

RESEARCH PAPER

Variation in sclerophylly among Iberian populations of *Quercus coccifera* L. is associated with genetic differentiation across contrasting environments

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ABSTRACT

Evergreen oaks are an emblematic element of the Mediterranean vegetation and have a leaf phenotype that seems to have remained unchanged since the Miocene. We hypothesise that variation of the sclerophyll phenotype among Iberian populations of *Quercus coccifera* is partly due to an ulterior process of ecotypic differentiation. We analysed the genetic structure of nine Iberian populations using ISSR fingerprints, and their leaf phenotypes using mean and intracanalopy plasticity values of eight morphological (leaf angle, area, spinescence, lobation and specific area) and biochemical traits (VAZ pool, chlorophyll and β -carotene content). Climate and soil were also characterised at the population sites. Significant genetic and phenotypic differences were found among populations and between NE Iberia and the rest of the populations of the peninsula. Mean phenotypes showed a strong and independent correlation with both genetic and geographic distances. Northeastern plants were smaller, less plastic, with smaller, spinier and thicker leaves, a phenotype consistent with the stressful conditions that prevailed in the steppe environments of the refugia within this geographic area during glaciations. These genetic, phenotypic, geographic and environmental patterns are consistent with previously reported palaeoecological and common evidence. Such consistency leads us to conclude that there has been a Quaternary divergence within the sclerophyllous syndrome that was at least partially driven by ecological factors.

INTRODUCTION

One of the characteristics of Mediterranean-type vegetation is the abundance of sclerophyll elements. Rigid, evergreen leaves covered with thick cuticles (*i.e.* sclerophylly) characterise the shrubs and trees that dominate most ecosystems in all five regions of the world that have a Mediterranean climate (Blondel & Aronson 1999). This congruency among geographically and evolutionarily distant plant forms has been historically interpreted as a case of convergent selection (Dunn *et al.* 1976). However, it

has been proved that sclerophylly is not an adaptation to Mediterranean conditions (Herrera 1992; Verdú *et al.* 2003). Many modern sclerophylls appear to descend from Tertiary, tropical-like ancestors that exhibited similar leaf syndromes (Palamarev 1989). Among them, the sclerophyllous oaks of the Western Mediterranean basin (holm oak: *Quercus ilex* L., cork oak: *Quercus suber* L. and kermes oak: *Quercus coccifera* L.) have foliar features analogous to those of fossil taxa that occupied similar ranges during the Tertiary. In particular, *Q. coccifera* leaves are strikingly similar to those of certain *Quercus* species

dating back to the Miocene (*Quercus mediterranea*; Palamarev 1989; Uzunova *et al.* 1997).

Climatic oscillations during the Quaternary Period forced major and repeated latitudinal and altitudinal shifts in the distribution of those taxa that survived (Hewitt 2004). Sclerophyllous oaks experienced range contractions and expansions, which likely involved demographic consequences, population extinctions, mutation spread and alleles lost in bottlenecks and founder effects. As a result, these oaks exhibit complex genetic diversity patterns, mainly due to post-glacial lineage admixtures, utterly complicated by interspecific hybridisation, particularly in the case of *Q. ilex* and *Q. coccifera* (Jiménez *et al.* 2004; Rubio de Casas *et al.* 2007a). The genetic consequences of such range modifications have been extensively studied for several taxa (see for instance López de Heredia *et al.* 2005; Rubio de Casas *et al.* 2006), but the phenotypic outcomes remain to be assessed. Regardless of the underlying causal processes, sclerophylls are successful under present-day conditions, and their leaf functional syndrome has been proved to withstand the extremes of the Mediterranean climate (*e.g.* Larcher 2000; Martínez-Ferri *et al.* 2000, 2004; Chaves *et al.* 2002).

The apparent stasis of sclerophylly through the Quaternary environmental and genetic rearrangements, together with the current functional significance of this syndrome (Salleo & Nardini 2000), could be taken as a case of exaptation (Gould & Vrba 1982). According to this interpretation, the intraspecific phenotypic variation observed among current populations of sclerophyllous *Quercus* species (Rubio de Casas *et al.* 2007b; Sánchez-Vilas & Retuerto 2007) would reflect either the expression of phenotypic plasticity in response to the local environment, and/or the emergence during the Quaternary of new variants by neutral genetic processes, such as founder effects, bottlenecks or genetic drift. Alternatively, a role of phenotypic plasticity as an elicitor of a recent ecotypic differentiation can be proposed for these plants (Balaguer *et al.* 2001). According to this latter hypothesis, if plasticity is mediating the genetic divergence, then phenotypic differences should be associated with environmental and genetic variation. In the present study, we tried to determine if that is the case and if variation found within the sclerophyllous syndrome is consistent with population genetic structure and environmental conditions. To test this, we used Iberian populations of *Q. coccifera* growing in a variety of environments. We compared population genetic patterns (obtained with Inter Simple Sequence Repeat, ISSR, fingerprinting), with environmental (climatic and edaphic) data, and with variations in leaf pigments and morphology, to address the following specific questions:

1 Is genetic variability geographically structured? The long life span and anemophily of the study species suggest limited genetic differentiation. However, the existence of glacial refugia seems to have facilitated allopatry (Lumaret *et al.* 2002; Olalde *et al.* 2002; Petit *et al.* 2003; López de Heredia *et al.* 2007).

2 In an affirmative case, is the geographic pattern sufficiently explained by neutral genetic processes? Correlation between geographic and genetic distances would suggest a stepwise post-glacial colonisation structure. Although this pattern may have been blurred by the repeated episodes of colonisation, population admixture and isolation, it seems unlikely that these processes solely explain a close match between genetic and environmental patterns.

3 Do genetically distinct populations exhibit phenotypic differences? It has been reported that the phenotype in *Q. coccifera* is remarkably consistent in spite of the high genetic variance (Rubio de Casas *et al.* 2007a). If, however, there is a significant correlation between the variations in neutral genetic and leaf traits, in consonance with differences in the local environment, this would lend support to the existence of genetically differentiated ecotypes.

MATERIALS AND METHODS

Field sites and plant material

Nine populations were sampled encompassing all the habitats of *Quercus coccifera* L in the Iberian Peninsula (Fig. 1; Cañellas & San Miguel 2003). Three 50 × 50 m plots were marked out at seven localities. Five individuals per plot were randomly chosen. In the other two localities (SAL and ARR), vegetation was too dense and five plants were chosen randomly on three transects across plots of the same size. A 27 × 27 matrix of geographic distances among plots (GEO) was produced. The distance between plots was assumed to be either 50 m within a population, or equal to the distance between populations. In order to gather comparable data, all sampling was conducted in midsummer, when plants are assumed to be in drought-induced aestivation (Cañellas & San Miguel 2003). Only plants bearing fruit were included in the study to avoid juveniles. We considered the individual crown as the functional unit. Measurements and samples were taken on fully expanded, current-year foliage in two exposures to span the phenotypic variability expressed by the same individual (*i.e.* intracanalopy leaf plasticity): south-facing, fully exposed (sun) and north-facing, in-crown (shade) leaves.

Environmental data

Climatic data included monthly 30-year maximum, minimum, mean temperatures and rainfall (Spanish National Institute of Meteorology). Soil data included pH and total N, P, K, Ca, Mg, Na, Zn and Mn. These data are available from the authors upon request. Climatic and edaphic data were combined into a single matrix of nine rows (one for each *Q. coccifera* population) and 57 columns containing the meteorological data and soil characteristics. This matrix was used after standardisation of variables to produce a dissimilarity matrix based on Euclidean distances among plots (ENV hereafter). A



Fig. 1. Distribution of the kermes oak (*Quercus coccifera* L.) in the Iberian Peninsula and location of the studied populations. The dashed line indicates the divide between the two genetically distinct geographic areas (see text for details).

Population	Location	Latitude (°N)	Longitude (°W)	Altitude
1 AIN	Ainsa, Huesca, Spain	42°26'19"	0°5'6"	1549 m asl
2 ARJ	Aranjuez, Madrid, Spain	40°0'2"	3°36'27"	579 m asl
3 ARR	Serra da Arrábida, Setúbal, Portugal	38°27'17"	9°0'62"	312 m asl
4 CAR	Cardeña, Córdoba, Spain	38°21'46"	4°19'20"	581 m asl
5 CÑV	Cañada de Verich, Teruel, Spain	40°52'46"	0°6'79"	822 m asl
6 FAC	Facinas, Cádiz, Spain	36°9'46"	5°40'9"	118 m asl
7 GAR	Gargallo, Teruel, Spain	40°51'87"	0°33'38"	1018 m asl
8 SAL	El Saler, Valencia, Spain	39°22'50"	0°19'40"	104 m asl
9 TAR	Tarazona, Zaragoza, Spain	41°50'35"	1°38'92"	694 m asl

constant distance among plots from the same population (0.01) was assumed, while the distance assigned to any two plots belonging to different populations was taken to be the distance between populations.

Genetic sampling and analyses

Leaf material was collected in the field from the first three individuals of each plot (*i.e.* nine populations \times three plots \times three individuals; $n = 81$). Samples were sterilized and analysed as described by Rubio de Casas *et al.* (2007a). Briefly, the primers 834 (AG)₈YT and 818 (CA)₈G (University of British Columbia Biotechnology Laboratory) were amplified using a thermal cycler Gene Amp PCR System 9700 (PE Applied Biosystems). Negative controls and replicates were included to verify repeatability of results. Gel electrophoresis and visualisation of the fragments was performed using precast polyacrylamide gels (Applied Biosystems) with a 20-bp ladder (Ez-load™ 20 bp Molecular Ruler, BIO-RAD Laboratories, Hercules, CA, USA). The silver-stained gels were

scanned and only fragments between 180 and 1000 bp were scored with Quantity One® quantitation software (BIO-RAD Laboratories). The two ISSR primers generated a total of 287 bands. The basic data structure consisted of a matrix of 81 rows and 288 columns, with one column identifying the individual and 287 columns describing the presence or absence of each of the bands. The ISSR data were then subjected to analyses of molecular variance (AMOVA) with ARLEQUIN, v 2.00 to test genetic structure at population level (Schneider *et al.* 2000) and with the Bayesian method suggested by Holsinger *et al.* (2002) with HICKORY (Holsinger & Lewis 2003). HICKORY was run with the default parameters except for the burn-in period (50,000), number of generations (500,000) and thinning (100). HICKORY enables the estimation of significance in population differentiation by comparing the Deviation Information Criterion (DIC) of the full model (no *a priori* assumption on population differentiation) with that of a model with the same parameters but with no population differentiation, defined as $\theta^B = 0$, with θ^B being the Bayesian analogue of

F_{ST} . If the DIC of the full model is at least six units smaller than that of the $\theta^B = 0$ model, then significant differences among populations can be assumed. However, HICKORY results have to be interpreted cautiously to ensure that the difference in DIC is because the full model fits the data better and not because of a difference in model complexity. That can be estimated by comparing D_{bar} (model fit) and pD (model complexity) of both models, ensuring that $D_{bar} + pD$ of the full model $< D_{bar} + pD$ of the $\theta^B = 0$ model (Holsinger & Wallace 2004). To explore the partitioning of the genetic variance into different geographic and genetic distinguishable areas, we used SAMOVA (Dupanloup *et al.* 2002) with number of groups (K) $2 \leq K \leq 8$. A matrix containing pairwise F_{ST} s for each pair of plots was computed with ARLEQUIN.

Leaf morphology and pigments

Leaf angle, area, specific leaf area (SLA), lobation index (ILB) and leaf spininess (SPIN) were calculated for a total of 2700 leaves (*i.e.* nine populations \times three plots \times five individuals; $n = 135$ each value calculated from two canopy positions \times 10 replicates). Leaf angle was measured in the field using a protractor on 10 mature leaves per exposure. Leaf lobation index and spininess were calculated as in Rubio de Casas *et al.* (2007b); the former as the difference between the measured perimeter and that of the ellipse that circumscribes the leaf blade, estimated by the YNOT formula (Maertens & Rousseau 2000) and divided by the measured perimeter, and the latter as the ratio between the number of marginal leaf spines and the perimeter of the circumscribing ellipse. Three pooled leaf samples of each exposure from the first three individuals of each plot were taken at noon and immediately stored in liquid nitrogen until their pigments were analysed (*i.e.* nine populations \times three plots \times three individuals; $n = 81$ each value calculated from two canopy positions \times three replicates; Martínez-Ferri *et al.* 2000). Briefly, chlorophylls and carotenoids were separated by HPLC (Waters Corp., Milford, MA, USA), following pigment extraction in cool acetone. The peaks were identified and quantified with pure commercial standards (VKI, Hørsholm, Denmark). Three light-responsive pigment pools were used in the analyses: total chlorophyll on a leaf area basis, and xanthophyll cycle pigments (VAZ) and β -carotene on a chlorophyll content basis. Every trait was described by intracanalopy plasticity (the unsigned difference between the mean for the trait in shade minus that in sun), and the arithmetic mean for each individual crown. Foliar data were averaged per plant, plants grouped in plots, plots nested in populations, and populations nested within the two geographic areas revealed by genetic analysis (see below). Differences between populations and/or areas for each of the characters were then assessed by fitting generalised linear models. Except when otherwise indicated, models were fitted with a Gaussian distribution and an identity link function. Dissimilarity matrices based

on Euclidean distances between plots were calculated using the eight traits after standardisation, one for means (PHEN matrix) and another for intracanalopy plasticity values (PLAS matrix). In order to explore the geographic pattern in the phenotypic data, we performed a principal components analysis (PCA) using the average across plots of both the mean value and intracanalopy plasticity of each morphological and pigment trait for each of the nine populations. Because of the heterogeneous nature of the leaf features, the PCA was performed on standardised variables.

Geographic, environmental, genetic (estimated with population pairwise F_{ST} values) and phenotypic distances are available from the authors upon request. The relationships among these matrices were investigated with simple and partial Mantel tests using the 'ecodist' package in R (Mantel 1967; Goslee & Urban 2007). If the genetic structure is due to isolation by distance, then genetic and geographic distances should be correlated. If all the observed differences among populations are due to environmental variation, then the correlation of phenotypic differences with environmental conditions should be significant even when accounting for genetic differences. Conversely, if the observed population variability is due to genetic divergence, then the correlation between genetic and phenotypic differences should remain significant even after eliminating environmental effects. These comparisons are also informative of the degree of adaptive differentiation reflected by phenotypic differences. For instance, if populations 'less distant' environmentally (inhabiting more similar environments) are more similar to each other in their mean phenotypes than to more 'environmentally distant' populations (*i.e.* if there is a correlation between environmental and mean phenotypic differences), whereas the same environmentally similar populations are not on average more genetically similar to each other than to more distant populations (*i.e.* no correlation between genetic and environmental differentiation), then this would be consistent with adaptive differentiation among populations. Spatial autocorrelation was controlled for in all partial Mantel tests by maintaining the matrix of geographic distances (GEO) as a partial. The significance of the correlations was assessed with 10,000 permutations.

RESULTS

Population genetic structure and environmental conditions

Most of the genetic diversity was distributed at the intrapopulation level and only around 5% of the total variation was due to population differentiation ($\theta^B = 5.43\%$; $F_{ST} = 5.12\%$). Inter-population differences were significant according to both AMOVA ($P < 0.0001$) and Bayesian inference approaches. The Deviance Information Criterion (DIC) values of the full model (DIC = 4902.88; $D_{bar} = 4316.90$; $pD = 585.98$) were more than six units smaller than those of the $\theta^B = 0$ model (DIC = 5505.89;

Dbar = 5233.54; $pD = 272.35$). This difference was because of the full model's better average fit (Dbar) and not to a reduced complexity of the full model compared to the $\theta^B = 0$ model (pD ; Holsinger & Wallace 2004). Genetic distinctiveness of populations was further supported by SAMOVA, according to which the grouping of populations that explained the highest variance percentage (3%) was $K = 8$. SAMOVA analyses also supported a geographic divide between the four NE populations (AIN, CÑV, GAR and TAR) and the remaining five populations (ARJ, ARR, CAR, FAC and SAL). Genetic differences between these two groups were found to be significant ($F_{CT} = 0.92\%$, $P = 0.016$) and every grouping with $K > 2$ broke each of the two groups down independently. The AMOVA showed levels of genetic variation in each of these two areas similar to that of the whole peninsula (Northeastern Iberia [NE] $F_{ST} = 4.77\%$, $P < 0.001$; Central and Southern Iberia [CS] $F_{ST} = 4.49\%$, $P < 0.001$).

The two geographic areas defined by the genetic analyses appeared to also be significantly distinct for 27 of the 57 environmental parameters (data not shown): mean and minimum temperatures of winter, spring and autumn months and maximum temperatures of winter months were colder in the northeast while summer rainfall and soil Ca content were higher.

Phenotypic differences among populations and between geographic areas

Mean phenotypic values and results of the nested generalised linear model of morphological and pigment traits are displayed in Table 1. All characters except leaf area and ILB differed significantly among populations, and all of them, except leaf angle and ILB, showed significant differences between geographic areas. The four populations from NE Iberia had smaller plants (1.1 *versus* 2.0 m plant height), with smaller (1.75 *versus* 4.4 cm² leaf area), spiner (2.2 *versus* 1.7 spines cm⁻¹) and thicker leaves (5.1 *versus* 6.3 m²·kg⁻¹ SLA). Five out of eight traits exhibited significantly lower values of intracanalopy plasticity in NE Iberia: leaf angle (18.9° *versus* 35.7°), leaf area (1.5 *versus* 4.9 cm²), ILB (0.060 *versus* 0.083), SLA (1.9 *versus* 3.4 m²·kg⁻¹) and β -carotene (16.6 *versus* 18.3 mmol·mol⁻¹). In a multivariate context, the first two axes of a PCA performed on the standardised average morphological and pigment traits of each of the nine investigated populations accounted for 45.81% and 19.15% of the total variance, respectively (Fig. 2). In the first PCA plane, populations were segregated along the first axis according to their assignment to each of the two geographic areas defined by the genetic analyses.

Correlation between genetic and phenotypic differences

Pair-wise matrix comparisons among multivariate dissimilarity matrices are shown in Table 2. In simple matrix comparisons, genetic differences were found to correlate only with differences in mean phenotypic values, while

environmental differences and geographic distances showed a strong correlation with between-population differences in mean phenotypes and intracanalopy plasticity. When correlations were simultaneously corrected for geographic distances and for environmental or genetic differences (Table 2B), no significant variation in the degree of matrix association was detected, except between environmental and mean phenotypic differences that ceased to be significantly correlated when geographic and genetic relatedness were simultaneously accounted for.

DISCUSSION

The significant phenotypic differences found within the sclerophyllous syndrome among populations of kermes oak (*Quercus coccifera* L.) were congruent with the genetic structure at the population and regional level. The simultaneous and independent correlation of mean leaf phenotypes with geographic distances and genetic differences supports the hypothesis that differences between populations are the result of adaptation to local conditions (Banta *et al.* 2007). This pattern is hardly explained solely by neutral genetic processes, but more likely by Quaternary divergence driven by ecological differences. These results also support previous reports of recent ecotypic differentiation based on experiments using saplings from a subset of populations of *Q. coccifera* (ARR, CÑV, GAR; Balaguer *et al.* 2001).

A small but significant genetic differentiation was observed among Iberian populations of *Q. coccifera*. Only slight differentiation at the population level was expected, given the long life span of the plant, its anemophily and the lack of isolation among populations (Hamrick & Godt 1996; Nybom 2004). Nevertheless, molecular differences proved to be significant in two contrasting statistical methods, in agreement with results obtained by other authors (Jiménez *et al.* 2004; López de Heredia *et al.* 2005). Above the population level, only the clustering of populations in NE Iberia, including four of the nine populations and CS Iberia, harbouring the other five, proved to be significant in terms of molecular variance. The regional split detected within the Iberian Peninsula was also observed by Lumaret *et al.* (2002) in holm oak. This congruency is not surprising given the high relatedness between the two species and that they both face the same limitations in gene flow and fruit dispersal (Jiménez *et al.* 2004; López de Heredia *et al.* 2007). Interspecific hybridisation might also have contributed to the overall structure. Apparently, there are no genetic barriers between *Q. coccifera* and other *Quercus* species (López de Heredia *et al.* 2005, 2007). However, Rubio de Casas *et al.* (2007a) showed that regional differences are unlikely to result from differential interspecific gene flow. Hybridisation, as a widespread phenomenon, did not seem to be related to the survival of *Q. coccifera* in suboptimal environments, contrary to what has been reported for other *Quercus* species (Petit *et al.* 2004).

Our results indicated that the present genetic structure does not seem to be the outcome of stepwise colonisation

Table 1. Descriptive statistics [mean (\pm SE)] and results of the generalised linear models (χ^2) performed among populations (A) and between geographic areas (B).

	(A) among populations											(B) between geographical areas			
	I. ARJ	I. ARR	I. CAR	I. FAC	I. SAL	II. AIN	II. CNV	II. GAR	II. TAR	χ^2	I. CS Iberia	II. NE Iberia	χ^2		
angle ($^\circ$) ^a	M 43.28 (\pm 2.03)	47.29 (\pm 1.06)	41.60 (\pm 1.10)	42.17 (\pm 0.86)	34.91 (\pm 1.70)	37.50 (\pm 1.63)	42.74 (\pm 1.67)	42.30 (\pm 1.91)	38.34 (\pm 1.69)	12.45***	41.85 (\pm 0.77)	40.22 (\pm 0.89)	1.58 n.s.		
n = 15	I 27.63 (\pm 3.01)	42.49 (\pm 3.26)	39.84 (\pm 1.96)	42.41 (\pm 1.62)	26.14 (\pm 2.75)	13.14 (\pm 1.90)	22.73 (\pm 3.24)	29.41 (\pm 2.48)	10.34 (\pm 2.12)	26.87***	35.70 (\pm 1.41)	18.91 (\pm 1.57)	51.19***		
ILB ^a n = 15	M 0.26 (\pm 0.01)	0.21 (\pm 0.01)	0.21 (\pm 0.01)	0.23 (\pm 0.01)	0.24 (\pm 0.01)	0.23 (\pm 0.01)	0.21 (\pm 0.01)	0.26 (\pm 0.02)	0.23 (\pm 0.01)	2.26 n.s.	0.23 (\pm 0.01)	0.23 (\pm 0.01)	0.02 n.s.		
I	0.09 (\pm 0.01)	0.11 (\pm 0.01)	0.06 (\pm 0.01)	0.05 (\pm 0.01)	0.07 (\pm 0.01)	0.04 (\pm 0.01)	0.06 (\pm 0.01)	0.07 (\pm 0.01)	0.07 (\pm 0.01)	0.57 n.s.	0.08 (\pm 0.00)	0.06 (\pm 0.00)	5.48*		
leaf area	M 3.30 (\pm 0.25)	5.42 (\pm 0.35)	5.47 (\pm 0.46)	3.98 (\pm 0.23)	3.80 (\pm 0.24)	1.64 (\pm 0.10)	1.40 (\pm 0.10)	2.08 (\pm 0.09)	1.87 (\pm 0.09)	0.12 n.s.	4.20 (\pm 0.15)	1.75 (\pm 0.06)	304.66***		
(cm ²) ^a n = 15	I 3.89 (\pm 0.37)	5.99 (\pm 0.59)	5.38 (\pm 0.59)	5.02 (\pm 0.38)	4.28 (\pm 0.33)	0.99 (\pm 0.13)	1.27 (\pm 0.17)	2.07 (\pm 0.18)	1.73 (\pm 0.18)	3.95**	4.91 (\pm 0.22)	1.52 (\pm 0.10)	209.97***		
SLA (m ² ·kg ⁻¹) ^a	M 5.18 (\pm 0.01)	7.06 (\pm 0.16)	6.82 (\pm 0.13)	6.32 (\pm 0.12)	6.25 (\pm 0.12)	4.80 (\pm 0.17)	5.37 (\pm 0.18)	5.52 (\pm 0.20)	4.74 (\pm 0.17)	9.91**	6.32 (\pm 0.10)	5.10 (\pm 0.10)	84.38***		
n = 15	I 2.95 (\pm 0.13)	3.53 (\pm 0.27)	3.48 (\pm 0.56)	3.86 (\pm 0.15)	3.01 (\pm 0.21)	1.85 (\pm 0.13)	1.76 (\pm 0.20)	2.16 (\pm 0.32)	1.80 (\pm 0.15)	1.31 n.s.	3.37 (\pm 0.09)	1.90 (\pm 0.11)	106.7***		
SPIN	M 1.72 (\pm 0.01)	1.50 (\pm 0.08)	1.41 (\pm 0.05)	2.07 (\pm 0.07)	1.94 (\pm 0.06)	2.13 (\pm 0.11)	2.63 (\pm 0.12)	2.25 (\pm 0.12)	1.74 (\pm 0.13)	11.32***	1.73 (\pm 0.04)	2.20 (\pm 0.07)	30.20***		
(spines cm ⁻¹) ^a	I 0.27 (\pm 0.01)	0.26 (\pm 0.01)	0.20 (\pm 0.01)	0.78 (\pm 0.01)	0.46 (\pm 0.01)	0.27 (\pm 0.01)	0.55 (\pm 0.01)	0.57 (\pm 0.01)	0.35 (\pm 0.01)	5.91*	0.40 (\pm 0.03)	0.44 (\pm 0.04)	0.23 n.s.		
n = 15	M 100.47 (\pm 2.51)	103.51 (\pm 4.23)	97.86 (\pm 2.22)	100.32 (\pm 3.32)	111.92 (\pm 3.59)	79.34 (\pm 1.31)	110.83 (\pm 3.21)	108.34 (\pm 2.44)	84.70 (\pm 1.67)	57.81***	97.21 (\pm 2.03)	103.93 (\pm 1.87)	10.15**		
β -car	(mmol·mol ⁻¹) ^b I 11.91 (\pm 3.36)	24.49 (\pm 6.24)	14.30 (\pm 3.07)	22.40 (\pm 5.10)	41.81 (\pm 11.28)	11.64 (\pm 2.51)	12.47 (\pm 5.65)	14.83 (\pm 3.47)	5.31 (\pm 1.75)	0.21 n.s.	18.31 (\pm 2.91)	16.62 (\pm 2.60)	9.40**		
n = 9	M 871.30 (\pm 33.21)	549.65 (\pm 28.34)	611.97 (\pm 28.74)	637.31 (\pm 33.56)	599.00 (\pm 51.72)	890.07 (\pm 48.50)	637.57 (\pm 35.21)	648.26 (\pm 51.28)	885.00 (\pm 40.51)	5.87*	742.04 (\pm 27.36)	637.87 (\pm 18.01)	-6.98**		
chl a + b	(μ mol·m ⁻²) ^b I 148.74 (\pm 51.85)	107.97 (\pm 35.80)	98.73 (\pm 20.94)	181.73 (\pm 45.66)	214.15 (\pm 65.52)	221.21 (\pm 31.60)	222.93 (\pm 51.06)	303.82 (\pm 69.95)	99.47 (\pm 27.40)	2.77 n.s.	166.39 (\pm 20.64)	196.76 (\pm 28.21)	1.72 n.s.		
n = 9	M 77.98 (\pm 2.36)	81.55 (\pm 3.73)	108.33 (\pm 8.24)	89.74 (\pm 3.23)	89.08 (\pm 4.93)	57.01 (\pm 2.70)	88.22 (\pm 3.68)	83.89 (\pm 2.61)	81.88 (\pm 2.79)	11.35***	77.91 (\pm 1.96)	94.86 (\pm 3.23)	8.61**		
VAZ	(mmol·mol ⁻¹) ^b I 28.33 (\pm 3.04)	36.80 (\pm 4.45)	37.95 (\pm 9.77)	22.61 (\pm 6.01)	38.05 (\pm 7.91)	14.55 (\pm 2.04)	31.69 (\pm 6.54)	34.93 (\pm 5.04)	28.86 (\pm 5.01)	2.82 n.s.	29.48 (\pm 2.28)	32.01 (\pm 4.04)	1.60 n.s.		
n = 9	M 2.25 (\pm 0.22)	2.03 (\pm 0.18)	2.53 (\pm 0.32)	1.31 (\pm 0.09)	1.83 (\pm 0.09)	1.16 (\pm 0.15)	0.74 (\pm 0.06)	1.32 (\pm 0.10)	1.14 (\pm 0.17)	86.75***	2.00 (\pm 0.10)	1.09 (\pm 0.07)	49.65***		
plant height	(m) n = 9														

Population means (M) and intracranopy plasticity values (I) were analysed separately. Sample sizes (n) are given below each variable. Roman numerals indicate geographical areas, i.e. I. 'CS Iberia' includes ARJ, ARR, CAR, FAC and SAL populations, whereas II. 'NE Iberia' includes AIN, CNV, GAR and TAR populations. For further details regarding variables see text.

^a Residual deviance df = 126; ^b Residual deviance df = 75. *P < 0.05; **P < 0.01; ***P < 0.001.

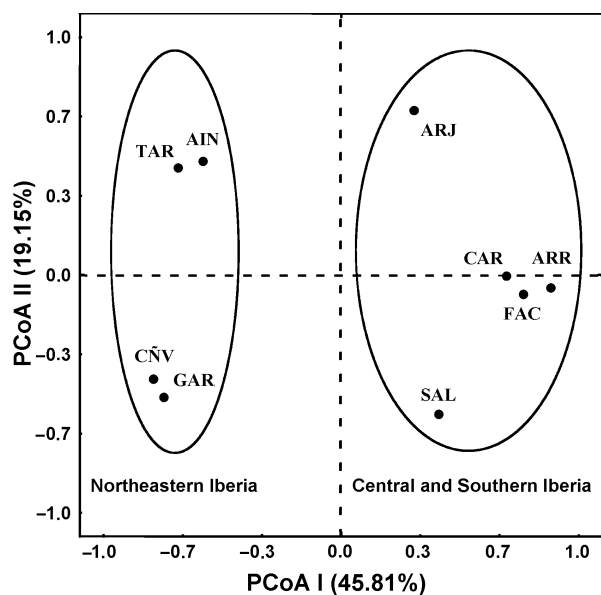


Fig. 2. Projection of the nine investigated populations on the first PCA plane based on mean values and intracranopy plasticities of eight morphological and pigment traits. The circles indicate geographic assignment to northeastern or central and southern Iberia. See Fig. 1 for labels.

from a single or a few refugia, since geographic and genetic distances were not correlated. The existence of several independent refugia within the Iberian Peninsula has probably enabled the maintenance of complex genetic patterns throughout the Holocene (see Jiménez *et al.* 2004; López de Heredia *et al.* 2007 for a thorough discussion). Differentiation among *Q. coccifera* populations was probably favoured by allopatry resulting from repeated confinement to glacial refugia during the Quaternary (Hewitt 2004). Previous reports agree that oaks originating from southern populations found secondary refugia in NE Iberia (Lumaret *et al.* 2002; Olalde *et al.* 2002; López de Heredia *et al.* 2007).

The genetic divergence observed between the NE and CS populations was concordant with a large environmental contrast. These geographic areas differed significantly in nearly 50% of the environmental (climatic and edaphic) variables measured. However, no correlation was found when genetic differences among populations were compared with differences in environmental conditions (Table 2). Thus, although environmental and genetic patterns of divergence partially overlap when the whole Iberian Peninsula is considered, there is not a clear relationship between them at the population scale. Ecologically driven gene flow reduction across the observed habitat boundary may follow a threshold pattern or is most likely accounted for by past events of isolation. Palaeoecological evidence has been found for the persistence of steppe environments during glaciations in NE Iberia (Ribera & Blasco-Zumeta 1998). Our results are

Table 2. Simple and partial correlation tests between geographic distances (GEO), population pairwise F_{ST} values (FST), differences in environmental characteristics (ENV), in phenotypic grand means (PHEN), and in intracranopy plasticity (PLAS). Significance was assessed by 10,000 randomisations. (A) Correlation between matrices. Pairwise correlations with geographic distances were assessed with simple Mantel tests, while partial Mantel tests controlling for GEO were used to account for spatial autocorrelation in all other comparisons. (B) Partial correlations corrected for environmental differences (above diagonal) and for genetic relatedness (below diagonal). Spatial autocorrelation was controlled for by including GEO in all comparisons.

	GEO	ENV	PLAS	PHEN	FST
(A)					
GEO	–				
ENV	0.581***	–			
PLAS	0.632***	0.282***	–		
PHEN	0.318***	0.153*	0.303***	–	
FST	0.041	0.111	0.044	0.310***	–
(B)					
GEO	–		0.420***	0.260**	–0.0192
ENV	0.581***	–	–	–	–
PLAS	0.631***	0.283***	–	0.274***	–0.015
PHEN	0.323***	0.131	0.316***	–	0.309***
FST	–	–	–	–	–

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

consistent with this scenario; the phenotypes of NE populations correspond to harsher climatic conditions. The divide between the two genetically distinct geographic areas also emerged from the analysis of phenotypic features. Plants from the NE were shorter, with smaller, thicker and spiner leaves (Table 1). This character syndrome is often associated with stressful and/or disturbance-prone environments (cf. Ackerly *et al.* 2002; Bond & Midgley 2003). Among other stress factors, the increase in frost frequency, particularly limiting for *Quercus coccifera* (Martínez-Ferri *et al.* 2004), is a determining factor in NE Iberia. Moreover, plants from this geographic area exhibited less plasticity than those from the rest of Iberia, as is often the case when plant development is limited by ecological conditions (Valladares *et al.* 2007).

Phenotypic plasticity was highly correlated with mean phenotypes, even after correcting for genetic and environmental distances (Table 2). Consistent with mean phenotypes, genetic traits and environmental factors, phenotypic plasticity also exhibited different values across the boundary between NE and CS populations. However, phenotypic plasticity was differentially correlated with environmental variation across sites, regardless of genetic affinities among populations. Given this degree of environmental responsiveness, it seems reasonable to expect that the initial survival of colonising lineages of *Q. coccifera* in the steppe conditions of NE Iberia were facilitated by the expression of phenotypic plasticity to enable the occupation of suboptimal niches. Previous reports have suggested that this role of plasticity might have fostered

adaptive change (Cavender-Bares & Wilczek 2003), in particular during the Quaternary climate changes (Ackerly 2003).

Ultimately, the variation observed within and among populations in vegetative phenotypes, coupled with the levels of genetic variation detected with neutral genetic markers, denotes the occurrence of microevolutionary processes in *Q. coccifera*. Although the leaf syndrome in this species is pre-Mediterranean (Verdú *et al.* 2003), results presented here support the hypothesis that trait evolution and population divergence have been shaped by the taxon's biogeographic Quaternary history.

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