (see Piotti et al. 2012 for a similar procedure). For this purpose, five shoots around the plant crown were selected. Vegetative phenology was recorded using a subjective scale from 1 (no presence of vegetative buds) to 5 (new leaves totally expanded) in a single point during the vegetative growth season. Thus, we captured the vegetative stage of maternal families in mid spring (Díaz-Fernández 2000).

Statistical analyses I: genetic diversity and structure based on molecular markers

Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium (LD) were tested at each population using Genepop v4.0 (Rousset 2008). We calculated expected heterozygosity (H_e) and inbreeding coefficient (F_{IS}) for each population using ARLEQUIN, v3.11 (Excoffier et al. 2005) and Genepop v4.0, respectively (Rousset 2008). Measures of allelic richness (A) and number of private alleles (A_{priv}) were estimated at each population and later standardized for sample size using the program HP-Rare (Kalinowski 2005).

Genetic structure of populations was studied using STRUCTURE 2.3.1, a Bayesian model-based clustering method which assigns individuals to clusters based on their multilocus genotypes (Pritchard et al. 2000). We ran STRUCTURE assuming correlated allele frequencies and admixture. The maternal family structure was used as prior information to assist the clustering. Ten independent runs for each value of K (number of population clusters) were conducted. 10^6 MCMC cycles, following a burn-in period of 100,000 iterations were performed. The true number of clusters was obtained following Evanno et al. (2005).

A partition of the molecular variance among populations and among open-pollinated maternal families within populations was also performed using a hierarchical analysis of molecular variance (AMOVA) with ARLEQUIN version 3.11 (Excoffier et al. 2005). Pairwise genetic distances between maternal families (called here F_{SC}) were calculated and an UPGMA dendrogram was produced. We also assessed evidence for the contribution of each single locus to population structure. For this purpose, we performed analyses of the molecular variance (1) for each single locus and (2) for all loci excluding one locus in each analysis. As reported in Soto et al. (2003), some loci did not follow strictly the stepwise mutation model (SMM) proposed for microsatellite by Kimura and Ohta (1978) in cork oak, so that the infinite allele model (IAM) was used in the analysis of molecular variance.

Statistical analyses II: differences in functional traits and phenology among maternal families and their relationships with molecular markers

Linear mixed models were performed to test for differences among populations and maternal families within populations in functional traits and phenology. The model equation was:

$$Y_{ijkn} = \mu + H_n + B_i + P_j + F(P)_{jk} + BP_{ij} + E_{ijkn}$$

where Y_{ijkn} is the observed value for the variable considered in the n tree of the k open-pollinated family from the j population into the i block; μ is the general mean, H_n is the fixed effect of initial plant size for individual n, B_i is the fixed effect of the i block, P_j is the fixed effect of the j population, $F(P)_{jk}$ is the random effect of the k maternal family nested within the j population, BP_{ij} is the random effect of the interaction between block i and population j, and $Eijkn$ is the residual error for $Yijkn$. We used procedure PROC MIXED from the statistical package SAS 9.2 (SAS/STAT® Software; SAS Institute).

We also examined whether genetic differentiation in neutral markers showed association with functional traits and phenology. For this purpose, we calculated the average values for each maternal family and trait. Then, we constructed matrices of pairwise distances for each trait by estimating the absolute value of the subtraction between each pair of maternal families. Simple and partial Mantel tests were performed between phenotypic, phenological, and F_{SC} genetic matrices. Although low level of population structure for neutral markers was found in these three cork oak populations (see "Results" section), we followed the conservative criterion of analyzing the three populations separately. Mantel tests were performed using ZT software with 10,000 permutations (Bonnet and Van de Peer 2002).

Statistical analyses III: heritability of phenology

In order to investigate the potential genetic control of phenology, narrow-sense heritability was calculated for this trait within each population (Visscher et al. 2008). The narrow-sense heritability on an individual basis for each population was estimated as follows:

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2}$$

σ_a^2 represents the additive genetic within each population and σ_p^2 is the total phenotypic variance (total variance of

random effects plus residual term). Additive variance was estimated as:

$$\sigma_a^2 = \frac{1}{2\theta} \times \sigma_m^2$$

where σ_m^2 represents the variance given by the maternal family within each population and θ is the coancestry coefficient, which was obtained for each population using molecular markers (see Gaspar et al 2009 for a similar procedure). Variance components and covariance-variance matrices for estimates of heritability were obtained implementing a mixed model for phenology (see text above). Standard errors of the heritabilities were estimated by the DELTA method (see Lynch and Walsh 1998 for details).

Results

Inter- and intrapopulation genetic differentiation in molecular markers

The mean number of alleles per locus was 6.2, and ranged from 4 (*Qp*ZAG46) to 9 (*Qr*ZAG15). One locus (*Qr*ZAG11) deviated from Hardy-Weinberg equilibrium (HWE), but the F_{ST} value did not significantly vary with the probability of departure from HWE (*Qr*ZAG11 F_{ST}=−0.002 P=0.533; general F_{ST}=0.029, P<0.001). After FDR corrections, there was no significant linkage disequilibrium for each locus pair across populations (data not shown). The levels of observed and expected heterozygosity were fairly high across the whole sample (mean H_O=0.47, H_E=0.57; Table 2) and they displayed low level of population variation (Table 2). The F_{IS} ranged from −0.01 to 0.05 and was not significantly different from zero for any population (Table 2).

Bayesian clustering analysis with STRUCTURE revealed weak, but significant, population structure. Results of our structure analyses showed maximum ΔK for K=2 (Table S1, Fig. S1), reasonably separating Portuguese and Moroccan populations (Fig. 1a). The Spanish population showed intermediate genetic assignment between these two populations (Fig. 1a). Another relevant result derived from STRUCTURE analysis was the observation that certain maternal families from a given population presented a high assignment to other population. Examples of this pattern can be found in Portuguese maternal family P43, which was assigned to cluster 2 (Morocco-Spain), or Moroccan maternal family M25, which was assigned to cluster 1 (Portugal; Fig. 1b).

Table 2 Genetic diversity statistics of cork oak populations using six microsatellite loci. Measures of allelic richness (A) and number of private alleles (A_{priv}) were estimated at each population and later standardized for sample size using the program HP-Rare (Kalinowski 2004, 2005)

Population	n	A	A_{priv}	H_O	H_E	F_{IS}
Portugal	79	4.8	0.58	0.49 (0.14)	0.58 (0.16)	0.047[a]
Spain	89	3.9	0.21	0.44 (0.21)	0.53 (0.22)	−0.013[a]
Morocco	96	4.5	0.80	0.50 (0.21)	0.60 (0.20)	0.051[a]

Standard deviations are shown in parentheses

H_E and H_O expected and observed heterozygosities, F_{IS} fixation index, *Prob* associated probability to F_{IS}

[a] Not significant at 5 % level

The hierarchical analysis of molecular variance over all loci revealed that differences between populations accounted for only 2.27 % of total variation although this value was significantly different from zero (F_{ST}=0.023, P<0.001). In accordance with this, UPGMA also showed a weak genetic grouping of maternal families from the same populations (Fig. 1b). In contrast, the variance among maternal families within populations accounted for 7.12 % (F_{SC}=0.071, P<0.001) of the total genetic variance.

After removing any single locus from the analysis, the partition of the variance in its different components remained nearly invariable except when *Qp*ZAG46 was excluded (Table 3). The among-population differences for this single microsatellite were the highest of all loci (F_{ST}=0.092, P<0.001; Table 3) and its exclusion significantly diminished the general F_{ST} value (general F_{ST}=0.007, P=0.041; Table 3).

Pairwise distances among populations were quite low, but all of them were significantly different from zero (P<0.001). Pairwise F_{ST} ranged from 0.010 for La Almoraima-Aïn Rami to 0.042 for Aïn Rami-Alcáçer do Sal. Additionally, a considerable high number of pairwise F_{SC} distances among maternal families were also significant (481 out of 946). Genetic distances between certain lines from the same population were particularly high (Fig. 1b). For instance, pairwise genetic distance between the maternal families "M20" and "M25" from Morocco was 29.2 % (F_{SC}=0.292, Fig 1b). These results were nearly the same even excluding any single loci from the analyses (data not shown).

Relationships between molecular and phenotypic data

Mixed models results showed differences among populations in phenology, leaf size, PA, and marginal differences in SLA (Table 4). Differences among maternal families were also found in leaf size, WL, and CI and marginally in PA (Table 4). Phenology did not show differences among maternal families (Table 4). This result was due to the limited intrapopulation variation in this trait within the Portuguese population. In fact,

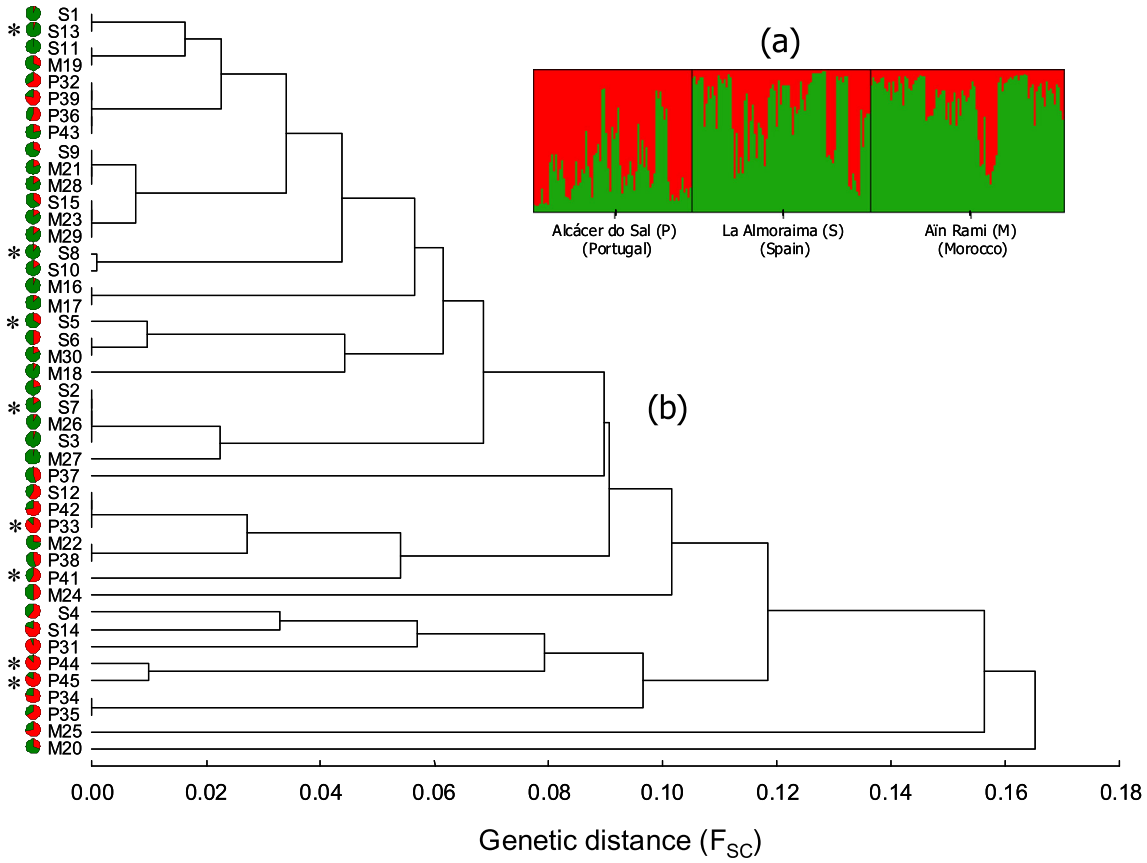

Fig. 1 *a* Bayesian clustering of six microsatellites across 264 individuals originating from the three studied populations. *Vertical bars* indicate the individual assignment to $K=2$ demes according to *STRUCTURE*. *b* UPGMA cluster analysis using F_{SC} distances obtained from six microsatellite data for 44 open-pollinated maternal families originating from three cork oak populations. *Circles* indicate maternal family frequencies of deme assignment from the *STRUCTURE* $K=2$. *Asterisks* indicate open-pollinated maternal families with percentage of full-sibs higher than 20 %

when populations were analyzed separately, Moroccan and Spanish populations showed high heritability values (see "Results" below).

Mantel tests showed that the matrices of distances of two leaf shape traits—CI and PA—were correlated to the genetic matrix for the Moroccan population (Table 5). Genetic matrix was also significantly correlated with among-maternal-family matrix of distances of phenology for the Moroccan population (Fig. 2, Table 5). Likewise, CI and PA were correlated to phenology in this population ($r=0.23$, $P=0.018$ for CI and $r=0.23$, $P=0.02$ for PA; Table 5). No significant correlations were observed in the other populations Table 5. Partial Matel tests showed that the positive correlation between the matrices of distances for phenology and CI became non-significant after conditioning on the genetic structure obtained using neutral markers (Table 6). In contrast, the correlation between phenology and PA ratio did not significantly change after correcting by genetic structure (Table 6).

Table 3 Single locus F_{ST} and general F_{ST} (estimated for all loci excluding the locus being considered) for six microsatellite. *P* values are shown

Locus	Single locus F_{ST}	*P*	General F_{ST}	*P*
*Qp*ZAG9	0.004	0.044	0.024	<0.001
*Qp*ZAG15	0.002	0.088	0.026	<0.001
*Qp*ZAG46	0.092	<0.001	0.007	0.041
*Qr*ZAG7	0.030	<0.001	0.020	<0.001
*Qr*ZAG11	−0.002	0.533	0.029	<0.001
*Qr*ZAG20	−0.004	0.647	0.029	<0.001

Heritability of phenology

Results derived from molecular analyses following Gaspar et al. (2009) showed that the percentage of full-sibs was 9.6 % in the progeny from Spain, 0 % in Morocco, and 13.0 % in Portugal. As a consequence, the additive genetic variance was estimated as $3.65\sigma^2_m$ for Spain, $4\sigma^2_m$ for Morocco, and $3.54\sigma^2_m$ for Portugal, where σ^2_m represents the variance given by the maternal family within each population (see Hardy et al. 2004 for details). Vegetative phenology showed high heritabilities for Moroccan ($h^2=0.80\pm0.18$) and Spanish ($h^2=0.68\pm0.16$) populations. In contrast, vegetative phenology had low heritability values in the Portuguese population ($h^2=0.23\pm0.08$).

Table 4 Significance levels from mixed models for phenology and leaf traits for plants from 45 maternal families from three cork oak populations

	Pop	Maternal family	Block	Pop × block	Plant size
Phenology	<0.001	0.264	<0.001	0.033	0.477
SLA	0.064	0.131	0.292	0.672	0.347
LS	<0.001	0.044	0.138	0.961	0.001
WL	0.718	0.003	<0.001	0.167	<0.001
CI	0.825	0.003	<0.001	0.278	0.019
PA	<0.001	0.074	0.001	0.966	0.006
$\Delta^{13}C$	0.483	0.457	<0.001	<0.001	0.011
N	0.549	0.195	<0.001	<0.001	<0.001

Fixed effects, population ("Pop") and block; random effect, maternal family (within population); covariate, plant size

LS leaf size, *WL* leaf width/length ratio, *CI* circularity index, *PA* perimeter square/area perimeter, *SLA* specific leaf area, $\Delta^{13}C$ 13-carbon isotope discrimination, *N* nitrogen leaf content per unit mass

Discussion

Genetic differentiation among populations

We found that the studied cork oak populations from Iberia and Morocco showed significant but low level of genetic differentiation in molecular markers (F_{ST}=0.023). However, the six microsatellites showed contrasting levels of genetic differentiation among populations. All microsatellites had F_{ST} values nearly zero except one of them, for which F_{ST}≈0.10. It has been long suggested that microsatellites with high F_{ST} values may be located in genomic regions that are under divergent selection (Clark et al. 2007). Here, we found that *Qp*ZAG46 was the one exhibiting high levels of genetic differentiation among populations. In fact, general F_{ST} significantly decreased (F_{ST}=0.007) after removing this microsatellite from the analyses. This microsatellite is considered highly useful to differentiate evergreen oak species, particularly in hybridizing zones, due to its high levels of genetic differentiation among species (Cavender-Bares and Pahlich 2009; Ortego and Bonal 2010). Our intraspecific studies on cork oak also showed high among-population genetic differentiation at this locus supporting the idea of previous studies that this locus is linked to genes encoding for adaptive traits in oaks (Ramírez-Valiente et al. 2009, 2010).

Table 5 Mantel tests of association among genetic distances (GEN) and maternal-line distances in phenology (PHEN), and functional leaf traits for each studied population. Columns represent *R* coefficients. Significant R coefficients (P < 0.05) are typed in bold. Significant values after FDR corrections are typed in italic. In order to control the inflation of type I error derived from repeated testing, the false discovery rate (FDR, the expected proportion of tests erroneously declared as significant) criterion was applied

Population			Matrices	
Spain	Morocco	Portugal		
−0.09	***0.27***	0.04	GEN	PHEN
−0.07	0.17	−0.16	GEN	LS
0.07	0.14	−0.19	GEN	WL
−0.11	***0.31***	0.14	GEN	CI
−0.01	***0.32***	−0.17	GEN	PA
0.07	0.15	−0.13	GEN	SLA
0.05	−0.06	0.03	GEN	$\Delta^{13}C$
−0.10	0.06	0.24	GEN	N

LS leaf size, *WL* leaf width/length ratio, *CI* circularity index, *PA* perimeter square/area perimeter, *SLA* specific leaf area, $\Delta^{13}C$ 13-carbon isotope discrimination, *N* nitrogen leaf content per unit mass

Genetic differentiation within populations

Molecular analyses using six microsatellite markers revealed high genetic differentiation among open-pollinated maternal families within cork oak populations. Differentiation between certain maternal families from the same population reached

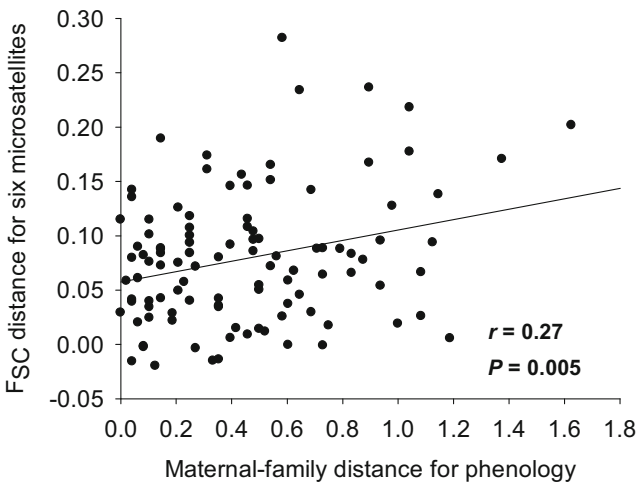

Fig. 2 Correlation between maternal family distance for phenology and F_{ST} distance for microsatellites for 15 maternal families originating from Morocco. The Pearson's correlation coefficient (*r*) and the significance level are shown

Table 6 Partial Mantel's tests of association between phenology (PHEN) and functional traits controlling for the effects of neutral genetic distances for Moroccan maternal families (GEN)

Matrices			*R* statistic	*P*
PHEN	LS	GEN	**0.334**	0.013[a]
PHEN	WL	GEN	0.207	0.079[b]
PHEN	CI	GEN	0.173	0.125
PHEN	PA	GEN	**0.297**	0.017[a]
PHEN	SLA	GEN	**−0.376**	0.003[c]
PHEN	Δ^{13}C	GEN	0.116	0.235
PHEN	N	GEN	0.323	0.051[b]

LS leaf size, *WL* leaf width/length ratio, *CI* circularity index, *PA* perimeter square/area perimeter, *SLA* specific leaf area, $\Delta^{13}C$ 13-carbon isotope discrimination, *N* nitrogen leaf content per unit mass

[a] Significant values

[b] Marginally nonsignificant values. Significance levels were corrected by FDR criterion

values of 29.2 %. This observation contrasts with the limited genetic differentiation among populations found in the entire distribution range of the species, even considering long-distance populations (Toumi and Lumaret 1998). In fact, in our previous study on Iberian cork oak populations using the same six microsatellites, the highest differentiation value was 11.7 %, observed among two populations separated 400 km in distance and 1,200 m in altitude (Ramírez-Valiente et al. 2009).

Several researchers have detected significant genetic differentiation in neutral molecular markers at small spatial scale for a broad number of organisms including forest tree species such as conifers (Senneville et al. 2001; González-Martínez et al. 2010) and oaks (Alberto et al. 2010). Restricted gene flow due to limited pollen dispersal has been suggested as the main force causing genetic structure for neutral markers within plant populations. Limitations to pollen flow can be created at small distances due to physical barriers such as topography, slope, orientation, and microrefugia or by temporal restrictions due to flowering phenology disparities among genotypes (Schuster et al. 1989; Cavender-Bares and Pahlich 2009).

We could not examine reproductive phenology in the mother trees due to the fact that they were not marked during seed collection. Here, we used vegetative phenology in their progeny because of the observation of high correlation with both male and female flowering across years (r=0.77, P<0.0001; r=0.71, P<0.0001, respectively) (correlations elaborated from Díaz-Fernández (2000) collected in 10 mother trees measured across three consecutive years) and their common genetic control (see Rohde and Bhalerao 2007; Horvath 2009; Piotti et al. 2012). Phenology showed a moderate heritability in Morocco and its pairwise distance matrix was strongly correlated with matrix of molecular data in this population. These results suggest that possible differences in phenology could prevent gene exchange among genotypes, promoting high genetic differentiation among maternal families (Fox 2003; Rosvall and Mullin 2003; Soularue and Kremer 2012).

Significant correlations were also observed between distance matrices of neutral markers, phenology, and two traits of leaf shape—circularity index and PA ratio. Correlations between adaptive traits and neutral makers have been observed in many other studies (see McKay and Latta 2002 for a review). Merilä and Crnokrak (2001) proposed one possible explanation for the origin of such phenotypic-genetic relationships. Since allozyme, microsatellite and other neutral genetic markers are not always freely recombining with the rest of the genome, Merilä and Crnokrak (2001) suggested that small amounts of linkage with quantitative trait loci can lead to significant phenotypic-genetic correlations. Results from this and previous studies show that one of the studied loci (*Qp*ZAG46) is potentially linked to adaptive leaf traits and fitness in oaks, which could be a potential explanation for the observed correlations between leaf shape traits and molecular markers. However, in our study such correlations were still observable when this locus, putatively under selective forces, was excluded from the analyses. Another alternative mechanism that Merilä and Crnokrak (2001) suggested to explain the origin of phenotypic-genetic correlations was the existence of a common factor driving the divergence in both neutral and functional traits. In this study, the matrices of both neutral markers and leaf shape traits were correlated to the matrix of vegetative phenology. As stated above, vegetative phenology is correlated to flowering phenology in cork oak and presents a moderate heritability. Therefore, an alternative explanation could be that genotypes with different patterns of phenology present less probability of gene exchange and as a consequence, their offspring becomes more differentiated in functional traits. To test this hypothesis, we performed partial Mantel tests between the matrices of phenology and leaf traits by correcting for the genetic structure. The rationale of this is that if phenology and leaf traits are not significantly correlated after accounting for the neutral genetic structure, then neutral evolutionary processes would explain the correlations among phenotypes. We found that the correlation between phenology and CI (circularity index of the leaf) significantly decreased after correcting by the genetic structure agreed with the expectations of a neutral origin (e.g., gene flow restrictions) of the maternal family differences in CI. n contrast, the correlation between phenology and PA was not explained by genetic structure in neutral markers, which suggests that other processes (e.g., disruptive selection) could be behind the differentiation among maternal families in this trait.

Finally, the limited number of individuals per maternal family (five to eight individuals) that we used for genetic analyses could have also affected the intrapopulation pattern of differentiation. Recent studies have suggested that 25–30 individuals per genetic unit (i.e., population, maternal family,

etc.) based on microsatellite allele frequencies are needed to obtain accurate estimates of the genetic parameters and genetic divergence (Hale et al. 2012). Some authors have pointed out that when the level of polymorphism is limited, sample size can be even as low as 10 individuals per population (Leberg 2002). Our study species, cork oak, presents considerably lower heterozygosity compared to other sympatric oak species (Soto et al. 2007). Nevertheless, we cannot rule out that the low number of individuals sampled in each maternal family has contributed to partially inflate the genetic differences among maternal families.

Summarizing, our study reveals high genetic differences in neutral markers and leaf traits among open-pollinated maternal families within cork oak populations. Differences between certain maternal families from the same stand were higher than those observed between long-distance populations. Such an intrapopulation structure in functional traits seem have been caused by neutral evolution (e.g., leaf circularity index), selective processes (e.g., leaf size and SLA), or both (e.g., PA). More generally, our results reinforce the importance of intrapopulation studies in long-lived plants under an evolutionary context.

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Data archiving statement Phenological, functional, and microsatellite data will be archived in the DRYAD. JARV has already registered, and the dataset will be submitted using his personal account in case the manuscript is considered for publication.

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