

Factors affecting cork oak growth under dry conditions: local adaptation and contrasting additive genetic variance within populations

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Abstract Increased drought severity is expected in the Mediterranean Basin over the twenty-first century, but our understanding of the potential of most forest tree species to cope with it remains uncertain. In this study, (1) we examined the potential effects of long-term selection and the capacity to respond to future changes in selective pressures in three populations of cork oak (*Quercus suber* L.). For this purpose, we evaluated the response to dry conditions of 45 open-pollinated trees originating from populations in Morocco, Portugal, and Spain. Growth, leaf

size, specific leaf area (SLA), carbon isotope discrimination ($\Delta^{13}\text{C}$), leaf nitrogen content (N_{mass}), and total chlorophyll content (Chl_{mass}) were measured in 9-year-old plants. (2) We also investigated the relationships between functional traits and aboveground growth by regression models. Plants presenting larger and more sclerophyllous leaves (low SLA and high leaf thickness) exhibited higher growths, with results suggesting that these traits are subjected to divergent selection in this species. Heritability estimates were moderately high for $\Delta^{13}\text{C}$ (0.43 ± 0.25 – 0.83 ± 0.31) and stem diameter (0.40 ± 0.15 – 0.71 ± 0.28) for the tree populations. For the rest of the traits (except for annual growth), heritability values varied among populations, particularly for height, leaf size, leaf thickness, and N_{mass} . Our results suggest that natural selection has led to local adaptations and has also affected the genetic variance intrapopulation in these cork oak populations, although studies with a higher number of populations should be carried out across different years. Additionally, the absence of significant genetic correlations and the fact that correlated traits did not undergo opposing selection provided little evidence for constraints on evolution caused by genetic correlations.

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Introduction

Summer drought is the most important ecological filter for plant establishment, long-term survival, and growth in Mediterranean ecosystems (Fotelli et al. 2000; Ackerly 2004; Lloret et al. 2004). Thus, traits related to water economy and growth, such as water use efficiency, leaf

sclerophylly, and leaf nitrogen content, are expected to be of primary importance in determining plant fitness under water-limited conditions (Dudley 1996; Heschel et al. 2002, 2004; Ludwig et al. 2004). The importance of these traits is likely to be even reinforced in the future due to the expected increase in the severity and duration of drought periods in the Mediterranean Basin (IPCC 2007). In the short term, the capacity of species to cope with these changing environmental conditions relies on their phenotypic plasticity as well as on their intraspecific genetic variation for adaptive traits (Ward et al. 2000; Ward and Kelly 2004; Marchin et al. 2008). In the long term, this response will also depend on both the possibility to migrate to favorable environments and the adaptability of the functional traits to the new conditions (Geber and Dawson 1993; Ward and Kelly 2004; Parmesan 2006). While there is abundant information of individual plant responses to changing abiotic factors by acclimation and plasticity, much less is known about the capacity of plants to evolve, particularly so in the case of long-living species such as trees (Petit and Hampe 2006).

The potential for adaptive evolution of quantitative traits depends on the amplitude of their additive genetic variance as well as the genetic covariances among traits (Falconer and Mackay 1996; Roff 1997). Thus, the evolvability of a trait can be estimated by two genetic parameters: heritability and the genetic correlations with other traits. Heritability determines the potential for evolutionary changes and the rate at which a trait can respond to selection (Falconer and Mackay 1996). Genetic correlations among traits and between each trait and fitness can also determine the evolutionary potential of the traits (e.g., Cheverud 1984; Arnold 1992). For instance, if two positively correlated traits are selected for in opposing directions (correlations between each trait and fitness have a different sign), then the adaptive response to selection would be constrained (Roff 1997; Campbell 1997; Cotter et al. 2004).

Natural selection and evolutionary potential of reproductive and morphological traits have been widely investigated (e.g., Leiss et al. 2004; Giles et al. 2006; Hodgins and Barrett 2008; Kaczorowski et al. 2008), but relatively few studies have focused on physiological traits related to drought tolerance (Geber and Griffen 2003), and hitherto we are not aware of any study with Mediterranean oaks. Cork oak (*Quercus suber* L.) is a Mediterranean evergreen tree species that displays intermediate tolerance to drought between the drought-tolerant evergreen oaks, such as *Quercus ilex* and *Quercus coccifera* (David et al. 2007; Peguero-Pina et al. 2009), and the more mesic deciduous ones, such as *Quercus faginea* and *Quercus pyrenaica* (Mediavilla and Escudero 2003). It is expected to be severely affected by climate change due to the increased intensity and duration of the drought periods expected for this region (see “Discussion” in Ramírez-Valiente et al.

2009a) and also because of its particularly high sensitivity to drought in the early stages of development (Aranda et al. 2005, 2007). Although factors affecting seedling survival have been studied in this species, factors affecting other fitness components are currently unknown. Additionally, it is known that cork oak populations differ in survival, growth, and functional traits related to drought stress tolerance (Gandour et al. 2007; Ramírez-Valiente et al. 2009a; b, 2010a, b), but there is scant knowledge of its intrapopulation genetic diversity, additive genetic variance, and genetic correlations among traits, so the potential for adaptive evolution of the species under a rapidly changing climate remains uncertain.

The first objective of this study was to estimate the interpopulation and intrapopulation genetic divergence, the genetic correlations among traits, and the heritabilities of a set of ecophysiological traits in order to evaluate the potential effects of the long-term selection on wild cork oak populations. For this purpose, we analyzed selected leaf ecophysiological traits during an extremely dry year in 9-year-old plants established under common environmental conditions. Plants were sampled from 45 open-pollinated families from three populations. The traits selected were specific leaf area (SLA), leaf size, leaf nitrogen content per leaf mass, and carbon isotope discrimination as a surrogate of water use efficiency (Farquhar et al. 1989). Based on previous findings (Ramírez-Valiente et al. 2009b, 2010a, b), we expected that the contrasting conditions regarding temperature and rainfall endured by the different populations have led to local adaptation. Likewise, we expected that the reproductive strategy of cork oak as a long-living species with outcrossing and anemophily, coupled with a high spatial and temporal heterogeneity in the environmental conditions within the populations, has led to high levels of intrapopulation genetic variance and, thus, relatively high capacity of response to future changes in selective pressures (Hamrick 2004).

The second objective of the study was to explore the relationships between the selected leaf traits and above-ground growth. We expected faster growth in cork oak individuals with lower $\Delta^{13}\text{C}$ (higher WUE) under dry conditions (Dudley 1996). Likewise, we expected that individuals with lower SLA and higher leaf thickness (more sclerophyllous leaves) and small leaves were favored in a dry environment, as sclerophylly reduces transpiration rate and improves drought resistance (Nobel 1999; Gratani and Varone 2006; Niinemets et al. 2007).

Material and methods

Study site

The study was carried out in a common garden of cork oak (*Q. suber* L.) established in the “Sierra de Andújar” Natural

Park (38° 21' 54" N, 3° 51' 40" W, 560 m a.s.l.), which is located within the main core of the cork oak natural range. The aspect of the plot is N–NE, and the slopes range from 3% to 13%. Climate is typical of Mediterranean regions, characterized by dry warm summers and moderately cold winters. Mean annual rainfall and temperature are 617 mm and 14.7°C, respectively. The average temperature for the most contrasting months is 5.9°C in January and 25.3°C in July (data for the interval 1987–2005, taken from the closest climatic station to the plot, located about 4 km from the common garden). 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).

Experimental design and climate during the studied year

Three populations and 22 mother trees per population were sampled for the experiment. The three populations studied are within the main area of distribution of cork oak, and they represent three sites with contrasting severity of the dry season (Table 1, Fig. 1). In these areas, cork oak forms extensive, mostly pure forests, which are rare in the Mediterranean basin. Thus, the effects of hybridization events with *Q. ilex* appearing in mixed forests (Burgarella et al. 2009) and inbreeding depression phenomena potentially present in marginal populations, which could potentially affect fitness, are presumably excluded or at least minimized in our study. Mother trees were randomly selected within each population. They were separated at least 150 m among each other, which is an enough distance to avoid familial structures (Soto et al. 2007). Seeds were collected from native stands during the autumn and winter of the year 1996. Seeds were sown in the beginning of 1997 and grown in a nursery for a year under standard conditions of water and nutrient availability to minimize the potential maternal effects. Then, they were planted in the field during the spring of 1998. The common garden followed a design of randomized complete blocks. Fifteen

open-pollinated families per population (from the 22 open-pollinated families established in the original common garden setting) and eight blocks were sampled for this study (total $N=360$ plants=8 saplings per family×15 families per population×3 population).

The ecophysiological study extended over 2005, a particularly dry year with just 306 mm of total annual rainfall (50.5% lower than the 40-year average). The spring and summer were especially dry. Precipitation was 36.3 mm from April to September, whereas the average value is 152.6 mm. Thus, precipitation during April–September of the 2005 was 76% lower than the average. The studied year was also a warm year with 15.3°C of annual temperature (0.6°C higher than the average). Spring and summer mean temperatures were particularly warm (14.2°C and 25.5°C, respectively) which were 1.7°C warmer than the average values for these seasons (12.5°C and 23.8°C, respectively).

Parameters studied

Growth parameters were measured in autumn to ensure that the vegetative season, which mainly extends across spring and summer, had finished. Diameter was measured at the base of the plants with calipers, and total height was measured with a pole ruler by considering the length from the bottom to the top of the main axis of each tree. The annual growth was estimated with a ruler from six shoots of the spring flush located in the top third of the crown and covering six different compass orientations.

Twelve sun spring leaves were sampled from three of the six shoots selected for annual growth measurements (N-, SE-, and SW-oriented branches). Leaves were scanned, and their individual leaf area was estimated by image analysis (software WINFOLIA v. 2002, Régent, Quebec, Canada). Leaf thickness was estimated in one leaf per orientation using a caliper. The total chlorophyll content of one leaf per plant was analyzed following extraction with dimethyl sulfoxide at 60°C for 4 h (Wellburn 1994). The remaining

Table 1 Location and climatic characterization of the assayed populations

Population	Country	Latitude (N)	Longitude (W)	Altitude (m)	Pa (mm)	Ps (mm)	T (°C)	MMH (°C)	mmc (°C)	PET (mm)	Xi
Alcácer do Sal (S)	Portugal	38° 22'	8° 32'	30	709	31	16.3	27.8	7.8	793.3	115.9
La Almoraima (A)	Spain	36° 16'	5° 22'	118	813	20	17.4	29.0	7.5	842.4	131.3
Aïn Rami (R)	Morocco	35° 04'	5° 12'	425	865 ^a	10 ^a	17.4 ^a	32.7	4.8 ^a	862.6	169.4

Pa annual precipitation, *Ps* summer precipitation, *T* mean annual temperature, *MMH* mean maximum temperature of the hottest month, *mmc* mean minimum temperature of the coldest month, *PET* potential evapotranspiration estimated by Thornthwaite (1948) method, *Xi* xerothermic index (estimated following Grossmann et al. (2002), using the formula: $Xi = \sum (2TM - PM)$ if $2TM > PM$ or $Xi = 0$ if $2TM \leq PM$, where *TM* is the monthly mean of the maximum and minimum temperatures and *PM* is the monthly precipitation)

^aData obtained from a climatic model (Hijmans et al. 2005)

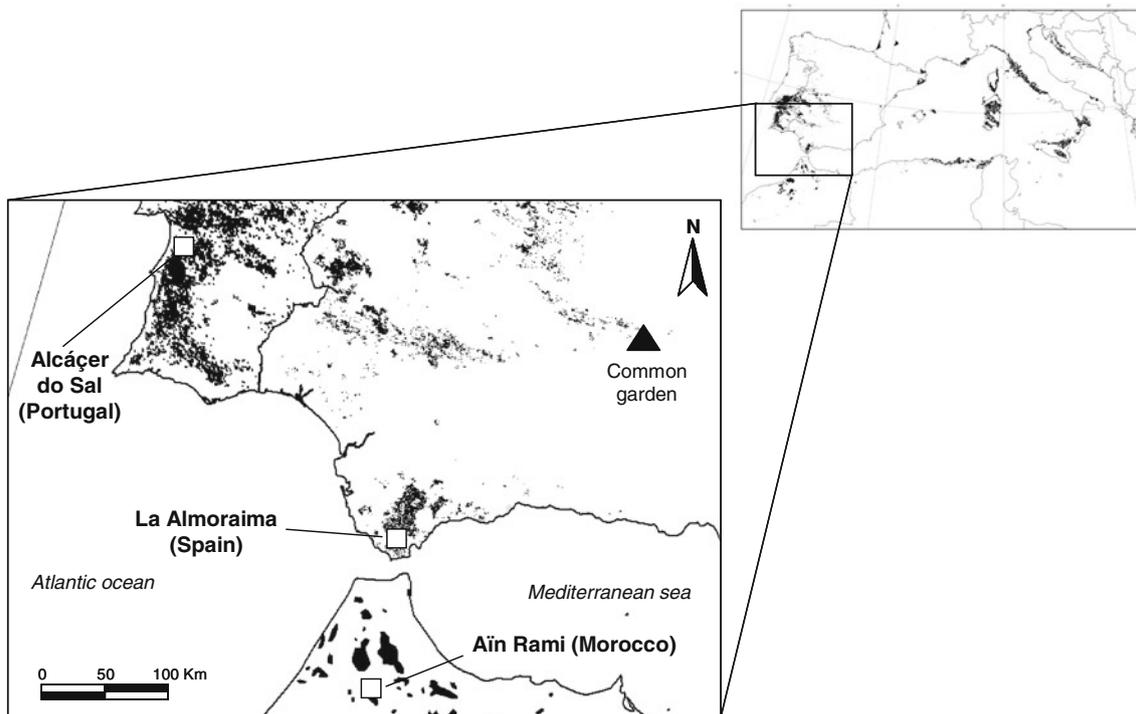


Fig. 1 Location of the three sampled populations of *Q. suber* and common garden site (*triangle*) within the distribution area of the species. Map adapted from Aronson et al. (2009)

leaves were oven-dried at 65°C to a constant weight after scanning. SLA was estimated in one leaf per orientation using the ratio of dry weight to leaf area of one leaf. The rest of the dry material (eight leaves per plant) was ground in a ball mill. This was used to determine the isotopic composition of C¹³ ($\delta^{13}\text{C}$). The isotopic ratio of ¹³C/¹²C was determined on a mass spectrometer at the *Instituto Andaluz de Ciencias de la Tierra* (Thermo Finnigan Delta Plus XL). Two internal standards were used for analysis after every ten leaf samples: sugar ($\delta^{13}\text{C}=-12.29\%$) and phthalic acid ($\delta^{13}\text{C}=-30.63\%$). The repeated analysis of these standards yielded an SD of less than 0.1‰.

The insensitivity of the isotopic signal and nitrogen leaf content to shoot orientation was established by performing an exploratory sampling analyzing the effect of shoot orientation within the sampled plants. This previous test legitimized the pooling of foliar material for the three orientations within the same plant for $\delta^{13}\text{C}$ and N analyses because no significant differences were observed among shoots for the same plant. Besides, any doubts about contamination caused by the method of grinding, related to abrasion of the plastic walls of the Eppendorfs by the mills were resolved by comparing the $\delta^{13}\text{C}$ values of subsamples from the same leaf ground to a fine powder with a ball mill (previous inclusion of the leaf material in a plastic Eppendorf with the stainless steel balls) and with a pottery mortar and pestle (Fig. S1). The slope of the

resultant regression of the two subsamples was 0.999; the intercept was not significantly different from 0 and R^2 was 0.98, indicating that there was no systematic contamination from the plastic during the grinding process (Fig. S1). Given the high number of samples, the ball mill method was selected for grinding the rest of the leaf material. The $\delta^{13}\text{C}$ values were used to estimate the isotopic discrimination ($\Delta^{13}\text{C}$) as (1) (Farquhar et al. 1982):

$$\Delta(\text{‰}) = (\delta_a - \delta_p) / (1 + \delta_p/1000) \quad (1)$$

where δ_p (‰) refers to $\delta^{13}\text{C}$ of bulk leaf material, and δ_a is the $\delta^{13}\text{C}$ of the air acting as carbon source (it was assumed to be -7.9%). Nitrogen content was analyzed for the same leaves by the Kjeldahl method (AOAC 1990). Briefly, samples (~0.15 mg of dried leaves) were subjected to an acid digestion by H₂SO₄ (96%) at 400°C during 1 h and 30 min. After that, samples were distilled in a basic environment (NaOH at 40%). Finally, a titration of the samples was carried out using HCl 0.05 N in a solution of H₃BO₃.

Statistical analyses

Nine linear models (one per variable) were performed to analyze the family and population effect on each variable. Both genetic and environmental maternal effects (e.g., acorn mass) appear to exert their greatest effect on cork

oak performance during the early years of the seedling growth (Quero et al. 2007, 2008), but their influence rapidly diminishes with age (Ramírez-Valiente et al. 2009a). In order to avoid possible differences among populations caused by different initial sapling growth due to maternal effects and/or environmental factors in the common garden (Shaw and Byers 1998; Casper et al. 2005), we measured plant size before the 2005 growing season and we included it in the model as a covariate. 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 overall 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 family nested within the j population; BP_{ij} is the random effect of the interaction between block i and population j ; and E_{ijkn} is the residual error for Y_{ijkn} . The traits annual shoot growth, leaf size, and specific leaf area were log-transformed to meet the assumptions of normality and homoskedasticity of variance. Variance analyses were carried out using the procedure PROC MIXED of the statistical package SAS 9.1 (SAS/STAT® Software; SAS Institute), with type III sum of squares and the restricted maximum likelihood method that renders unbiased estimates. Since the evolution of the traits in response to natural selection depends on standing genetic variation and covariation among traits within populations, the genetic parameters, heritabilities, and genetic correlations were estimated for each population separately. For this purpose, the variance components were calculated for each population separately by performing nine linear models (one per variable) per each population, where the equation was:

$$Y_{ikn} = \mu + H_n + B_i + F_k + E_{ikn}$$

where Y_{ikn} is the observed value for the variable considered in the n tree of the k open-pollinated family 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; F_k is the random effect of the k family; and E_{ikn} is the residual error for Y_{ikn} . PROC VARCOMP of SAS 9.1 was used to calculate the variance components (SAS/STAT® Software; SAS Institute). The narrow-sense heritability on an individual-tree basis for each population was calculated as follows:

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2} = \frac{4\sigma_f^2}{\sigma_f^2 + \sigma_e^2}$$

where σ_a^2 represents the additive genetic variance within the given population, σ_p^2 is the total phenotypic variance in that

population, σ_f^2 is the variance given by the family within each population, and σ_e^2 is the residual variance. The additive genetic variance within the population was estimated as $4\sigma_f^2$ because a preliminary study with microsatellite markers suggests a low correlated paternity in cork oak populations and a very low percentage of full sibs in the progenies.

Genetic correlations between traits were also estimated for each population using the formula:

$$r_{A_{12}} = \frac{\sigma_{A_1A_2}}{\sqrt{\sigma_{A_1}^2 \cdot \sigma_{A_2}^2}}$$

where $r_{A_{12}}$ is the genetic correlation between traits 1 and 2, $\sigma_{A_1A_2}$ is the covariance between trait 1 and trait 2, and $\sigma_{A_1}^2$ and $\sigma_{A_2}^2$ are the additive genetic variance of traits 1 and 2, respectively.

Standard errors and the 95% confidence intervals for the heritabilities and genetic correlations were estimated by the DELTA method to explore if their values were statistically different from zero and to test for differences among populations in the values of these parameters (see Lynch and Walsh 1998 for details).

Additionally, we investigated the potential direct and total (direct + indirect) relationship between functional traits and aboveground growth in dry conditions. For this purpose, we constructed four regression models sensu Lande and Arnold (1983), where aboveground growth was included as dependent variable and phenotypic traits were included as independent variables in the models. We used shoot growth as a surrogate of plant performance during a dry year for cork oak saplings because (1) annual growth is an important component of total growth that can explain final differences in long-term size, which has been documented to be related to age of first reproduction and acorn weight in oaks (Guyette et al. 2004). (2) Besides, there was no mortality of any plant during the study year despite the low rainfalls occurring in the common garden. In fact, survival is very high during the early stages of the life cycle in cork oak plants, but it rapidly diminishes with age (Ramírez-Valiente et al. 2009a).

Models were

$$W = \text{constant} + S_n \times X_n + \text{error} \quad (\text{model I})$$

$$W = \text{constant} + S_n \times X_n + 1/2 \times C_n \times X_n^2 + \text{error} \quad (\text{model II})$$

$$W = \text{constant} + \beta_1 \times X_1 + \beta_2 \times X_2 + \dots + \text{error} \quad (\text{model III})$$

$$W = \text{constant} + \beta_1 \times X_1 + 1/2 \times \gamma_{11} \times X_1^2 + \beta_2 \times X_2 + 1/2 \times \gamma_{22} \times X_2^2 + \gamma_{12} \times X_1 \times X_2 + \dots + \text{error} \quad (\text{model IV})$$

where W is aboveground growth, estimated as the average growth in six spring shoots around the tree crown, X_1 , X_2 , ...

X_n represent the trait value for the traits 1, 2, ... n . Model I was constructed to obtain S (called “linear selection differential” sensu, Lande and Arnold 1983) for each single trait. This parameter indicates the linear relationship between a trait and shoot growth, taking into account both direct and indirect effects. Likewise, we implemented a second model (model II) in order to explore for a potential quadratic relationship between the traits and shoot growth. C (called “nonlinear selection differential” sensu, Lande and Arnold 1983) is the term which indicates the nonlinear relationship between a trait and shoot growth, taking into account both direct and indirect effects. Both models I and II were performed for each trait separately, where the analyzed trait is represented by X_n .

Model III was constructed to obtain β (called “linear selection gradient” sensu, Lande and Arnold 1983) for each trait. This parameter indicates the linear direct relationship between each single trait and shoot growth. Analogously, to explore for nonlinear direct relationships between each trait and shoot growth, a second-degree multiple polynomial regression was performed (model IV). In model IV, γ s (called nonlinear phenotypic gradients sensu, Lande and Arnold 1983) indicated the direct quadratic relationship between the traits and shoot growth (e.g., γ_{11} , γ_{22} ...) and the relationship between the two-way interaction terms (e.g., X_1X_2 ...) and growth (e.g., γ_{12} ...). Models III and IV (multitrait regressions) were run just once, for all traits jointly.

Following Lande and Arnold (1983), growth values were relativized in the regression models by dividing each single growth value by mean growth. Likewise, traits were standardized to a mean=0 and variance=1 [i.e., standardized value = (value–mean)/SD]. The γ and C final values for quadratic terms were obtained by multiplying by two the coefficients resulting from the regression analysis (see Lande and Arnold 1983; Stinchcombe et al. 2008). Phenotypic relationships between traits were studied by Pearson correlations between all pair traits. To eliminate the possibility that spurious significant phenotypic differentials, gradients, and correlations arise from population differences in phenotypic traits, we included in the regression and correlation analyses the population structure (i.e., population was included as a categorical independent factor). However, we found no evidence of the effect of population structure on regression and correlation results, so population factor was finally removed from the analyses.

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 to all multiple tests along the different statistical tests. The FDR was controlled at each p level using a standard step-up procedure (see Benjamini and Hochberg 1995; García 2004).

Results

Interpopulation and intrapopulation differentiation

There was high among-family variation in carbon isotopic discrimination ($\Delta^{13}\text{C}$) ($P=0.007$) (Table 2, Fig. S2) and moderately high variation in basal diameter ($P=0.016$) and total height ($P=0.030$). Additionally, there was also marginal among-family variation ($P<0.10$) in annual shoot growth, leaf size, and leaf thickness (Table 2). Of all the traits measured, only height, SLA, and leaf size exhibited significant population divergence (Tables 2 and 3). The interpopulation differences in leaf size were especially large (Fig. S2, Table 3). Plants from the Moroccan population showed the highest values for height, leaf size, and SLA (Table 3).

Narrow-sense heritability estimates ($h^2 \pm \text{SE}$) were moderately high in the three populations studied for diameter (0.40 ± 0.15 – 0.71 ± 0.28) and $\Delta^{13}\text{C}$ (0.43 ± 0.25 – 0.83 ± 0.31) (Table 4). Heritability estimates for leaf chlorophyll content per mass (0.00 – 0.07 ± 0.19), SLA (0.00 – 0.28 ± 0.22), and annual growth (0.00 ± 0.19 – 0.37 ± 0.23) were similarly low for all populations (Table 4). For the rest of the traits (height, leaf size, leaf thickness, and N_{mass}), heritability estimates varied among populations (Table 4). Portuguese population exhibited the highest heritability estimates for height (1.00 ± 0.31), leaf size (0.61 ± 0.27), and N_{mass} (0.69 ± 0.30) and the lowest ones for leaf thickness (0.00) (Table 4). Spanish population presented the highest heritability value for leaf thickness (0.67 ± 0.28).

Association between traits and growth

There were significant positive phenotypic linear differential ($S=0.31$, $P<0.001$) and gradient ($\beta=0.20$, $P<0.001$) for leaf size, indicating that plants with larger leaves presented larger annual shoot growth (Table 5). Likewise, SLA showed a negative linear association with annual shoot growth ($S=-0.34$, $P<0.001$, $\beta=-0.33$, $P<0.001$) (Table 5) and a positive quadratic association with annual growth ($C=0.13$, $P<0.01$), indicating that plants with lower SLA showed larger annual shoot growth. Leaf thickness exhibited also a positive linear relationship with growth ($S=0.25$, $P<0.001$) although quadratic term was not significant ($C=0.04$, $P=0.516$). Otherwise, $\Delta^{13}\text{C}$ and nitrogen content per mass showed no linear association with annual growth (Table 5). However, $\Delta^{13}\text{C}$ showed a weak negative quadratic differential ($C=-0.16$, $P<0.05$). Plants exhibiting lower leaf chlorophyll content had larger annual shoot growth ($S=-0.21$, $P<0.001$ (Table 5)). Significant nonlinear gradients (γ) were not detected for any trait (data not shown).

The most relevant results from the phenotypic correlation analyses were the observation of a negative correlation

Table 2 Results of the mixed models for each studied variable

Variable	Population <i>F</i> value	Family (population) <i>Z</i> value	Block <i>F</i> value	Population × block <i>Z</i> value	Initial height <i>F</i> value
Height	5.47*	2.13*	12.22***	0.14	–
Diameter	1.61	2.41**	6.75**	0.16	–
Annual growth	1.16	1.54****	2.78*	0.25	8.81**
$\Delta^{13}\text{C}$	1.53	2.68**	16.17***	0.87	3.69****
SLA	4.05*	0.93	4.46**	0.00	15.21**
Leaf size	34.94***	1.38****	2.94*	0.00	11.91**
Leaf thickness	3.22****	1.55****	54.91***	0.22	26.38***
N_{mass}	1.63	1.16	1.66	1.45****	2.19
Chl_{mass}	1.84	0.00	4.21*	0.78	17.80***

Initial height was included in the model as a covariate for all traits except for height and diameter. Italicized text indicates statistically significant differences after FDR corrections. See text for details

$\Delta^{13}\text{C}$ carbon isotope discrimination, *SLA* specific leaf area, N_{mass} nitrogen leaf content per unit mass, Chl_{mass} total chlorophyll leaf content per unit mass
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.10$ (marginally nonsignificant values)

between $\Delta^{13}\text{C}$ and N_{mass} ($r = -0.22$, $P < 0.001$) and $\Delta^{13}\text{C}$ and leaf thickness ($r = 0.30$, $P < 0.001$). Leaf thickness and SLA were also negatively correlated ($r = -0.58$, $P < 0.001$). All the phenotypic correlations between different traits are given in Table 6.

Discussion

Among-population and intrapopulation differentiation: local adaptation and evolutionary capacity of functional traits

Populations differed significantly for SLA, height, and leaf size and marginally for leaf thickness. Since the number of studied populations was low, we could not explore the

causes of this population differentiation in functional traits. Results obtained in previous studies suggested that the differences among cork oak populations in these traits were mainly due to divergent selection imposed by temperature and rainfall variation (Ramírez-Valiente et al. 2010a, b). Alternatively, other forces such as neutral evolutionary processes (e.g., founder effect, genetic drift, etc.) potentially could have also caused differences among populations in these phenotypic traits. However, studies using molecular markers do not support this hypothesis (see Toumi and Lumaret 1998; Jiménez et al. 1999; Ramírez-Valiente et al. 2009b).

The three cork oak populations studied showed moderately high heritabilities for diameter and $\Delta^{13}\text{C}$ caused by high intrapopulation genetic variance in these traits. These

Table 3 Means \pm standard error for the ecophysiological traits and fitness component (shoot growth) measured in 15 open-pollinated families from three populations

Variable	Alcácer do Sal	La Almoraima	Añ Rami
Height (cm)	75.0 \pm 6.8–164.4 \pm 9.5a	92.0 \pm 10.4–132.5 \pm 17.4a	113.9 \pm 13.3–150.3 \pm 17.0b
Diameter (cm)	3.03 \pm 0.44–5.66 \pm 0.14	3.44 \pm 0.56–5.51 \pm 0.45	3.58 \pm 0.50–5.91 \pm 0.49
Shoot growth (cm)	3.41 \pm 1.02–14.73 \pm 4.82	3.26 \pm 0.72–10.94 \pm 2.31	5.71 \pm 1.00–14.95 \pm 4.37
$\Delta^{13}\text{C}$ (‰)	19.35 \pm 0.30–20.64 \pm 0.26	19.11 \pm 0.37–20.29 \pm 0.45	19.11 \pm 0.28–20.19 \pm 0.38
SLA (m ² kg ⁻¹)	5.44 \pm 0.20–6.03 \pm 0.29a	5.11 \pm 0.13–6.23 \pm 0.36ab	5.38 \pm 0.21–6.27 \pm 0.30b
Leaf size (cm ²)	1.40 \pm 0.15–2.39 \pm 0.22a	1.77 \pm 0.23–2.83 \pm 0.29b	2.22 \pm 0.22–3.49 \pm 0.53c
Leaf thickness (mm)	0.24 \pm 0.02–0.27 \pm 0.02	0.23 \pm 0.02–0.27 \pm 0.02	0.22 \pm 0.02–0.26 \pm 0.02
Nitrogen _{mass} (mg g ⁻¹)	14.50 \pm 0.73–17.44 \pm 0.61	15.09 \pm 0.58–16.56 \pm 0.31	15.22 \pm 0.44–17.40 \pm 0.65
Total Chlorophylls _{mass} (mg g ⁻¹)	2.68 \pm 0.21–3.60 \pm 0.34	2.64 \pm 0.22–3.40 \pm 0.36	2.74 \pm 0.26–3.60 \pm 0.36

The minimum and maximum mean values of the open-pollinated families of each of the three populations studied are given. Significant differences among populations after FDR corrections (see text for details) are shown in italics. Homogeneous populations at 95% confidence are indicated by the same letter for trait showing significant differences among populations

Table 4 Narrow-sense heritabilities (standard error) of the phenotypic variables for each cork oak population

	Alcacer do Sal	La Almoraima	Ain Rami
Height	1.00 (0.31)	0.00 (0.00)	0.40 (0.22)
Diameter	0.71 (0.28)	0.47 (0.24)	0.40 (0.15)
Annual growth	0.00 (0.16)	0.37 (0.23)	0.20 (0.20)
$\Delta^{13}\text{C}$	0.71 (0.30)	0.83 (0.31)	0.43 (0.25)
SLA	0.00 (0.00)	0.24 (0.21)	0.28 (0.22)
Leaf size	0.61 (0.27)	0.12 (0.18)	0.01 (0.16)
Leaf thickness	0.00 (0.00)	0.67 (0.28)	0.33 (0.22)
N_{mass}	0.69 (0.30)	0.00 (0.00)	0.33 (0.26)
Chl_{mass}	0.07 (0.19)	0.00 (0.00)	0.00 (0.00)

Heritability values significantly different from zero were typed in bold. Heritability values showing significant differences among populations after FDR correction were in italics

$\Delta^{13}\text{C}$ carbon isotope discrimination, *SLA* specific leaf area, N_{mass} nitrogen leaf content per unit mass, Chl_{mass} total chlorophyll leaf content per unit mass

results were similar to those reported for other forest tree species (e.g., Johnsen et al. 1999; Matzner et al. 2001; Prasolova et al. 2001; Xu et al. 2003; Lauteri et al. 2004). Since heritabilities were significant for these traits, they are likely to respond to natural selection in the three studied cork oak populations. Conversely, the low intrapopulation genetic variance for chlorophyll content per mass and SLA suggests that these traits present low evolutionary potential, and thus, their future responses to selection can be limited (e.g., Kaczorowski et al. 2008). The heritability estimates of the remaining characters (except annual growth) varied among populations due to differences in the level of additive genetic variance within the populations. The differences were particularly remarkable for height and leaf size, two traits under possible divergent selection as discussed above. Although studies with a higher number of populations should be carried out across different years, our results suggest that the divergent selective pressures have led to local adaptations but also have shaped the level of genetic variance in these traits in the three cork oak populations.

Additionally, we found a low number of significant genetic correlations (Table S1 of supplementary material). Genetic correlations among traits may constrain the adaptive response to selection if two positively correlated traits are selected for in opposing directions (Roff 1997; Campbell 1997; Cotter et al. 2004). The facts that (1) a limited number of genetic correlations were significant and (2) positively correlated traits did not undergo opposing selection in our analyses (see Table 5 and Table S1) provide little evidence for constraints on evolution caused by genetic correlations.

Relationships between ecophysiological traits and growth in a dry year

Our study showed that there was a strong negative relationship between SLA and growth, which was used as a surrogate of fitness. Plants with higher leaf thickness also had greater growth (significantly positive *S*) although phenotypic gradient (β) for this trait was not significant. β indicates the association between a trait and growth after removing the effects of the associations among traits (see “Material and methods”). The absence of significant β for leaf thickness was probably due to the strong correlation between this trait and SLA.

The relationship between SLA and shoot growth is similar to that found in a previous study in dry conditions (Ramírez-Valiente et al. 2010a). Higher SLA is associated with higher leaf area ratio values and higher photosynthetic capacity in interspecific studies and, thus, higher potential of carbon gain and growth rate (Reich et al. 1997, 1998; Cornelissen et al. 1998; Shipley 2006). However, lower SLA has fitness benefits in dry environments when comparing intraspecific variability since more sclerophyllous leaves allow a more conservative water use, maintaining photosynthetic activity and carbon gain over a longer period of time (Dudley 1996). Therefore, this strategy would fit what is expected for drought-tolerant species such as evergreen Mediterranean oaks (Leiva and Fernández-Alés 1998; Gratani et al. 2003). On the other hand, there was a weak negative quadratic relationship between $\Delta^{13}\text{C}$ and growth. This was in agreement with that found in a recent study where there was no relationship between $\Delta^{13}\text{C}$ and growth in dry conditions but there was a strong positive association between $\Delta^{13}\text{C}$ and growth under wetter

Table 5 Phenotypic linear differentials (*S*), linear gradients (β), and quadratic differentials (*C*) for standardized plant ecophysiological traits and relativized annual shoot growth

Trait	<i>S</i>	β	<i>C</i>
$\Delta^{13}\text{C}$	0.07	0.07	-0.16*
SLA	<i>-0.34***</i>	<i>-0.33***</i>	<i>0.13**</i>
Thickness	<i>0.25***</i>	-0.01	0.04
Leaf size	<i>0.31***</i>	<i>0.20***</i>	-0.02
N_{mass}	0.01	0.06	-0.03
Chl_{mass}	<i>-0.21***</i>	-0.08	0.00

Significant nonlinear gradients (γ) were not detected for any trait (data not shown). Italicized text indicates phenotypic differential and gradients significantly different from zero after FDR corrections. See text for details

$\Delta^{13}\text{C}$ carbon isotope discrimination, *SLA* specific leaf area, N_{mass} nitrogen leaf content per unit mass, Chl_{mass} total chlorophyll leaf content per unit mass

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

Table 6 Phenotypic correlations between ecophysiological traits

Variable	$\Delta^{13}\text{C}$	SLA	Leaf size	Thickness	N_{mass}
SLA	-0.03				
Leaf size	-0.12*	0.09			
Thickness	<i>0.30***</i>	<i>-0.58***</i>	0.00		
N_{mass}	<i>-0.22***</i>	<i>0.21***</i>	<i>0.26***</i>	<i>-0.17**</i>	
Chl_{mass}	0.12	<i>0.27***</i>	<i>-0.34***</i>	0.04	<i>0.14*</i>

Italicized text indicates significant correlations after FDR corrections. See text for details

$\Delta^{13}\text{C}$ carbon isotope discrimination, *SLA* specific leaf area, N_{mass} nitrogen leaf content per unit mass, Chl_{mass} total chlorophyll leaf content per unit mass

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

conditions (Ramírez-Valiente et al. 2010a). Although further research is needed, this observation could be explained by the higher relative importance of N_{mass} on $\Delta^{13}\text{C}$ under high water availability as observed in Ramírez-Valiente et al. (2010a) and highlights the importance of nonconservative water use during wet years for enhanced growth.

Another relevant result of our study was the positive relationship between leaf size and growth. Larger leaves have been suggested to confer fitness benefits in some environments because, for the same number of leaves, a larger leaf size increases the photosynthetic area and, thus, the potential for an increased carbon gain (Horn 1971; Givnish 1979; Gates 1980). In the present study, we did not measure total number of leaves and we do not know the total leaf area of each plant. However, there are evidences showing that leaf size is significantly and positively correlated to total leaf area in cork oak (Jiménez-Anca 2000), which does suggest that larger leaves allow greater potential photosynthetic area in this species. Nevertheless, a greater leaf area of the plant also involves increased transpiration. Thus, the positive effects of larger leaves on growth are contrary to expectations from water loss theory in plants living in low-water-availability environments. Several studies have observed the positive effect of increased leaf size on fitness even in dry environments (e.g., Dudley 1996; Donovan et al. 2007) although its importance is usually reduced when water availability decreases (Dudley 1996). Thus, maybe the growth benefits of a larger leaf size (and, thus, a higher total leaf area) override the potential disadvantages of increased water losses in the present study (Niinemets et al. 2007).

Finally, we found a negative relationship between chlorophyll content per mass and growth (a significantly negative S). Higher chlorophyll content per mass increases the photosynthetic capacity per unit mass and, thus, the potential carbon gain and the relative growth rate (Yoder 1992). Hence, a negative association between chlorophyll content and growth is contrary to expectations. No significant phenotypic gradient (β) was observed for

chlorophyll content. Hence, the negative differential (S) for chlorophyll content was probably due to the correlation with other traits such as SLA and leaf size, which were strongly associated to growth. However, even if height indicates to some extent fitness in forest tree species, some caution should be considered in the interpretation of the relationships between ecophysiological traits and growth. There could be also ontogenic reasons for correlations between growth and other traits as well as physiological ones.

In conclusion, cork oak plants originated from different populations varied in SLA and leaf size measured under dry conditions. They also significantly differed in total height. Populations showed significant differences in the heritability estimates for leaf thickness, leaf size, height, and nitrogen leaf content per mass. Our results suggested that the divergent selective pressures have led to local adaptations and have also shaped the level of genetic variance within the studied cork oak populations, although studies with a higher number of populations should be carried out across different years. Leaf size, leaf thickness, and SLA were traits influencing growth under dry conditions in cork oak saplings. Specifically, larger leaf sizes and sclerophyllous leaves (lower SLA and higher leaf thickness) had growth benefits for cork oak saplings under dry conditions. Finally, the absence of significant genetic correlations and the fact that correlated traits did not undergo opposing selection provided evidence for little constraints on evolution caused by genetic correlations.

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