

# Extended Executive Summary

**Thesis title: Phenotypic plasticity and intraspecific genetic variation in *Quercus suber*: Evaluation using ecophysiological traits and molecular markers**

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## Abstract

The goal of the present PhD Thesis was to assess the intra-specific variability and phenotypic plasticity in cork oak (*Quercus suber*) in a set of phenotypic traits related to drought tolerance, seed size and growth. For this purpose, traits were measured in plants established in common garden experiments during climatically contrasting years.

There was a positive relationship between acorn mass/size and seedling height and survival during the early stages of the life cycle. Both acorn size and seedling survival were positively correlated to drought severity at the place of origin (measured by a xerothermic index) suggesting that population differences were likely due to local adaptation processes. This finding reveals that not all populations are equally vulnerable to increase to drought considering the seedling stage.

Nine-year old plants were phenotypically characterized. This analysis showed that individuals originated from the wettest populations exhibited the higher specific leaf area (SLA), nitrogen content per mass ( $N_m$ ) and total chlorophyll content per mass ( $Chl_m$ ). Conversely, populations originated from the driest and coldest sites presented the lowest values for those traits. These results suggest that drought and temperature

are important selective agents for cork oak. Likewise, plants originated from wet populations showed low water use efficiency (WUE) during a wet year but there were no population differences during a dry year. These results suggest that plants from wet populations are better capable to take advantage of wet conditions.

Among-population genetic differentiation ( $Q_{ST}$ ) for height growth,  $N_m$ , SLA and leaf size were higher than among-population genetic differentiation for molecular markers measured by six nuclear microsatellites ( $F_{ST}$ ). Thus, population divergence in these phenotypic traits is likely due to divergent selection (and not by neutral evolutionary forces). This result reinforces the importance for conservation programs of forest genetic resources to focus research efforts to studies of the distribution in the genetic diversity in phenotypic traits related with fitness, and the fact molecular markers such as microsatellites could be a poor predictor of phenotypic trait variation.

There were differences in height growth, stem diameter, WUE, SLA and leaf size among open-pollinated maternal lines. Differences among maternal lines for some of these traits were even higher than those observed among populations. The high within populations variability for certain traits, which otherwise showed a moderate additive genetic variation, could be a reliable mechanism of cork oak populations to buffer the negative effects of climatic change.

Finally, cork oak plants showed smaller, more sclerophyllous leaves (lower SLA,  $N_m$  and  $Chl_m$ ) and higher WUE in a dry year than in a wet year. These results indicate that these traits present a high phenotypic plasticity to water availability in cork oak. There was also population x year interaction for shoot growth and a correlation between the difference in shoot growth among years and annual mean temperature at the place of origin, suggesting a possible strategy of taking advantage of favourable conditions for the warmer populations. The high plasticity for most of the morphological and biochemical leaf traits highlights its role as one of the main evolutionary mechanisms for long-living plant organisms to cope with environmental changes.

## General Introduction

The Mediterranean basin is one of the European regions expected to be most affected by climate change, with temperatures expected to increase at least 2-4°C, over the next century (Christensen et al. 2007). Precipitation is expected to decrease during summer and autumn, but to increase in winter (Déqué et al. 1998), with an overall decrease in water availability due to increased evapotranspiration, especially during the summer. If this change is as rapid as expected, forest climatic zone boundaries could move more rapidly than forest tree species can migrate (Davis and Zabinski 1992, Higgins and Harte 2006), so the survival of forest tree species will increasingly depend on their capacity of adaptation to the novel conditions (Davis and Shaw 2001, Etterson and Shaw 2001). The potential of species to respond to these rapid changes will depend on their phenotypic plasticity, the intra-specific genetic variation for adaptive traits or both (Marchin et al., 2008).

Cork oak is one of the keystone forest tree species in these ecosystems. This sclerophyllous species 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 semi-marcescent ones, such as *Quercus faginea* and *Quercus pyrenaica* (Mediavilla and Escudero 2003). Low population divergence has been documented in cork oak for molecular markers (Toumi & Lumaret 1998, Jimenez et al. 1999, Lumaret et al. 2005). In contrast, population differentiation has been observed for growth form (Gandour et al. 2007) and traits related to tolerance to low temperatures (Aranda et al. 2005). However, there is scant knowledge of intraspecific genetic differentiation in traits related to drought tolerance and water economy. Furthermore, the role of phenotypic plasticity and the ability of this species to cope with the environmental changes have not been also explored so far.

The main goals of this PhD thesis were: (1) to estimate the inter- and intra-population genetic divergence in seed size, survival, growth and a set of leaf ecophysiological traits controlling carbon uptake and water loss and (2) to study the

phenotypic plasticity to water availability of these phenotypic traits in order to explore both the potential effects of the long-term selection on wild cork oak populations and the capacity of this species to respond to environmental changes. For this purpose, we analysed seed size, survival, growth and a set of leaf ecophysiological traits in climatically contrasting years in plants established in four common garden trials located in two different sites. Additionally, in order to explore the forces driving the potential genetic divergence (i.e. natural selection or neutral evolution), 195 cork oak saplings were genotyped using six microsatellite markers.

Cork oak is widely distributed in the western Mediterranean basin and occupies environments of contrasted temperature and rainfall (Díaz-Fernández et al. 1995, Aronson et al. 2009). We hypothesized that differential selective pressures have led to population genetic differentiation in this oak species as a result of adaptation to the local environment (Kawecki and Ebert 2004, Savolainen et al. 2007). Likewise, we hypothesized 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 (Hamrick 2004). Finally, as observed in other Mediterranean oak species, cork oak was expected to respond to environmental heterogeneity in space and time by exhibiting certain level of phenotypic plasticity to water availability (e.g. Gratani et al. 2003, Gimeno et al. 2009).

## Detailed Overview

### 1. Differences in juvenile survival under increasing drought are mediated by seed size in cork oak (*Quercus suber* L.).

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#### Introduction

Seed size is one of the most important traits influencing the early stages of the life cycle of the plant. The importance of seed size in governing the fitness of progeny has been supported by extensive empirical evidence (Gomez 2004), several studies have found that larger seeds help seedlings to endure drought (Leishman and Westoby 1994, Seiwa 2000), and promote germination (Milberg et al. 2000, Pearson et al. 2002), emergence (Castro 1999), growth and survival (Seiwa 2000, Baraloto et al. 2005). Species and population-level variation in this trait can be due to local adaptation. Seed size has been observed to be an adaptive trait associated with environmental variables, such as latitude (Yakimowski and Ecker 2007), altitude (Yamada and Miyaura 2005), temperature (Murray et al. 2004) and water availability (Parciak 2002) but also with dispersal strategy and growth form (Moles and Westoby 2004a).

Selection pressure on seed size acting through offspring fitness is widely accepted (Rees 1997, Sakai et al. 1998, Leishman et al. 2000, Messina and Fox 2001 and references therein). The importance of seed size lies in the influence that it exerts on seedling performance during establishment, especially under both competitive and resource-limiting conditions (Moles and Westoby 2004b).

Models predicting suitable habitat for Iberian forest tree species under future climate change scenarios indicate that the range of *Q. suber* could decrease drastically

in southern and inland Spain although it should persist along the southwestern coasts (Benito Garzón 2008). It could also extend its distribution northwards and to higher altitudes benefiting from a potential increase in winter temperatures (Benito Garzón 2008). However, those models that predict species redistribution with climate change largely ignore within-species genetic variation (Hampe and Petit 2005). Genetic variation at the population level may extend or curtail the predicted range of a species more than previously assumed (Higgins and Harte 2006).

Our overall goal was to determine the capacity of *Q. suber* to cope with expected climate change by comparing population-level differences in survival in a common garden. Our specific objectives were (1) to quantify population-level variation in survival under dry conditions, (2) to estimate the influence of acorn size on the response of *Q. suber* to local climatic conditions and (3) to identify which populations would be the most susceptible to the climate change expected for the region.

## **Material and methods**

A sample of seeds (from 28-49 per population) was used to characterize the average acorn mass and size on a dry mass basis for each population. Then, seeds were carried to a nursery, where they were sown. Seedlings were grown for a year under optimum conditions prior to transplanting to the study site. Measurements of seedling height before planting were carried out on a sample of thirty individuals within each population. A one-way ANOVA was performed to test for differences in seed mass and width and length among populations. Another one-way ANOVA was used to evaluate differences in seedling height. Spearman's correlation analysis was carried out to test for association between acorn mass and seedling height.

### *Climate in the common-garden and sites of origin*

The study was carried out during the four years directly following planting in a common garden. Climatic data for trial and the site of each parent population were

obtained from their nearest meteorological station. A xerothermic index ( $X_i$ ) was calculated from the climatic data, following Grossmann et al. (2002), using the formula:  $X_i = \sum (2TM - P)$  if  $2TM > P$  or  $X_i = 0$  if  $2TM \leq P$ , where TM is the monthly mean of the maximum and minimum temperatures and P is the monthly precipitation.

#### *Survival, seed effects and increasing droughts*

Survival was assessed every autumn for four years. We recorded “annual survival” each year and “final survival” in the last year of the study. Linear logistic models were constructed for final and annual survival.

To test whether seed size was maternally affected or locally adapted non-parametric Spearman’s correlations were performed between acorn traits and climate at the site of origin on the parent population. If subject to maternal effects, we expected that seed size was strongly influenced by the climate in the year of ripening and collection at the population-origin site, and if seed size was due to local adaptation we should find a stronger relationship between it and average climate.

To examine whether populations from drier sites responded better under dry conditions, we implemented non-parametric Spearman’s correlations between final and annual survivals, the xerothermic index of the native stands and geographic data. Seedling height and average acorn size from each population were included in these analyses in order to examine whether these traits affect survival.

To help us consider how these populations might respond to climate change, we determined the expected future climate for the studied populations over the three time periods (2011-2040, 2041-2070 and 2071-2080) by extrapolation of numeric data calculated using the model CGMC2 developed by the Canadian Climate Modelling Centre (Flato et al. 2000). As anthropogenic emissions of CO<sub>2</sub> have increased more than those proposed by any recent model (Canadell et al. 2007), we estimated the expected future climate under the A2 scenario. This family of scenarios describes a global socio-economic situation that would cause the highest increases in the

anthropogenic emissions of carbon dioxide and other greenhouse gases (IPCC, 2007). We calculated the expected future xerothermic indices for each period and population.

## Results

*Quercus suber* seedlings in our common-garden trial exhibited variation in survival at the population level. Populations from the wettest sites had the smallest average seed size and the lowest final survival under the dry conditions at the trial site. During the four-year experiment, conditions at the common-garden site were drier than those typically recorded over much of species current range (xerothermic index  $\approx 140$ ). However, these values are still far milder than the expected conditions in the southern and central Iberian Peninsula (xerothermic index  $\approx 210$ ) and are more extreme than those expected for the northern sites (xerothermic index  $\approx 80$ ).

Our study makes three important findings. The first is that there is a geographical pattern in a phenotypic trait (seed size) related to fitness in *Q. suber*. Our second finding is that *Q. suber* seedlings originating from sites characterized by more intensive droughts exhibited significantly higher survival rates than those from regions where drought is less severe. This pattern shows that populations differ in their tolerance to drought stress, and could be caused by differences in physiological and morphological traits as consequence of an adaptation to local environmental conditions. Nevertheless, (1) larger mean acorn size of seedlings sourced from sites with high summer drought and at southern latitudes, (2) the positive relationship between acorn traits and seedling height and (3) higher survival rates of larger seedlings during the first years of growth, suggest that selection favors bigger seeds at the harshest sites.

Several studies have reported that bigger acorns lead to increased seedling growth (Buckley 1982, Stock et al. 1990, Tripathi and Khan 1990, Osunkoja et al. 1994) and higher root/shoot ratios (Buckley 1982, Lloret et al. 1999), which can improve the seedling performance by developing and maintaining deep and extensive root systems

accessing water from deep within the soil profile (Dell and Havel 1989). Selection for big acorns as a consequence of selection for tall seedlings is likely to be stronger in water-limiting environments, indeed, a positive relationship among acorn size, seedling height and summer survival has been reported for other Mediterranean oak species (Gomez 2004). Thus, local adaptation through natural selection can drive acorn size divergence.

The third major finding of this study is that not all populations are equally vulnerable to climate change. Under the ongoing climate change, all Iberian *Q. suber* populations are expected to incur more intensive drought periods, but Northern populations are poorly adapted to dry conditions (low survival in the common garden and small acorn size). Theoretically, under a drier future climate these populations would not survive well. However, the xerothermic index at the population sites are expected to remain low (low drought intensity) during the 21<sup>st</sup> Century. Moreover, if the change in climate for these populations is as slow and gradual as predicted, they might be able to adapt to the drier environmental conditions. Southern populations are the best adapted to dry conditions (big acorns and high survival rates in the common garden). Owing to their relatively good adaptation to dry conditions, and the moderate climate change expected in these areas, these populations are expected not to be seriously affected under the future climate scenarios. The outlook for inland, continental populations is not as bright as for northern and southern ones. These populations are intermediately adapted to dry conditions (moderate/high survival rates in the common garden and medium-sized acorns). However, drastic and fast increases in summer droughts are expected to occur in these regions, so these central populations may not have much time to adapt to more arid conditions and could be substantially reduced.

The potential of the tree species to respond to the climate change will not only depend on the genetic variation in adaptive traits at among-population level, but also on the genetic variation at within-population level (Hamrick 2004). Thus, intrapopulation

divergence in adaptive traits should be analyzed in the future to better understand the potential of this species to cope with climate change.

## **2. Geographic variation in cork oak leaf traits reveals significant drought adaptations across climatically contrasting populations.**

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### **Introduction**

Contrasting climatic conditions generally exert different selective pressures which can lead to ecotypic differentiation even in forest tree species, which generally present high gene flow among populations (Savolainen et al. 2007 and references therein). Inter-population divergence has been documented in cork oak for growth form (Gandour et al. 2007) and traits related to tolerance to low temperatures (Aranda et al. 2005).

The aim of the study was to assess the population divergence in a set of ecophysiological traits related to drought tolerance and growth across fifteen cork oak populations spanning over most of the distribution range of the species within the Mediterranean Basin. These populations originate from sites with contrasting climates, which allows for the evaluation of whether differences in leaf attributes could have been caused by local adaptation through natural selection. The experiment was performed during an extremely dry year with juvenile plants established in a common garden in 1998. The traits measured were specific leaf area, leaf thickness, leaf area, leaf nitrogen content per leaf mass, total chlorophyll content per mass and carbon isotope discrimination as a surrogate of water use efficiency (Farquhar et al. 1989). These traits were selected because they have been broadly documented to be involved in drought responses in many species (e.g. Dudley 1996, Casper et al. 2005, Heschel and Riginos 2005, Donovan et al. 2007).

We hypothesise that a severe summer drought elicits differential responses among populations of cork oak mediated by key leaf traits. Thus, we expect inter-

population differentiation in drought related traits as a result of adaptation to the local climate at the origin of the studied populations. Owing to the reported sensitivity of the species to low temperatures (Aranda et al. 2005), differences in minimum temperatures across populations might have also exerted local adaptations, so we also expected population differences that could have been induced by the impact of freezing temperatures and cold snaps in their native sites.

## **Material and methods**

### *Common-garden design*

The study was performed on fifteen populations distributed along the natural range of cork oak in the Mediterranean basin. The studied populations spanned over a wide range of climatic conditions. The measurements of quantitative traits were carried out in a common garden where the populations were assayed. The trial was established in western Spain at Monfragüe National Park (39° 51' N, 6° 1' W, 375 m, Cáceres), which is located within the core of the cork oak natural range (Aronson et al. 2009). The climate of this area is typically Mediterranean characterized by cool winters and hot and dry summers. The average annual precipitation is 755.2 mm and the average annual temperature is 17.2°C for the period 1964-2005 at Serradilla meteorological station, 9 km apart from the common garden site (AEMET, National Meteorological Agency).

### *Phenotypic measurements*

The ecophysiological study extended over 2005, an unusually dry year with only 468.7 mm of total annual rainfall (38% lower than the forty-year average). The summer was extremely dry this year. Precipitation was 27.2 mm from June to October, whereas the average value is 85.2 mm. Thus, precipitation during June-October of 2005 was 68.1% lower than the average. Summer was also hot (28.0 °C) that is 0.8 °C warmer than the average values for this season (27.2 °C).

Functional leaf traits potentially related to drought tolerance ( $\Delta^{13}\text{C}$ , leaf area, SLA, leaf thickness,  $N_{\text{mass}}$ ,  $\text{Chl}_{\text{mass}}$ ) and annual shoot growth were measured. One plant per population was sampled within each block.

### *Data Analyses*

In order to determine the possible variation among populations and whether it could be explained by adaptation to climate, we proceeded as follows:

(1) Since populations extend across a wide range of climatic conditions and the environmental differences among the sites of origin were not explained by a single climatic variable, we performed a PCA in order to group the populations according to the degree of climatic similarity. Variables included in the analyses were: annual precipitation, summer precipitation, annual mean temperature, winter minimum temperature, summer maximum temperature.

(2) Seven General Linear Models (GLMs) (one per variable) were constructed for functional leaf traits and annual shoot growth, including climatic groups as fixed factor. Likewise, population (within climatic group) was included to explain genetic differences due to adaptation to other local environmental factors different from climate (e.g. soil properties, nutrients availability, pressures from herbivory, etc.).

### **Results**

Cork oak showed high inter-population divergence in all the traits studied except for  $\Delta^{13}\text{C}$  during an extremely dry and warm year in a common garden. Cork oak populations originating from rainy sites, particularly during summer (climatic groups III and V) exhibited significantly thinner leaves and higher SLA than the other populations. For a large fraction of plant species, a positive association between SLA and rainfall/water availability has been documented (e.g. Marchin et al. 2008), including oaks (e.g. Bruschi et al. 2003, Gratani et al. 2003).

Low SLA has been suggested to be adaptive in water-limited environments because thick or dense leaves provide a good structural reinforcement to reduce water loss (Maximov 1929, Oertli 1989). Another non-excluding theory suggests that low SLA may be adaptive because it is negatively correlated to physiological traits such as water use efficiency (WUE) (Lamont et al. 2002). The absence of correlation between SLA and WUE in the present study suggests that low SLA is likely adaptive for cork oak in dry environments due to reduced water loss of plants with thick leaves. Nevertheless, the negative correlation between SLA and WUE observed in other studies also supports a potential adaptive role of SLA in water-limited habitats caused by its negative relationship with WUE. Thus, the significant differences in SLA found among populations in the common garden and the association between SLA and precipitation at the place of origin, suggest that inter-population divergence could be at least partly due to an adaptation to local rainfall.

Cork oaks originating from cool sites (mainly characterized by cold winters) exhibited significantly thicker leaves and lower SLA than the rest of the populations. Thicker leaves (lower SLA) usually present more cell wall material per unit leaf volume and more cell layers (Atkin et al. 2006). Low SLA could be adaptive in colder environments because more cell layers in leaves reduce the effects of chilling temperatures on leaves by slowing down the rate of freezing (Ball et al. 2002). Differences among cork oak populations in cold tolerance have been reported, with populations originated from cold sites documented to be less sensitive to cold stress (Aranda et al. 2005). Leaf longevity and leaf mass per area (the inverse of SLA) have been reported to be positively correlated to freezing sensitivity in oak species, including cork oak (Cavender-Bares et al. 2005), suggesting that the leaf characteristics of the populations from “cool” sites (low SLA and high leaf thickness) could have been influenced by adaptations to low temperatures at the places of origin.

Cork oak populations also varied significantly in leaf nitrogen ( $N_{\text{mass}}$ ) and total chlorophyll content per mass ( $\text{Chl}_{\text{mass}}$ ). The pattern of inter-population divergence for

$N_{\text{mass}}$  and  $\text{Chl}_{\text{mass}}$  was similar to that for SLA. So, cork oak populations originating from rainy sites exhibited the highest  $N_{\text{mass}}$  and  $\text{Chl}_{\text{mass}}$  values whereas populations from cool and dry places showed the lowest ones. In fact,  $N_{\text{mass}}$  and SLA or leaf thickness were positively correlated. These results agreed with previous findings on a broad range of plant species (e.g. Reich et al. 1997, Wright et al. 2005) including cork oak (Quero et al., 2006). This association between  $N_{\text{mass}}$  and SLA or leaf thickness has been commonly explained in terms of selection against the combination of high  $N_{\text{mass}}$  and thickness (low SLA), because self-shading of chloroplasts and diffusion limitations of  $\text{CO}_2$  in the thicker leaves (with lower SLA) can limit the effective photosynthetic use of high  $N_{\text{mass}}$  concentrations (Reich et al. 1998, Quero et al. 2006).

Leaf area (LA) was also a trait which showed highly significant differences among cork oak populations. However, the percentage of the variance explained by climatic group was very low (< 1%) versus the percentage of the variance explained by population within climatic group (~ 25 %). Plants originated from Moroccan populations had the largest leaves. Population divergence of a widespread species may be influenced by different selective pressures imposed by different ecological factors but also by neutral evolutionary processes such as genetic drift, founder effects or bottlenecks (White et al. 2007). Thus, the unusually large leaves of Moroccan populations could be the result of some sort of local adaptive process, neutral evolution or both.

### **3. Phenotypic plasticity and local adaptation in leaf ecophysiological traits of climatically contrasting cork oak populations under different water availabilities.**

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#### **Introduction**

Forest tree species distributed across a wide range of environmental conditions are submitted to differential selective pressures. Long-term selection can lead to development of morphological and physiological adaptations to the local environment, generating ecotypic differentiation in important functional traits (Kawecki and Ebert 2004, Savolainen et al. 2007). Additionally, forest tree species can cope with this environmental variability if their traits exhibit phenotypic plasticity, i.e. ability to express alternative phenotypes in response to environmental variation (Bradshaw 1965, Sultan 1987, Lortie and Aarssen 1996). These two non-excluding strategies allow species to cope with environmental heterogeneity and shed light on the adaptive role of phenotypic traits in tree species. Thus, if a given trait is adaptive for a particular species, it is expected to show ecotypic differentiation caused by divergent selection pressures, a large phenotypic plasticity to cope with the environmental heterogeneity or both (Valladares et al. 2000, 2007). Nevertheless, to conclusively support the hypothesis that a trait is adaptive in a given environment, it is necessary to demonstrate that the trait is related to plant performance (e.g. fruit production, vegetative biomass, growth) in that environment and also that this association is weak or absent in other environments where the trait is not expected to be adaptive (e.g. Dudley 1996, Donovan et al. 2007).

Cork oak is a sclerophyllous tree species with a wide distribution in the western Mediterranean basin that occupies environments of contrasted temperature and rainfall (Díaz-Fernández et al. 1995, Aronson et al. 2009). Thus, it represents a suitable species to study processes of local adaptation. Population divergence has been

documented in cork oak for growth form (Gandour et al. 2007) and traits related to tolerance to low temperatures (Aranda et al. 2005). However, to our knowledge, the role of phenotypic plasticity on the ability of this species to cope with the environmental heterogeneity has not been explored so far.

The aim of this study was to evaluate the importance of local adaptation and phenotypic plasticity in key functional leaf traits potentially involved in cork oak's drought tolerance. For this purpose, we analyzed selected leaf ecophysiological traits in nine year-old plants established under common environmental conditions during two years with contrasting annual and summer precipitation. Plants were sampled from thirteen populations characterized by different climates. The traits selected were specific leaf area, leaf size, leaf nitrogen content per leaf mass and carbon isotope discrimination as a surrogate of water use efficiency (Farquhar et al. 1989). These traits have been broadly documented to be involved in drought responses and they have been reported to be under natural selection in dry environments (e.g. Dudley 1996a, Casper et al. 2005, Heschel and Riginos 2005, Donovan et al. 2007).

## **Material and methods**

The study was conducted in a common garden of cork oak. Thirteen populations were essayed, spanning over a wide range of climatic conditions. The study was carried out eight years after plantation in the field. Saplings were phenotypically characterized in two years with contrasting climatic conditions: 2005 (dry) and 2006 (wet). The differences in amount of annual and summer rainfall were particularly remarkable. So, annual rainfall was 468.7 mm in 2005 versus 644.5 mm in 2006.

Annual growth was measured in each plant. Additionally, we measured several phenotypic traits potentially related to drought tolerance: leaf size, specific leaf area (SLA), total chlorophyll content, nitrogen leaf content ( $N_{\text{mass}}$ ) and carbon isotope discrimination ( $\Delta^{13}\text{C}$ ).

### *Statistical analysis*

In order to examine the effects of genetic differentiation in functional leaf trait, to explore the possible climatic influence on population divergence (local adaptation) and to investigate the phenotypic plasticity in response to rainfall variation, we performed five repeated-measures ANOVA (one per variable) where year was the repeated factor (subject effect, see model below). Since populations varied over a wide climatic range and the environmental differences among the places of origin were not explained by a single climatic variable, we grouped the populations according to the degree of climatic similarity ("climatic group"). Climatic group were included as a categorical factor in the variance analyses. Differences in traits among climatic groups indicate genetic differences explained by adaptation to different climates. Likewise, the term population (within climatic group) was included in order to test for genetic differences due to adaptation to other local environmental factors different from climate (e.g. soil properties, nutrients availability, pressures from herbivory, etc.). The interaction term climatic group x year was used to evaluate potential differences in plasticity among climatic groups.

Additionally, we investigated the potential relationship between functional traits and aboveground growth, as the performance variable, under contrasting climatic conditions in order to assess the potential adaptive value of these traits for cork oak under different water availabilities. For this purpose, we constructed regression models *sensu* Lande and Arnold (1983).

## **Results**

### *Adaptation to environment: population divergence and phenotypic plasticity*

Populations differed significantly in all traits associated to plant growth (leaf size, specific leaf area and carbon isotope discrimination). The population divergence in leaf traits was consistent with that expected for populations differing in temperatures

and precipitation pattern. Cork oak populations originating from rainy summer and winter sites exhibited higher SLA and  $\Delta^{13}\text{C}$  than the remaining populations. The SLA and  $\Delta^{13}\text{C}$  commonly decreases with decreasing rainfall in forest tree species (e.g. Bussotti et al. 2002, Schulze et al. 2006, Marchin et al. 2008). A decreasing pattern of SLA and  $\Delta^{13}\text{C}$  with rainfall has been recently reported in cork oak by Gouveia and Freitas (2009). However, the trait measurements in that study were performed “*in situ*” at the places of origin of the trees. Thus, the environment and genetic effect could not be differentiated. In the present study, the large significant differences in SLA among climatic groups together with their association to the amount of summer precipitation at the place of origin, suggest that among-population differentiation could be at least partly due to an adaptation to different patterns of rainfall.

In contrast, despite the fact that cork oak populations belonging to climatic groups V (rainy summers) and III (rainy and mild winters) exhibited higher SLA and  $\Delta^{13}\text{C}$  than those from other climatic groups, they exhibited significant differences in leaf size. Trees from rainy summer sites (V), also characterized by cool annual and winter temperatures had much smaller leaves than those trees from the humid winter sites, which present mild/warm annual and winter temperatures. This suggests (1) that leaf size is probably adapted to the different temperature patterns and growth seasons experienced by the different populations, and, what is more important, (2) that leaf size and SLA can evolve separately.

Leaf size and SLA varied across years evidencing a high phenotypic plasticity in these traits. Besides, both these and the rest of the traits varied as was expected for years with different water availabilities. Thus, plants had larger leaves, higher SLA and  $\Delta^{13}\text{C}$  in the wet year (2006) than in the dry one (2005), which agrees with the results obtained from the phenotypic analyses based on Lande and Arnold (1983) and with the observed pattern of population and climate divergence.

Finally, the genotype x environment interaction was very weak for all traits, suggesting low population divergence in plasticity for this species as also observed in

*Quercus ilex* a closely related species (Gimeno et al. 2009). However, this term was significant for shoot growth (a performance variable). A plausible explanation for this result could be the potential differences in plasticity among populations in non-measured traits. In fact, studies on other evergreen Mediterranean oaks have reported differences in plasticity in some morphological and physiological traits which have not been studied here (e.g. Balaguer et al. 2001, Gratani et al. 2003). Besides, we observed a positive correlation between population difference in shoot growth between the wet (2006) and the dry year (2005) with annual mean temperature at the place of origin. In fact, populations from warmer sites grew more than populations from cooler sites under wet conditions (year 2006) but not under dry ones (year 2005). These results suggest that “warm” populations take more advantage of favourable conditions than “cool” ones as observed in other temperate forest tree species (e.g. Lauteri et al. 2004).

#### *Association between ecophysiological traits and growth under dry and wet conditions*

There were significant differences in the phenotypic gradient and differentials of SLA across years. Plants with more sclerophyllous leaves (lower SLA) grew more during the dry year (2005), but not during the wet one (2006). Higher SLA is associated with higher LAR values and higher photosynthetic capacity, and thus, higher potential of carbon gain and growth rate in inter-specific studies (Reich 1998, Shipley 2006). However, lower SLA has been reported to provide fitness benefits in dry environments since more sclerophyllous leaves allow the plant to avoid an excess of water use, maintaining photosynthetic activity and carbon gain over a longer time period (Dudley, 1996). This strategy fits to expectations for drought tolerant species such as evergreen Mediterranean oaks (Leiva and Fernández-Alés 1998, Bussotti et al. 2002).

There are many selection studies in the literature on SLA and other indices of sclerophylly such as leaf thickness under different irradiance regimes, soil properties, plant densities and herbivory pressures (e.g. Steinger et al. 2003, Avramov et al. 2007,

Saldaña et al. 2007). However, to our knowledge, there are few studies aimed at analysing the phenotypic gradients and differentials for these traits in response to water availability (e.g. Etterson 2004, Agrawal et al. 2008). Etterson (2004) showed increasing negative gradients and differentials for SLA along an aridity gradient for *Chamaecrista fasciculata*. In other words, they found that plants presenting more sclerophyllous leaves had increased fitness along an aridity gradient. As a consequence, they suggested that climate alters selection regimens for this trait in their studied species. Although we have not studied the associations of traits with fitness itself, but aboveground growth, the facts that (1) the association between SLA and growth show differences across years, (2) SLA decreases with summer and annual rainfall, and (3) populations with higher rainfalls at the places of origin present higher SLA, suggest that divergence in water availability causes differential selection for SLA in cork oak.

Although there were no significant differences across years in the phenotypic gradients and differentials for the rest of the traits, marginal differences ( $P = 0.08$ ) in partial correlation coefficients of carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) between the wet (2006) and the dry year (2005) were observed. In fact, there were both direct and indirect positive associations between higher  $\Delta^{13}\text{C}$  (lower WUE) and growth in the wet year (2006), but no under dry conditions (year 2005). There is a variety of studies showing different kinds of relationships between WUE and aboveground growth/biomass in dry environments, from positive (e.g. Dudley 1996, Ludwig et al. 2004) and neutral (e.g. Ludwig et al. 2004) to negative relationships (e.g. Donovan et al. 2007). Similar variety of findings is also reported for wet conditions (e.g. Dudley 1996, Agrawal et al. 2008). Donovan et al. (2007) argued a dehydration strategy as a possible explanation for the negative relationship between WUE and aboveground biomass in the annual *Helianthus anomalous*. Heschel and Riginos (2005) suggested that lower WUE may favour fitness traits when plants experience drought early in the vegetative season. In the present study, the results for  $\Delta^{13}\text{C}$  were not completely

consistent with those observed for SLA, because plants with more sclerophyllous leaves (lower SLA) grew more during the dry year (2005), but not during the wet one (2006). In fact, there was a positive correlation between SLA and  $\Delta^{13}\text{C}$  in both years. Thus, we would have expected that lower  $\Delta^{13}\text{C}$  had growth benefits in dry conditions (year 2005) but not in wet ones (year 2006). Possible explanations for this apparent discrepancy need further research, but they could be related with consequences of SLA on leaf life span (Cavender-Bares et al. 2005), vegetative phenology (Heschel and Riginos 2005, Donovan et al. 2007) and changes in the relationships nitrogen-photosynthesis-WUE due to variation of SLA with water availability (Reich et al. 1998).

In conclusion, our study shows phenotypic differentiation in SLA, leaf size and  $\Delta^{13}\text{C}$  among cork oak populations associated to summer and annual rainfall at the places of origin. SLA and leaf size positively responded to an increase in summer and rainfalls in the common garden, indicating phenotypic plasticity in these traits. There was also genotype x environment interaction for shoot growth, and a correlation between the difference in shoot growth among years and annual mean temperature, suggesting a more opportunistic strategy of taking advantage of favourable conditions for the warmer populations. Finally, plants with lower SLA presented larger aboveground growth in a dry year (2005) whereas those with lower water use efficiency showed larger growth under wetter conditions (year 2006). Finally, we observed that plants with larger leaf sizes had higher growth benefits in both years. Altogether, our results suggest an adaptive value of SLA and LS for cork oak under a Mediterranean climate, and their potentially important role for dealing with varying temperature and rainfall regimes through both local adaptation and phenotypic plasticity.

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

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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 (White *et al.* 2007). Elucidating the causes of the intraespecific genetic differentiation is a central theme in evolutionary biology (e.g. Merilä and Crnokrak 2001, Reed and Frankham 2001). However, the ascertainment of the relative importance of neutral evolutionary processes and natural selection as determinants of divergence among populations is complex (Merilä and Crnokrak 2001). This point is typically solved by the comparison of the  $F_{ST}$  and  $Q_{ST}$  statistics (e.g. Latta *et al.* 2003, Volis *et al.* 2005). Wright's (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 and 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, what 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).

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. Thirteen cork oak populations, covering a vast climatic gradient of rainfall and temperature were studied.

Specifically, we 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 and 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 and Crnokrak 2001, Reed and Frankham 2001), by pairwise comparisons of population differentiation in  $F_{ST}$  and  $Q_{ST}$  estimates.

## **Material and methods**

### *Quantitative traits*

The measurements for the quantitative traits were carried out in 20 plants per population. 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. 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 ( $\Delta^{13}C$ ).

### *Molecular markers*

Fifteen plants per population (195 plants in total) were genotyped using six (GA)<sub>n</sub> nuclear microsatellites 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).

Differentiation among populations ( $F_{ST}$ ) was estimated using ARLEQUIN, v3.11 (Excoffier *et al.* 2005). 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 geographic 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).

### *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 ( $\sigma_B^2$ ) and the within-population ( $\sigma_W^2$ ) components as follows:

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

where  $V_\alpha$  is the variance of the population component,  $V_\epsilon$  is the residual variance and  $h^2$  is the heritability (Lande 1992). The variance components for the estimate of  $Q_{ST}$  for each trait ( $V_\alpha$ ,  $V_\epsilon$ ) were estimated by performing seven GLMs.

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 analyzed during the same year.

#### *Comparison of molecular and quantitative data*

To detect differences of means between  $F_{ST}$  and  $Q_{ST}$  values, tests based on the standard deviation confidence intervals for bootstrap samples were done (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.

#### Results:

Relatively low multilocus  $F_{ST}$  was obtained, 0.032 (0.003), showing that around the 97% of the total variation is present within population. This result is consistent with the low differentiation value reported for the region by Toumi and 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 bias of  $Q_{ST}$  estimates in the analysis. Higher values of  $Q_{ST}$  respect to  $F_{ST}$  have been found in different forest tree species (e.g. Hamrick 2004), as well as in other organisms (Merilä and Crnokrak 2001, McKay and Latta 2002). 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 as compared to neutral evolutionary processes for leaf nitrogen content, leaf size and SLA in a dry year suggests that these traits are subjected to diversifying selection. Conversely, 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.

#### *F<sub>ST</sub> as predictor of Q<sub>ST</sub>*

Pairwise  $F_{ST}$  values were slightly correlated with altitude and geographic 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 were 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 comparisons were excluded from the analyses. The location of Haza de Lino (HZ), at 1300 meters 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. However, the affinity between  $Q_{ST}$  for leaf size and  $F_{ST}$  for neutral

markers can not be explained by the effect of the geography. Another potential mechanism suggested by Merilä and Crnokrak (2001) was based on that not all microsatellite loci are completely freely recombining with the rest of the genome. We found that the correlation between  $F_{ST}$  and  $Q_{ST}$  for leaf size was exclusively caused by the microsatellite *QpZAG46*. 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, 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 can not be rejected.

## **5. Natural selection on cork oak: allele frequency reveals divergent selection in cork oak populations along a temperature cline.**

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### **Introduction**

Plants can respond to environmental heterogeneity by either exhibiting large phenotypic plasticity, i.e. great ability to express alternative phenotypes in response to environmental variation (Bradshaw 1965, Valladares et al. 2000, 2007), showing adaptations to their local environment (Kawecki and Ebert 2004, Savolainen et al. 2007), or both. Among-population differentiation in functional traits along an environmental gradient is one of the most widespread examples of local adaptation in wild populations. This process, known as adaptive divergence, generates genetic clines, which may be evident in allele diversity and the frequency of markers associated with genes encoding for adaptive traits (Huestis and Marshall 2006, Mullen and Hoekstra 2008). These patterns of correlation between molecular diversity and the environment have been reported in several plant species including grasses (e.g. Kelly et al. 2003, Mitton and Duran 2004, Jump et al. 2006). However, although there are many examples of clines in allele frequencies in natural populations and sometimes the selective agent responsible for causing population genetic structure is even known, a precise understanding of the association between genetic markers and functional traits is often lacking in the context of natural selection (e.g. Mitton and Duran 2004, Jump et al. 2006). In fact, the genetic effects (e.g. overdominance, dominance, additive effects, etc.) underlying such associations are often ignored in these studies. Developing mechanistic hypotheses for such associations enables the evolutionary consequences of natural selection on the populations to be investigated. For instance, if alleles at a given locus (related to an adaptive trait) exhibit overdominance, the fitness of an heterozygous individual will be greater than either homozygote, resulting in the long-

term persistence of multiple alleles in a population even under strong selective pressures (Mitton 1997, Schmidt and Rand 2001). In contrast, if homozygous individuals exhibit higher fitness than heterozygotes at a given locus (e.g. by additive effects), natural selection eventually will lead to fixation of the favoured allele in the population (unless some form of balancing selection is acting) (Falconer and Mackay 1996, White et al. 2007).

In the present study, we have investigated the putative linkage of the six nuclear microsatellites to genes coding for adaptive traits. We examined the potential genetic effects underlying these associations: overdominance (heterozygote superiority), dominance and additive effects. We also examined whether there was a spatial pattern in the allele frequency of the loci associated with adaptive traits in thirteen cork oak populations characterized by climatic differences at their place of origin.

## **Material and methods**

### *Phenotypic analysis*

Phenotypic characterization was carried out in a common garden. A total of 15 plants from 13 cork oak populations were selected. We measured total height, diameter and annual growth during 2005. Besides, we measured several phenotypic traits potentially related to drought tolerance: leaf size, specific leaf area (SLA), nitrogen leaf content ( $N_{\text{mass}}$ ) and carbon isotope discrimination ( $\Delta^{13}\text{C}$ ).

### *Molecular analysis*

The 195 individuals phenotypically characterized were genotyped with six nuclear microsatellites transferred to cork oak 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). DNA was extracted from leaves following the method described by Doyle and Doyle (1990). Amplification and scoring were performed following Soto et al. (2003, 2007).

### *Statistical analyses*

We explored associations between phenotypic traits and molecular markers. For this purpose, (1) we evaluated the influence of single-locus heterozygosity on growth and ecophysiological traits by constructing General Linear Models (GLMs) (one per trait) where phenotypic traits were the dependent variables and heterozygosity/homozygosity at each locus was fitted as fixed effect with two levels (e.g. Savolainen and Hedrick 1995). Thus, in this first analysis we exclusively distinguished between heterozygous and homozygous individuals without considering the genotype of specific alleles. (2) We examined the effect of the single-locus genotype on phenotypic traits. For that, we first constructed GLMs, where genotype of each locus was included as categorical variable and where the dependent variables were the different quantitative traits. Then, for those loci where genotype was associated with phenotypic traits, we implemented GLMs where the presence/absence of each allele was included as categorical variable with two levels and phenotypic traits as dependent variables.

We also calculated the population frequencies for the alleles of those loci that were related to phenotypic traits. Then, we constructed Generalized Linear Models (GLZs) with binomial error structure and log link function to test for differences in allele frequencies among populations. Likewise, we examined associations between allele frequencies and population average trait values. Finally, to evaluate the effects of climate as a selective agent, we explored the associations between allele frequencies and local climatic conditions (e.g. Mitton and Duran 2004).

## **Results**

### *Linkage disequilibrium and genotypic effects*

There were clear differences in leaf size and growth correlated to the genotype at locus *QpZAG46*, but no other relationship for the rest of the variables and loci.

Genotype at locus *QpZAG46* explained 13.1 and 3.6 % of the phenotypic variance in height growth and leaf size, respectively. Homozygous individuals for allele *QpZAG46-188* (where 188 means the number of the base pairs) had the largest leaves and the highest growth. Likewise, after controlling for population structure and initial height (this only for leaf size), we found that the presence of allele *QpZAG46-188* was positively associated with eight-year growth in height whereas *QpZAG46-192* allele had a negative association with it. Allele *QpZAG46-188* was also marginally associated to a larger leaf size, whereas individuals carrying *QpZAG46-190* had significantly smaller leaves.

These results suggest a possible linkage between locus *QpZAG46* and genes coding for leaf size and growth. Our results agree with other studies on oaks, which report that *QpZAG46* belongs to the same linkage group as *QrZAG87* and *QpZAG119* (Scotti-Saintagne et al. 2004, Brendel et al. 2008), close to a QTL coding for leaf size (Brendel et al. 2008).

#### *Adaptive differentiation and evolutionary response to climate change of cork oak populations*

Cork oak populations were exceptionally differentiated at the locus *QpZAG46*. The population frequency of allele *QpZAG46-188* was positively correlated with AMT at the place of origin, whereas the population probability of the presence of alleles *QpZAG46-190* and *QpZAG46-192* increased as annual and winter temperatures dropped. These results, together with the fact that *QpZAG46*-alleles had different effects on adaptive traits, suggest that temperature is a relevant selective agent for cork oak and its selective pressures lead to the development of local adaptations (e.g. Aranda et al. 2005). In view of these results and given that constraints to gene flow among populations are weak (Toumi and Lumaret 1998, Jiménez et al. 1999), adaptive pressures can be expected to be the main factor responsible of genetic differentiation at the locus.

## **6. Factors affecting cork oak growth under dry conditions: local adaptation and contrasting additive genetic variance intra-populations.**

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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 morphology and biochemistry are expected to be of primary importance in determining plant fitness in these water-limited environments (Dudley 1996, Heschel et al. 2002, Heschel et al. 2004, Ludwig et al. 2004). Their importance is likely to be reinforced in the future due to the expected increase in the severity and duration of drought periods in this region (IPCC 2007). In the short-term, the capacity of species to cope with these changing environmental conditions is reliant on their phenotypic plasticity as well as on their intra-specific genetic variation for adaptive traits (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). 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 for 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). 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 example, 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, Cotter et al. 2004).

The objectives of this study were: (1) to estimate the inter- and intra-population genetic divergence, the genetic correlations among traits and the heritabilities of a set of six 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 in nine-year-old plants established under common environmental conditions during an extremely dry year. (2) To explore the relationships between the selected leaf traits and aboveground growth as a surrogate of plant fitness in a dry year.

## **Material and methods**

### *Phenotypic characterization*

The study was carried out in a common garden of cork oak (*Quercus suber* L.) over 2005, a particularly dry year with just 306 mm of total annual rainfall (50.5% lower than the forty-year average). Eight plants from forty five open-pollinated families originating from three different populations were sampled for this study. Total height, basal diameter and annual growth were measured in each plant. Additionally, we measured several phenotypic traits potentially related to drought tolerance: leaf size, specific leaf area (SLA), leaf thickness, total chlorophyll content, nitrogen leaf content ( $N_{\text{mass}}$ ) and carbon isotope discrimination ( $\Delta^{13}\text{C}$ ).

Nine linear models (one per variable) were performed to analyze the family and population effect on each variable. 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 populations separately.

On the other hand, we investigated the potential relationship between functional traits and aboveground growth in dry conditions. For this purpose, we constructed regression models *sensu* Lande and Arnold (1983). Phenotypic relationships between traits were also studied by Pearson correlations between all pair traits.

## **Results**

### *Among- and intra-population differentiation: evolutionary capacity of functional traits*

The three studied cork oak populations showed moderately high heritabilities for total height, diameter and  $\Delta^{13}\text{C}$  caused by high intra-population genetic variance in these traits. Since heritabilities were significant for these traits, they are likely to respond to natural selection in the three studied cork oak populations. Conversely, the heritabilities for the remaining characters varied among populations due to differences in the level of additive genetic variance within the populations and do not by differences in the total phenotypic variance. The differences were particularly remarkable for SLA, leaf thickness and leaf size, three traits under possible divergent selection as discussed above. Taken together, these 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. The absence of significant genetic correlations and the fact that correlated traits did not undergo opposing selection in our analyses providing 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. Lower SLA has fitness benefits in dry environments since more sclerophyllous leaves allows the plant to avoid an excess of water use, maintaining photosynthetic activity and carbon gain over a longer period of time (Dudley 1996). This strategy fits to the expectations for drought tolerant species such as evergreen Mediterranean oaks (Leiva and Fernández-Alés 1998, Gratani et al. 2003). Nevertheless, water use efficiency (WUE) (inferred from carbon isotopic discrimination) was found here not to affect shoot growth. This was unexpected because  $\Delta^{13}\text{C}$  has been documented to decrease with the increase in water stress in cork oak (Aranda et al. 2007) and WUE has been reported to be mainly mediated by the response of stomatal conductance to water vapour in oaks (Quero et al. 2006). We do not have an explanation for this unexpected result, but the negative phenotypic correlation between SLA and WUE observed in cork oak (Aranda et al. 2007, Gouveia et al. 2009), and the absence of such correlation in the present study, could be related to the absence of any relationship between WUE and shoot growth in a dry year.

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 (Givnish 1979). 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. Many studies have observed the positive effect of 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).

In conclusion, our results suggest that leaf size, leaf thickness, SLA and chlorophyll content are important traits for cork oak saplings growth under dry conditions. Specifically, larger and more sclerophyllous leaves rendered growth benefits. Populations differed significantly in leaf thickness, SLA, and particularly in leaf size. Populations also showed significant differences in heritabilities in these traits, suggesting that the divergent selective pressures have led to local adaptations but also have shaped the level of genetic variance in the studied cork oak populations. Finally, the absence of significant genetic correlations and the fact that correlated traits did not undergo opposing selection providing little evidence for constraints on evolution caused by genetic correlations.

## General conclusions

- Cork oak seedlings originating from larger seeds presented higher growth and survival rates during the first years of development. Likewise, seed size and survival rate were correlated to the severity of drought season at the place of origin, which suggests that population divergence is probably due to local adaptation.
- Cork oak saplings originating from wet populations exhibited the highest values of specific leaf area (SLA), nitrogen content ( $N_m$ ) and total chlorophylls ( $Chl_m$ ). In contrast, populations originating from cold and dry sites showed the lowest values of SLA,  $N_m$  and  $Chl_m$  (i.e. more sclerophyllous leaves). These results point that drought but also low temperatures in winter are important selective agents for this oak species.
- Iberian populations presented low genetic differentiation in molecular markers (3.2%). The population originating from “Las Alpujarras” (Granada, southern Spain) was however highly differentiated from the rest of Iberian populations. This population is located at 1300m a.s.l. and 200 km from the main cork oak distribution range, which could have prevented gene exchange with other populations.
- Population genetic differentiation in total height,  $N_m$ , SLA and leaf size were significantly higher than that observed in molecular markers suggesting that population divergence in these traits is probably due to differential selective pressures.
- There was a strong correlation between population distances in leaf size and growth and genetic distances at locus *QpZAG46*. Besides, there was a strong correlation between genotype at this locus and these phenotypic traits. These results suggest that this locus is near genes encoding for leaf size and growth.
- Cork oak saplings carrying allele *QpZAG46-188* showed higher growth and larger leaves, whereas those individuals presenting alleles *QpZAG46-190* and

QpZAG46-192 exhibited lower growth and smaller leaves. Allele frequencies at this locus were correlated to annual mean temperature and minimum winter temperatures, suggesting that temperature exerts strong selective pressures on cork oak populations.

- There were high differences among maternal lines in height, diameter,  $^{13}\text{C}$ -carbon isotope discrimination, specific leaf area and leaf size, indicating high intrapopulation genetic variation in these traits.
- Cork oak saplings exhibited smaller leaves, more sclerophyllous leaves (lower SLA,  $N_m$ , and  $\text{Chl}_m$ ) and higher water use efficiency in a dry year than in a wet year, revealing high phenotypic plasticity of these traits to water availability.
- There was a significant interaction population  $\times$  year for annual growth. Besides, there was a positive correlation between growth differences between the wet year and the dry year ( $\Delta$  growth) and annual mean temperature, suggesting that “warm” populations take advantage of favourable conditions than “cool” populations.

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