

Elucidating the role of genetic drift and natural selection in cork oak differentiation regarding drought tolerance

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Abstract

Drought is the main selection agent in Mediterranean ecosystems and it has been suggested as an important evolutionary force responsible for population diversification in these types of environments. However, population divergence in quantitative traits can be driven by either natural selection, genetic drift or both. To investigate the roles of these forces on among-population divergence in ecophysiological traits related to drought tolerance (carbon isotope discrimination, specific leaf area, leaf size and leaf nitrogen content), we compared molecular and quantitative genetic differentiation in a common garden experiment including thirteen cork oak (*Quercus suber* L.) populations across a gradient of rainfall and temperature. Population differentiation for height, specific leaf area, leaf size and nitrogen leaf content measured during a dry year far exceeded the molecular differentiation measured by six nuclear microsatellites. Populations from dry-cool sites showed the lowest nitrogen leaf content and the smallest and thickest leaves contrasting with those from humid-warm sites. These results suggest (i) these traits are subjected to divergence selection and (ii) the genetic differences among populations are partly due to climate adaptation. By contrast, the low among-population divergence found in basal diameter, annual growth and carbon isotopic discrimination (a surrogate for water use efficiency) suggests low or no divergence selection for these traits. Among-population differentiation for neutral markers was not a good predictor for differentiation regarding the quantitative traits studied here, except for leaf size. The correlation observed between the genetic differentiation for leaf size and that for molecular markers was exclusively due to the association between leaf size and the microsatellite *QpZAG46*, which suggests a possible linkage between *QpZAG46* and genes encoding for leaf size.

Keywords: adaptation, carbon isotope discrimination, drift, F_{ST} , leaf size, Q_{ST} , *Quercus suber*, selection

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Introduction

Population divergence of a widespread species may be influenced by either different selective pressures

imposed by different ecological environments, neutral evolutionary processes, or both (Still *et al.* 2005). Elucidating the causes of the intraspecific genetic differentiation is a central theme in evolutionary biology (e.g. Merilä & Crnokrak 2001; Reed & Frankham 2001; Latta 2003; Cano *et al.* 2004). However, the ascertainment of the relative importance of neutral evolutionary

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processes and natural selection as determinants of divergence among populations is complex (Merilä & Crnokrak 2001). This point is typically solved by the comparison of the F_{ST} and Q_{ST} statistics (e.g. Latta 2003; Saint-Laurent *et al.* 2003; Volis *et al.* 2005). Wright (1951) F_{ST} makes use of neutral genetic markers to estimate the divergence between populations caused by drift and mutations (Steane *et al.* 2006). Likewise, Q_{ST} measures the level of population genetic structure in quantitative traits by quantifying the proportion of total variation that occurs between populations (Spitze 1993). Any significant difference between F_{ST} and Q_{ST} among populations assumed to be in drift-migration equilibrium is thought to be attributable to the effects of natural selection (Merilä & Crnokrak 2001). Higher relative divergence in quantitative traits than in neutral markers ($Q_{ST} > F_{ST}$) suggests a spatially divergent selection, which favours different genotypes in different populations, whereas the opposite ($Q_{ST} < F_{ST}$) suggests that the same genotypes are favoured in different populations, which suggests spatially homogenizing selection. If the two measures do not differ significantly, the population differences in quantitative traits could be attributed to genetic drift and other neutral evolutionary processes (Andersen *et al.* 2007).

Several studies in the last twenty years have explored natural selection on quantitative traits (e.g. Dudley 1996; Heschel & Riginos 2005; Donovan *et al.* 2007). However, the number of studied taxa has been very low (Kingsolver *et al.* 2001). The knowledge on this issue is particularly lacking for forest tree species, mainly due to the difficulty to obtain the genetic differentiation parameter for quantitative traits (Q_{ST}) in these species (Hamrick 2004). This fact is caused by the long-life cycle and the wide distribution area of these species, which hampers the performance of genetic experiments using enough populations and families to obtain accurate estimates of within- and among-population genetic variability (Geber & Griffen 2003). Besides, most of the studies estimating Q_{ST} values have focused on morphological, phenological or growth characters (e.g. Palo *et al.* 2003; Steane *et al.* 2006; Bower & Aitken 2008) and very few ones have centred on the analysis of physiological traits.

Summer drought is the most important selection agent in Mediterranean ecosystems and plants exhibit morphological and physiological adaptations to cope with environmental stress. Selection for drought tolerance has rarely been examined (Dudley 1996; Heschel & Riginos 2005; Donovan *et al.* 2007) and, to our knowledge, never in long-living Mediterranean species. Cork oak (*Quercus suber* L.) is one of the keystone forest tree species in these ecosystems. It occurs in a vast range of climatic conditions regarding rainfall and temperatures

(Díaz-Fernández *et al.* 1995). Relatively low divergence among populations has been reported for molecular markers (Toumi & Lumaret 1998; Jiménez *et al.* 1999) but population differentiation has been found in seed size, which is an important trait for tolerance to drought stress (Ramírez-Valiente *et al.* 2009).

The aim of this study is to evaluate the importance of neutral evolutionary processes and natural selection under dry conditions in the genetic differentiation among cork oak populations for quantitative traits related to water stress tolerance. We analysed phenotypic traits related to growth, morphology and physiology which were found to be under natural selection in some species (Dudley 1996; Heschel & Riginos 2005; Donovan *et al.* 2007). Moreover, we evaluated genetic differentiation in total height and basal diameter as estimates of the response to natural selection over a seven-year period. We were also interested in assessing whether the possible population divergence could be driven by adaptation to different environmental conditions. Thus, thirteen cork oak populations, covering a vast climatic gradient of rainfall and temperature, were studied. Selection has a greater effect for traits that are closely associated with fitness than for those that are not (Mousseau & Roff 1987), so our expectation is that growth traits (performance characters) present the highest population divergence.

Specifically, we have examined whether the divergence in quantitative traits exceeded that one in neutral markers ($Q_{ST} > F_{ST}$) as expected if the divergence in the quantitative traits was driven by natural selection (Merilä & Crnokrak 2001). We also tested whether the among-population divergence in quantitative traits measured under dry conditions could be caused by an adaptation to different rainfall and temperature conditions. On the other hand, we have tested the hypothesis that the degree of differentiation in molecular markers may be predictive of the degree of differentiation in quantitative traits (Merilä & Crnokrak 2001; Reed & Frankham 2001), by pairwise comparisons of population differentiation in F_{ST} and Q_{ST} estimates.

Materials and methods

Study site and common garden

The common garden experiment was established in Southern Spain, *La Sierra de Andújar* Natural Park (38°21'54"N, 3°51'40"W, 560 m, Jaen) (Fig. 1). The climate is typical dry-Mediterranean, characterized by hot and dry summers and cool winters. The average annual precipitation for the last 30 years is 617 mm and the average annual temperature is 14.7 °C (AEMET, National Meteorological Agency). The common garden

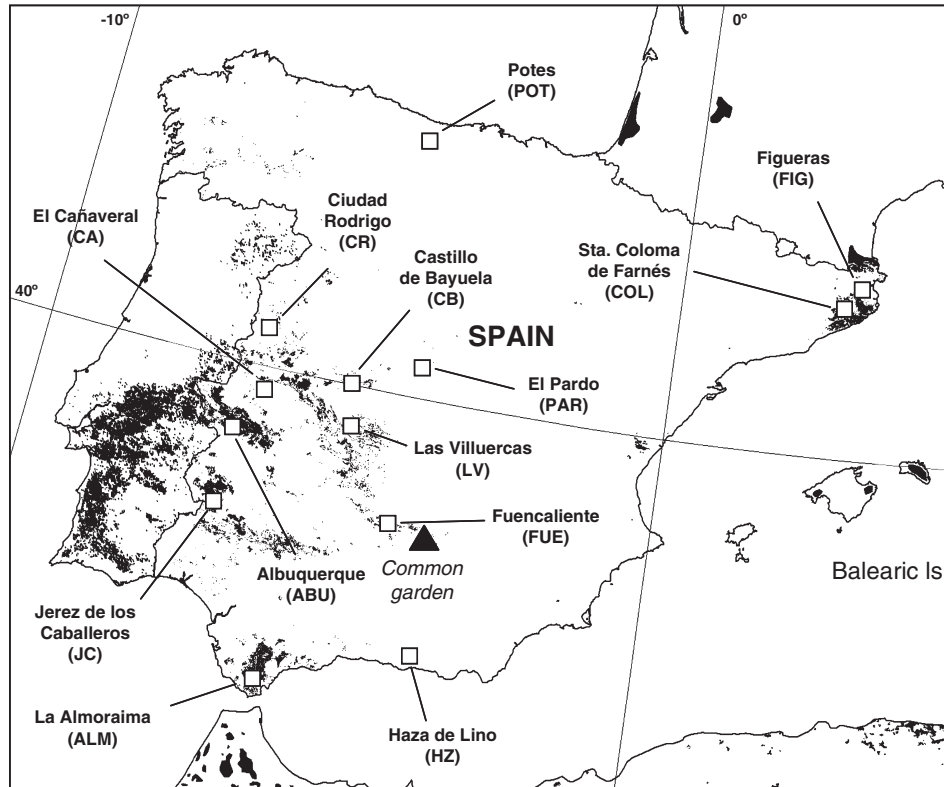


Fig. 1 Location of the sampled populations of *Quercus suber* and of the common garden site within the distribution area of cork oak species in the Iberian Peninsula. Map adapted from Aronson *et al.* (2009).

Table 1 Location and climatic characterization of the thirteen cork oak populations and the common garden site during the study year (2005) and over a seven-year period from the establishment of the common garden (1998–2005)

Code	Population	Nearest locality	Latitude	Longitude	Altitude (m)	Pa	Ps	T	MMH
POT	La Liebana region	Potes	43° 09'N	4° 37'W	270	736	94.6	13.2	26.0
FIG	Catalonian Pyrenees	Figueras	42° 24'N	2° 48'E	25	647	101.8	15.7	30.8
COL	Coastal catalonia	Santa Coloma de Farnés	41° 54'N	2° 30'E	175	805	159.8	15.6	30.5
CR	Salamanca	Ciudad Rodrigo	40° 35'N	6° 26'W	653	525	64.1	13.2	31.3
PAR	Guadarrama mountain range	El Pardo	40° 31'N	3° 45'W	750	474	55.6	13.5	31.4
CB	Tietar valley	Castillo de Bayuela	40° 07'N	4° 31'W	677	644	55.1	16.6	36.8
CA	North Caceres	El Cañaveral	39° 22'N	6° 22'W	362	672	42.7	16.8	35.0
LV	Las Villuercas region	Cañamero	39° 22'N	5° 21'W	600	1008	54.9	15.4	34.8
ABU	San Pedro hills	Alburquerque	39° 13'N	7° 13'W	500	643	37.6	15.4	31.3
FUE	Eastern Morena mountain range	Fuencaliente	38° 24'N	4° 16'W	670	432	38.9	14.8	33.3
JC	Western Morena mountain range	Jerez de los Caballeros	38° 13'N	6° 42'W	492	627	39.1	16.1	33.5
HZ	Las Alpujarras region	Haza de Lino	36° 47'N	3° 18'W	1300	607	24.5	13.0	29.3
ALM	Los Alcornocales National Park	Castellar de la Frontera	36° 16'N	5° 22'W	118	813	20.0	17.4	29.0
	Common garden site—2005		38° 22'N	3°52'W	560	306	2.6	15.3	34.4
	Common garden site—1998–2005		38° 22'N	3°52'W	560	562	8.2	14.2	34.5

Pa, annual precipitation (mm); Ps, summer precipitation (mm); T, mean annual temperature (°C); MMH, mean maximum temperature of the hottest month (°C).

followed a design of randomized complete blocks. Thirteen populations were essayed, spanning vast range of climatic conditions (Table 1). It included 30 blocks and four plants per population within each block. Seedlings

were planted following a 3 × 3 m square grid. Seeds were collected from native stands during the autumn and winter of the year 1996 from 20 to 30 trees per population, separated for more than 100 m to minimize the

risk of sampling in closely related trees. This distance is enough to avoid familial structures even in open wood formations of cork oak (Soto *et al.* 2007). Seeds were sown in the beginning of 1997 and grown in nursery for a year under standard conditions of high water and nutrient availability, and then planted in the field during the spring of 1998.

Molecular markers

Leaf material was collected during the spring of 2006 from 15 trees per population (195 plants in total). DNA was extracted from leaves following the method described by Doyle & Doyle (1990). A total of six (GA)_n nuclear microsatellites were used, transferred from other *Quercus* species: QpZAG9, QpZAG15, QpZAG46, developed in *Q. petraea* (Matts.) Liebl. (Steinkellner *et al.* 1997); QrZAG7, QrZAG11 and QrZAG20 developed in *Q. robur* L. (Kampfer *et al.* 1998). Amplification and scoring were performed following Soto *et al.* (2003, 2007). Standard genetic diversity parameters [allelic richness (A), expected heterozygosity or genetic diversity (H_e), observed heterozygosity (H_o) and fixation index (F_{IS})] were determined for each population by using GENETIX version 4.05 (Belkhir *et al.* 1996–2004). Deviation from Hardy–Weinberg equilibrium and linkage disequilibrium (LD) was tested using ARLEQUIN, version 3.11 (Excoffier *et al.* 2005). Differentiation among populations (F_{ST}) was estimated using the Infinite Allele Model (IAM) (Kimura & Crow 1964), as some of the loci did not follow strictly the Stepwise Mutation Model (SMM) (proposed for microsatellite by Kimura & Ohta 1978) in *Q. suber*, as reported in Soto *et al.* (2003) and Lorenzo (2006). An UPGMA dendrogram based on pairwise F_{ST} was produced.

Among-population genetic differentiation in neutral markers can be driven by constraints to gene flow between populations, which can be caused mainly (i) by a reproductive isolation by distance and (ii) by a reproductive isolation due to differences in phenology (e.g. Premoli 2003; Hirao & Kudo 2008). To estimate the proportion of genetic differentiation in neutral markers among populations explained by isolation by distance, a Mantel test of association was performed between the genetic matrix (GEN) and a geographical distance dissimilarity matrix (GEO). Likewise, altitude has been suggested as the main factor influencing phenology disparities between genetically close populations (e.g. Hegazy *et al.* 2008). Thus, to estimate the proportion of genetic differentiation in neutral markers caused by phenology differences, a Mantel test of association was performed between the genetic matrix (GEN) and an altitude distance dissimilarity matrix (ALT). As genetic distance is probably to correlate with both parameters

(altitude and geographical distance), two partial Mantel association tests were used to control for such matrices. A partial Mantel test analyses the correlation between two matrices while controlling for the effect of a third matrix. These statistical analyses were performed using ZT software with 10 000 permutations (Bonnet & Van de Peer 2002).

Quantitative traits

The measurements for the quantitative traits were carried out during 2005, a year characterized by extremely low rainfall (50.5% lower than the forty-year average, Table 1). A total of 20 blocks were selected and one plant per population was sampled within each block (13 populations \times 20 plants per population = 260 plants in total). Basal diameter growth and total height were measured as estimates of the accumulated growth. The annual growth of each plant was assessed through averaging the length of six shoots (with six different orientations) of the upper crown, all around the tree crown. We measured several phenotypic traits potentially related to drought tolerance and which have found to be under selection in dry conditions (Dudley 1996; Donovan *et al.* 2007): leaf size, specific leaf area (SLA), nitrogen leaf content (N_{mass}) and carbon isotope discrimination ($\Delta^{13}\text{C}$). SLA is a measure of the projected leaf area per unit of dry weight, and it has been found to be related to the photosynthetic capacity and stomatal conductance (Reich *et al.* 1997). $\Delta^{13}\text{C}$ is a surrogate of water use efficiency during the time for which the plant has fixed carbon (Farquhar *et al.* 1982; Farquhar & Richards 1984). Finally, leaf nitrogen, measured as the nitrogen mass per unit leaf mass (N_{mass}), is widely accepted to be associated to the inversion on leaf photosynthetic components (especially Rubisco), and thus, influencing on the photosynthetic capacity. The measurements were carried out in spring leaves from three orientations (N, SE, SW). The leaf size was measured using the software WINFOLIA version 2002 (Régent, Quebec, Canada). The leaves were dried in an oven at a constant temperature of 65 °C to a constant weight, after scanning, to estimate the specific leaf area (SLA) in one leaf per orientation. The rest of the dry material was used to analyse the isotopic composition of C^{13} and the nitrogen leaf content (mass based) after grinding them to fine powder using a ball mill.

Estimation of differentiation at quantitative traits

When random mating ($F_{IS} = 0$) and linkage equilibrium are assumed, the estimate of Q_{ST} for a trait for diploids can be calculated by partitioning the total additive

genetic variance into the among-population (σ_B^2) and the within-population (σ_W^2) components as follows:

$$Q_{ST} = \frac{\sigma_B^2}{\sigma_B^2 + 2 \cdot \sigma_W^2} = \frac{V_\alpha}{V_\alpha + 2 \cdot (h^2 \cdot V_\epsilon)}, \quad (\text{eqn 1})$$

where V_α is the variance of the population component, V_ϵ is the residual variance and h^2 is the heritability (Lande 1992). The variance components for the estimate of Q_{ST} for each trait (V_α , V_ϵ) were estimated by performing seven GLMs (one per trait) following the model:

$$Y_{jn} = \mu + H_n + P_j + E_{jn}$$

where Y_{jn} is the observed value for the variable considered in the n tree and the j population; μ the general mean, H_n the fixed effect of height for individual n ; P_j the fixed effect of the j population and E_{jn} is the residual error for Y_{jn} . Owing to the effect of the plant size on leaf morphology, structure, composition, growth and WUE (Casper *et al.* 2005), height was included in the linear models as a covariate when height and diameter were analysed.

The major drawback in Q_{ST} studies is the requirement of the designed experiment to estimate heritability. In our work, the employed heritability value (h^2) for each trait was obtained from a common garden experiment established also in 1998 at the same place, and where ecophysiological traits were analysed during the same year (see González-Martínez for a similar procedure). In this study, forty-five families from three cork oak origins were analysed. Narrow-sense heritability values were calculated as follow:

(1) First, seven linear mixed model analyses (one per trait) were performed to calculate the variance components for each factor. 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 the general mean, H_n the fixed effect of previous height for individual n , B_i the fixed effect of the i block; P_j the fixed effect of the j population; $F(P)_{jk}$ the random effect of the k family nested within the j population; BP_{ij} the random effect of the interaction between block i and population j and E_{ijkn} is the residual error for Y_{ijkn} .

(2) Second, the narrow-sense heritability values were estimated for each trait:

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2} = \frac{3\sigma_f^2}{\sigma_f^2 + \sigma_{rb}^2 + \sigma_e^2}$$

where σ_f^2 represents the variance given by the family within each population; σ_{rb}^2 is the variance component

for the interaction population \times block and σ_e^2 is the residual variance. The additive genetic variance was estimated as $3\sigma_f^2$. Using $3\sigma_f^2$, rather than $4\sigma_f^2$ is a more conservative estimate of additive genetic variance that adjusts for expected partial inbreeding in the natural stands (Rochon *et al.* 2007).

Additional estimates of Q_{ST} values were performed for the range limits (0.1–1) of heritability to evaluate the influence of within-population variability on the Q_{ST} value. A bootstrap resampling was carried out at individual level within population to estimate standard deviations for Q_{ST} estimations (1000 samples). All the analyses were performed using SAS 9.1 (SAS/STAT® Software, SAS Institute).

On the other hand, Whitlock (2008) argued that the average value of Q_{ST} could be biased (i) when the selected traits are chosen because they had been found to be under spatial divergent selection previously and (ii) when genes affecting traits are not genetically independent. The first source of bias was minimized because only some traits studied here have been found to be under different directional selection in cork oak (data not shown). To solve the second issue, several Q_{ST} averages were estimated using different combinations of traits genetically correlated (see Tables S1 and S2).

Comparison of molecular and quantitative data

To detect differences of mean values between F_{ST} and Q_{ST} values, tests based on the standard deviation confidence intervals for bootstrap samples were performed (Manly 1997). To estimate the proportion of genetic differentiation in quantitative traits explained by neutral evolutionary processes, seven Mantel's tests (one per trait) of association were performed between the quantitative genetic matrix for each trait matrix and the molecular genetic distance matrix.

Structure of the genetic variance at molecular markers and quantitative traits

Population divergence in quantitative traits can be driven by either natural selection, genetic drift or both. To ascertain the effects of these forces on population differentiation in quantitative traits, we followed the next steps (A–B):

(A) Owing that populations varied along a vast climatic range and the environmental differences among the places of origin were not explained by a single climatic variable, we performed a PCA to group the populations according to the degree of climatic similarity (called here 'climatype'). For this purpose, correlations between climatic variables were performed. Only those variables that were mathematically independent were

included in the analysis, i.e.: annual precipitation, summer precipitation, winter minimum temperature and summer maximum temperature.

(B) We determined the structure of the genetic variance for molecular markers (B1) and quantitative traits (B2). (B1) We conducted an analysis of the molecular variance (AMOVA) using ARLEQUIN, version 3.11 (Excoffier *et al.* 2005) for the hierarchical partitioning of the genetic diversity: among climatotypes (F_{CT}) and among populations within climatotypes (F_{SC}). (B2) Likewise, we performed a partition of the total additive genetic variance for quantitative traits: among climatotypes (called here ' Q_{CT} ') and among populations within climatotypes (called here ' Q_{SC} '). For this purpose, first, we constructed six GLMs (one per trait) using the procedure PROC GLM of the statistical package SAS 9.1 (SAS/STAT® Software; SAS Institute). The model equation was:

$$Y_{ijn} = \mu + H_n + C_i + P(C)_{ji} + E_{ijn}$$

where Y_{ijn} is the observed value for the variable considered in the n tree of the j population from the i climatotype; μ the general mean, H_n the fixed effect of previous height for individual n , C_i the fixed effect of the i climatotype; P_j the fixed effect of the j population nested within the i climatotype and E_{ijn} is the residual error for Y_{ijn} . We calculated the components of the variance for climatotype (V_α), population (climatotype) (V_β) and residual (V_ϵ) using PROC VARCOMP of SAS 9.1 (SAS/STAT® Software; SAS Institute). Analogously to the Q_{ST} estimate, when random mating ($F_{IS} = 0$) and linkage equilibrium are assumed, partitioning the genetic diversity for quantitative traits for diploids into the among-climatotype (Q_{CT}) and among-population within-climatotype (Q_{SC}) components was calculated as follow:

$$Q_{CT} = \frac{\sigma_A^2}{\sigma_A^2 + \sigma_B^2 + 2 \cdot \sigma_W^2} = \frac{V_\alpha}{V_\alpha + V_\beta + 2 \cdot (h^2 \cdot V_\epsilon)}$$

$$Q_{SC} = \frac{\sigma_B^2}{\sigma_A^2 + \sigma_B^2 + 2 \cdot \sigma_W^2} = \frac{V_\beta}{V_\alpha + V_\beta + 2 \cdot (h^2 \cdot V_\epsilon)}$$

where (σ_A^2) was the additive genetic variance for climatotype, (σ_B^2) the additive genetic variance for population within climatotype, (σ_W^2) the additive genetic variance within-population and h^2 is the narrow-sense heritability (see above for details of the heritability calculations) (Lande 1992). Thus, in drift-migration equilibrium, higher percentage of genetic variance at climatotype level explained by quantitative traits than that one by molecular markers ($Q_{CT} > F_{CT}$) suggests that population divergence is partly due to the adaptation to different climatic conditions. Likewise, whether the percentage of genetic variance at population within climatotype level

explained by quantitative traits is higher than that one by molecular markers ($Q_{SC} > F_{SC}$) would be indicative that the population divergence is partly due to a local adaptation to differential environmental conditions caused by other factors different to climate (e.g. soil type, herbivory...). On the contrary, whether $F_{CT} > Q_{CT}$ and/or $F_{SC} > Q_{SC}$ suggest that the same genotypes are favoured under different climatic and/or local environmental conditions, which would be indicative of spatially homogenizing selection (Merilä & Crnokrak 2001). The sources of bias were minimized following Whitlock (2008) (see the above procedure about the calculation of the Q_{ST} values for more detail).

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. The FDR was controlled at each p-level using a standard step-up procedure (see Benjamini & Hochberg 1995; García 2004).

Results

Genetic diversity and population differentiation based on molecular markers

The six microsatellite loci used in this study were not very polymorphic for cork oak, as shown in other studies (Soto *et al.* 2007). The total number of alleles detected for each locus varied from four ($QpZAG46$ and $QrZAG20$) to nine alleles ($QrZAG7$). However, despite the low diversity of the loci, the levels of observed and expected heterozygosity were fairly high across the whole sample (mean $H_o = 0.53$; $H_e = 0.56$, Table 2). The fixation index (F_{IS}) varied between populations from 0.152 to 0.091 but were not significantly different from zero for all populations. Most populations did not deviate significantly from Hardy-Weinberg equilibrium for all loci. The only exceptions occurred for just one locus (El Pardo for $QpZAG9$, Jerez de los Caballeros for $QrZAG11$, and Ciudad Rodrigo and Figueras for $QrZAG20$). Although some loci showed linkage disequilibrium (LD), no consistent pattern was found across all populations (data not shown).

Relatively low multilocus F_{ST} was obtained, 0.032 (0.003), although this value was significantly different from zero ($P \ll 0.0001$). Despite the low differentiation among cork oak populations, a quite high number of pairwise F_{ST} estimates were significantly different from zero (39 out of 78, results not shown). The highest numbers of significant pairwise F_{ST} estimators were found for Haza de Lino (HZ, 11) reflecting a clear differentiation of this population from the rest of Iberian populations (Fig. 2). Pairwise F_{ST} values were slightly

Table 2 Genetic diversity statistics of cork oak populations using six microsatellite loci

Population	Province	N	A	A_e	H_e	H_o	F_{IS}	Prob
POT	Cantabria	15	3.33 (1.37)	2.17	0.539 (0.114)	0.496 (0.056)	0.0790	ns
FIG	Gerona	15	3.33 (1.51)	2.12	0.528 (0.110)	0.487 (0.053)	0.0786	ns
COL	Barcelona	15	3.83 (1.83)	2.42	0.587 (0.100)	0.533 (0.053)	0.0914	ns
CR	Salamanca	15	3.50 (0.84)	2.54	0.606 (0.055)	0.595 (0.054)	0.0182	ns
PAR	Madrid	15	3.50 (0.55)	2.39	0.582 (0.081)	0.544 (0.056)	0.0653	ns
CB	Toledo	15	3.33 (0.52)	2.22	0.550 (0.045)	0.562 (0.053)	-0.0214	ns
CA	Cáceres	15	3.50 (1.05)	2.16	0.538 (0.077)	0.495 (0.054)	0.0800	ns
LV	Cáceres	15	3.83 (0.41)	2.16	0.537 (0.058)	0.507 (0.054)	0.0550	ns
ABU	Badajoz	15	3.17 (1.17)	2.09	0.521 (0.082)	0.600 (0.052)	-0.1515	ns
FUE	Ciudad Real	15	3.83 (1.60)	2.09	0.522 (0.107)	0.515 (0.053)	0.0124	ns
JC	Badajoz	15	3.33 (1.37)	2.09	0.522 (0.110)	0.500 (0.053)	0.0411	ns
HZ	Granada	15	3.50 (1.05)	1.99	0.498 (0.079)	0.458 (0.053)	0.0804	ns
ALM	Cádiz	15	3.33 (0.82)	2.11	0.526 (0.090)	0.517 (0.054)	0.0179	ns
Total		195	6.17 (2.14)	2.26	0.559 (0.082)	0.525 (0.015)	0.0637	ns

N, population size; A, allelic richness; A_e , effective number of alleles; H_e and H_o , expected and observed heterozygosities; F_{IS} , fixation index; Prob, associated probability to F_{IS} (ns, not significant at 5% level). Standard deviations in parentheses.

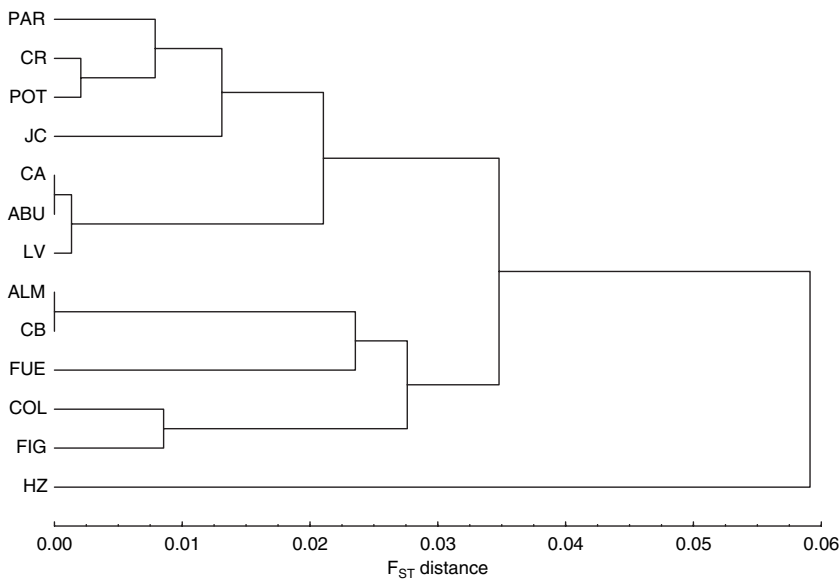


Fig. 2 UPGMA cluster analyses using F_{ST} distances obtained from six microsatellite data for thirteen cork oak populations in the Iberian Peninsula.

correlated with altitude and geographical closeness (Table 3). Correlation of F_{ST} with geographical closeness became significantly higher when altitude differences (as a surrogate of phenology disparities) were taken into account (Table 3).

Quantitative differentiation

Q_{ST} values ranged between 0.062 (0.044) for $\Delta^{13}C$ and 0.459 (0.076) for leaf size and averaged 0.190 (0.144) (Table 4). The average Q_{ST} value was significantly higher than F_{ST} 0.032 (0.003) even considering for the analysis only sets of traits genetically independent (Tables S1 and S2). This suggests that the discrepancies

in population affinities may be driven, at least partly, by diversifying selection. Although all traits examined displayed Q_{ST} values significantly higher than the average F_{ST} (Table 4), height, SLA, leaf size and nitrogen content had moderate-high values while basal diameter, annual growth and $\Delta^{13}C$ presented lower differentiation (Table 4).

The among-population Q_{ST} distances were significantly correlated with F_{ST} for annual growth and leaf size and marginally correlated for height, diameter and nitrogen leaf content (Table 3). However, when HZ pairwise were excluded from the analyses, all relationships between Q_{ST} and F_{ST} became nonsignificant except for annual growth and leaf size (data not

Table 3 Simple and partial Mantel's tests of association among neutral genetic distances, altitude, geographical distance and genetic distances of quantitative traits (QST)

Matrix				
A	B	C	R-statistic	P
GEN	ALT		0.283	0.051 m
GEN	GEO		0.288	0.055 m
GEN	ALT	GEO	0.295	0.052 m
GEN	GEO	ALT	0.299	0.027 *
HT	GEN		0.338	0.028 m
DT	GEN		0.256	0.046 m
GR	GEN		0.520	0.001 **
$\Delta^{13}\text{C}$	GEN		-0.016	0.503
SLA	GEN		0.127	0.203
NITR	GEN		0.414	0.053 m
LEAF	GEN		0.440	0.002 **

HT, height; DT, basal diameter; GR, Annual growth; $\Delta^{13}\text{C}$, ^{13}C -Carbon isotope discrimination; SLA, Specific leaf area; NITR, leaf nitrogen content per mass, LEAF, leaf size; GEO, geographical distance; ALT, altitude distance.

The pattern of association in the dependent matrix was compared with the predictor matrix B, while controlling for the effects of matrix C, using partial Mantel tests.

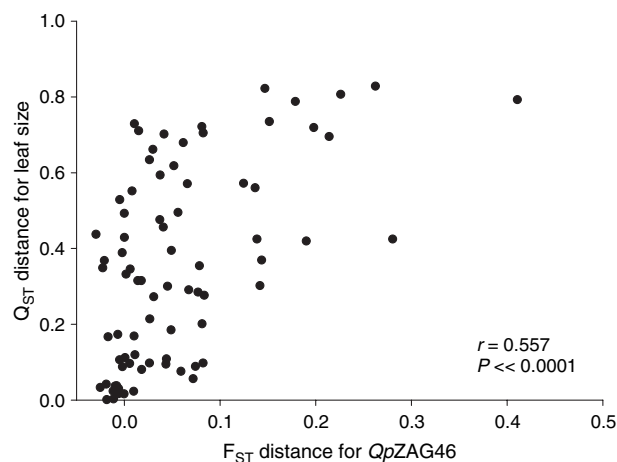
*Significant values, m Marginally nonsignificant values.

Significance levels were corrected by FDR criterion. See text for details.

shown). Furthermore, we found that the correlation between F_{ST} and Q_{ST} for leaf size was exclusively due to the microsatellite *QpZAG46* (Fig. 3).

Climatic adaptation or neutral evolution

The results from the PCA for climate similarity showed that variation in climate was explained by four PC Axis. We grouped populations in 'climatypes' according to the first two PC Axis because they explained most of the variance (71.4%) (Fig. 4). Populations were classified in five climatic groups (I-V) (See Table 1 for

**Fig. 3** Correlation between Q_{ST} distance for leaf size and F_{ST} distance for *QpZAG46*. The Pearson's correlation coefficient (r) and the significance level are shown.

population abbreviations): I-Typical Mediterranean—TM (CB, CA, ABU, JC), II-Cool Mediterranean—CM (CR, PAR, FUE, HZ), III-Mediterranean with humid and mild winters—MW (ALM), IV-Mediterranean with humid and cool winters—CW (LV) and V-Mediterranean with humid summers HS— (FIG, COL, POT) (Fig. 4). The results from the analyses of variance showed large significant differences between climatotypes in leaf size and specific leaf area (Table 5), indicating that leaf morphology is under a strong climatic control. Individuals from the climatic group III, characterized by mild and very humid winters, exhibited the largest and thinnest leaves on average (Table 5). Whereas, individuals from cool sites (climatype II) had the thickest leaves and together with the plants from humid-summer localities presented the smallest leaves on average (Table 5). There were also differences among populations within climatotypes in total height, leaf size and specific leaf area.

Table 4 Q_{ST} values (standard deviations in parentheses) for seven quantitative traits considering different heritability values

Variable	Q_{ST}^*	h^{2*}	$Q_{\text{ST}} (h^2 = 0.1)$	$Q_{\text{ST}} (h^2 = 1)$
Height	0.171 (0.065)	0.30 (0.15)	0.394 (0.113)	0.065 (0.027)
Basal diameter	0.076 (0.044)	0.37 (0.06)	0.253 (0.118)	0.036 (0.021)
Annual growth	0.067 (0.051)	0.19 (0.12)	0.167 (0.113)	0.022 (0.017)
Carbon isotope composition ($\Delta^{13}\text{C}$)	0.062 (0.044)	0.47 (0.16)	0.197 (0.122)	0.027 (0.020)
Specific leaf area (SLA)	0.278 (0.099)	0.10 (0.11)	0.442 (0.124)	0.080 (0.036)
Leaf size	0.459 (0.076)	0.16 (0.12)	0.577 (0.076)	0.125 (0.036)
Nitrogen	0.221 (0.097)	0.14 (0.12)	0.442 (0.124)	0.048 (0.026)

Q_{ST}^* , this value of Q_{ST} was estimated using the heritability value h^{2*} (SE) obtained from the analysis of a common garden studied during the same year and at the same localization that is followed in this study (see Material and methods for details)

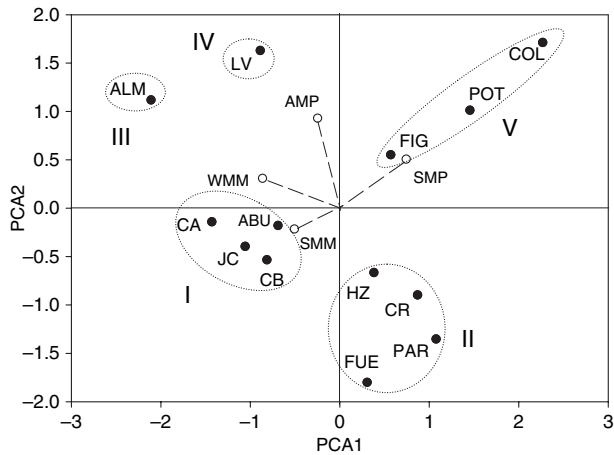


Fig. 4 Scores on PCA1 and PCA2 of thirteen populations of cork oak. The *x*- and *y*-axes are normalized with zero corresponding to the mean PC score. White dots represent the values of the climatic variables for the factor coordinates. AMP: annual mean precipitation, SMP: summer mean precipitation, WMM: winter mean minimum temperature, SMM: summer mean maximum temperature. Black dots indicate the values of the populations for the factor coordinates. Abbreviations correspond to the populations shown in Table 1. Dotted lines point out the climatically similar populations according to PCA values. Climatic groups (I–V): I-Typical Mediterranean—TM (CB, CA, ABU, JC), II-Cool Mediterranean—CM (CR, PAR, FUE, HZ), III-Mediterranean with humid and mild winters—MW (ALM), IV-Mediterranean with humid and cool winters—CW (LV) and V-Mediterranean with humid summers HS—(FIG, COL, POT).

All Q_{CT} values were higher than F_{CT} (0.007). However, whereas specific leaf area [$Q_{CT} = 0.162$ (0.135)], leaf size [0.082 (0.119)] and nitrogen leaf content 0.081 (0.079) displayed moderate values, the rest of the traits presented very low population differentiation explained by climate divergence (Table 6). All Q_{SC} values were also higher than F_{SC} (0.026). Likewise, population divergence depended on the considered trait. Leaf size, specific leaf area, nitrogen leaf content and height moderate-high Q_{SC} values (Table 6), indicating that

Table 6 Structure of the genetic variance for quantitative traits for two hierarchical levels: climatype (Q_{CT}) and population (climatype) (Q_{SC})

Variable	Climatype Q_{CT} (SD)	Population (climatype) Q_{SC} (SD)
Height	0.010 (0.024)	0.170 (0.060)
Basal diameter	0.010 (0.024)	0.079 (0.050)
Annual growth	0.028 (0.042)	0.073 (0.073)
Carbon isotope composition ($\Delta^{13}C$)	0.016 (0.021)	0.042 (0.036)
Specific leaf area (SLA)	0.162 (0.135)	0.309 (0.149)
Leaf size	0.082 (0.119)	0.390 (0.109)
Nitrogen	0.081 (0.079)	0.178 (0.107)

The values of Q_{CT} and Q_{SC} were estimated using the heritability values (Table 4) obtained from the analysis of a common garden experiment studied during the same year and at the same localization that is followed in this study (see Material and methods for details). Standard deviations are in parentheses.

besides climate, other differences in local environmental conditions could have caused the observed population divergence in these traits.

Discussion

The low F_{ST} values (0.032) point out to a limited differentiation between populations, showing that around 97% of the total variation is present within population. This result is consistent with the low differentiation value reported for the region by Toumi & Lumaret (1998) and Jiménez *et al.* (1999) using allozymes.

Comparison of the levels of differentiation of F_{ST} and Q_{ST}

Significant differences between F_{ST} and Q_{ST} were observed in this study even considering the sources of

Table 5 Mean \pm standard error for all climatypes for the seven studied traits. Significant differences among climatypes ($P < 0.05$) are in bold

Climatype	Height (cm)	Diameter (cm)	Growth (cm)	$\Delta^{13}C$ (‰)	SLA (cm ² g ⁻¹)	Leaf size (cm ²)	N_{mass} (mg g ⁻¹)
I-Typical Mediterranean (TM)	145.83 \pm 5.01	5.33 \pm 0.20	5.99 \pm 0.06	18.71 \pm 0.10	51.77 \pm 0.59	1.87 \pm 0.02	16.22 \pm 0.20
II-Cool Mediterranean (CM)	132.16 \pm 4.97	5.00 \pm 0.19	5.57 \pm 0.06	18.91 \pm 0.10	51.15 \pm 0.59	1.75 \pm 0.02	15.74 \pm 0.19
III-Humid and mild winters (MW)	139.05 \pm 9.88	5.00 \pm 0.39	6.61 \pm 0.11	18.87 \pm 0.19	55.58 \pm 1.16	2.18 \pm 0.04	16.14 \pm 0.38
IV-Humid and cool winters (CW)	156.90 \pm 9.88	5.90 \pm 0.39	6.40 \pm 0.11	18.61 \pm 0.19	53.36 \pm 1.17	2.00 \pm 0.04	16.57 \pm 0.38
V-Humid summers (HS)	140.10 \pm 5.81	5.26 \pm 0.23	5.71 \pm 0.06	18.79 \pm 0.11	53.92 \pm 0.70	1.66 \pm 0.02	16.37 \pm 0.37

$\Delta^{13}C$, 13- Carbon isotope discrimination; SLA, specific leaf area; N_{mass} , leaf nitrogen content per unit mass. Significant differences (after FDR corrections) among sites (a) and among climatypes were typed in bold.

bias of Q_{ST} estimates in the analysis. Higher values of Q_{ST} with respect to F_{ST} have been found in different forest tree species (e.g. Lascoux 1996; Kremer *et al.* 1997; Hamrick 2004), as well as in other organisms (Merilä & Crnokrak 2001; McKay & Latta 2002; Palo *et al.* 2003). However, while height, nitrogen on a mass basis, SLA and leaf size showed moderately high Q_{ST} values (0.171 (0.065)–0.459 (0.076), diameter, $\Delta^{13}C$ and annual growth presented low values (0.062 (0.044)–0.076 (0.044)). The high quantitative genetic differentiation among populations compared with neutral evolutionary processes for leaf nitrogen content, leaf size and SLA in a dry year suggests that these traits are subjected to diversify selection. The relative measures of among-population divergence, such as Q_{ST} , are inherently dependent on the extent of within-population diversity, and, consequently, on the heritability of the character, according to (1). However, in this study, the Q_{ST} values for these three traits were much higher than F_{ST} estimations, independently of the value of heritability adopted for obtaining Q_{ST} (Table 4). Hence, the acting of natural selection can be confirmed. Moreover, the larger among-climate variation for these quantitative traits (Q_{CT}) than that one for molecular markers (F_{CT}) suggests that the population divergence is partly due to the climate adaptation. Likewise, the higher population divergence within climatypes for quantitative traits (Q_{SC}) than for molecular markers (F_{SC}) also suggests that other selection pressures different to climate would be causing part of the population divergence, and the effects of this local adaptation on the population differentiation can be even larger than those due to the climatic conditions.

However, the possible biases in the estimate of F_{ST} and Q_{ST} (López-Fanjul *et al.* 2003; Whitlock 2008) and the low Q_{ST} values for $\Delta^{13}C$, diameter and annual growth traits did not enable to ascertain if divergence among populations for these traits is driven by selection or not. As mentioned above, the low population differentiation found for $\Delta^{13}C$ could be caused by high variation in within-population diversity (Lynch *et al.* 1999). In fact, when we used a low heritability value (0.1) [and consequently, low variation of within population diversity, according to (1)], Q_{ST} increased up to 0.197 (0.122) for this character (Table 4). This suggests that the low genetic among-population differentiation for $\Delta^{13}C$ in cork oak was partly due to the fact that cork oak populations can keep high levels of additive genetic variance for this trait (Ramírez-Valiente *et al.* 2006).

Genetic differentiation among populations for annual growth was also very low [$Q_{ST} = 0.067$ (0.051)]. However, this value measured in a dry year contrasted with the much higher value of the accumulated growth in height [$Q_{ST} = 0.171$ (0.065)]. The higher among-popula-

tion differentiation in height, which responds to climatic conditions during the entire period since plants were in field, regarding to annual growth, determined by the extremely dry conditions during 2005, may be due to the populations that differ in their ability to take advantage of the wet years. Nevertheless, studies under these more favourable conditions should be carried out for a better understanding how natural selection is acting on this species.

F_{ST} as predictor of Q_{ST}

Pairwise F_{ST} values were slightly correlated with altitude and geographical closeness. Once altitude (as a surrogate of phenology disparity) was taken into account the correlation of F_{ST} with geographical closeness became significantly higher. These results would support the idea that at least in some way there are possible physical and phenological constraints to gene flow among cork oak populations. On the other hand, the distance matrix of quantitative data derived from accumulated growth (height and diameter) and nitrogen leaf content was weakly correlated with distance matrix of molecular data, indicating some parallelisms between the data sets. Nevertheless, correlation became only marginal after considering the climatic distances. In this case, probably, the weak association between molecular and phenotypic traits reflects mainly in the divergence of Haza de Lino (HZ) from the remaining populations. In fact, there was no correlation when HZ pairwise were excluded from the analyses. The location of Haza de Lino (HZ), at 1300 m above sea level and 200 km from the main distribution area (Díaz-Fernández *et al.* 1995), could have prevented gene exchange with central populations promoting a higher differentiation of this population from the main core of the species range in the Iberian Peninsula. Likewise, studies on organelle DNA show a different evolutionary history of this population (López de Heredia *et al.* 2007). These events could have led to a genetic divergence in molecular markers; while the adaptation to an extreme environment for the species, especially low temperatures (Aranda *et al.* 2005), could have caused the divergence in quantitative traits.

A strong association for paired comparisons between leaf size and molecular values was found. Some researchers have detected a positive correlation between the magnitude of F_{ST} and Q_{ST} and have suggested that molecular markers may provide a conservative estimate of quantitative divergence (Pfrender & Lynch 2000; Merilä & Crnokrak 2001; Steinger *et al.* 2002). Merilä & Crnokrak (2001) suggested that this correlation can be caused by a common factor (such as geographical distance) driving the divergence both in F_{ST} and Q_{ST} . In

this study, F_{ST} for neutral markers was correlated with geographical distance. Genetic divergence in leaf size (Q_{ST}) was associated with two environmental variables (rainfall unpredictability and annual mean temperature), indicating divergence selection on this trait. However, both leaf size and environmental variables did not show any association with geographical distance. Thus, the affinity between Q_{ST} for leaf size and F_{ST} for neutral markers cannot be explained by the effect of the geography. Another potential mechanism suggested by Merilä & Crnokrak (2001) was based on that not all microsatellite loci are completely freely recombining with the rest of the genome. Agreeing with this explanation, Brendel *et al.* (2008) found that the microsatellite *QrZAG111* was close to a QTL for the $\delta^{13}C$ in *Quercus robur*. We found that the correlation between F_{ST} and Q_{ST} for leaf size was exclusively caused by the microsatellite *QpZAG46* (Fig. 3). This microsatellite was localized near to *QrZAG87* and *QpZAG119* in *Quercus petraea* (Scotti-Saintagne *et al.* 2004). These markers were also included in the same linkage group in *Quercus robur*, where QTLs coding for leaf size were observed (Brendel *et al.* 2008). Thus, although further investigation on this issue is deserved, the hypothesis that the correlation between F_{ST} for molecular markers and Q_{ST} for leaf size could be caused by linkage between molecular and quantitative trait loci that cannot be rejected.

Our study revealed (i) that there is diversifying selection for traits related to drought stress (SLA, leaf size and nitrogen leaf content). (ii) The climatic gradient explained partly the among-population differentiation, although, adaptation to local environmental conditions had even a larger effect on the population genetic divergence for these traits. These results suggest that in spite of the relatively low genetic diversity and weak among population divergence in molecular markers, cork oak is able to develop local adaptations in response to natural selection. (ii) Differentiation in *QpZAG46* was a good predictor of the differentiation in quantitative traits for leaf size and annual growth, which could be explained by a putative linkage between loci. Further research should be focused in this issue, as microsatellite *QpZAG46* could thus be affected by natural selection.

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This work forms part of Jose A. Ramírez-Valiente's PhD which is currently carrying out under the supervision of Ismael Aranda and Fernando Valladares. This is focused on the analysis of the local and evolutionary capacity of cork oak and other Mediterranean tree species by integrating molecular techniques and ecophysiological studies. Ismael Aranda is a functional plant ecologist working in the physiological response of forest tree species. Most of his studies are aimed to a better understanding of the ecological and evolutionary consequences of the functional diversity by different genetics backgrounds, ranging from the species-specific pattern to the intra-specific differentiation among populations. Fernando Valladares is a plant ecologist working in the integration of ecophysiological research with plant demography and fitness under global change scenarios. He is especially interested in the evaluation of the ecological and evolutionary significance of phenotype plasticity, as well as the ecological constraints acting on it. Alvaro Soto and Luis Gil are professors of genetic and plant anatomy in the department of "silvopascicultura" at the Polytechnique University of Madrid. Zaida R. Lorenzo is a postdoctoral fellow also at the Polytechnique University of Madrid. They share an interest in the study of genetic diversity, hybridization phenomena and conservation genetics in forest tree species.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Genetic correlations (S. E.) performed between the studied variables

Table S2 Average Q_{ST} values (and standard deviations) for all possible combinations of genetically independent traits (Table S1)

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