

# Sun and shade leaves of *Olea europaea* respond differently to plant size, light availability and genetic variation

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## Summary

1. Canopy plasticity, the expression of different leaf phenotypes within the crown of an individual tree has complex functional and evolutionary implications that remain to be thoroughly assessed. We hypothesized that it can lead to disparity in how leaves in different positions of the canopy change with allometric growth and population genetic structure.

2. Leaf phenotypes of the inner and outer canopy were estimated using eight morphological and physiological characters. All traits were measured under field conditions in six populations of *Olea europaea* and again in a common garden for a subset of the genotypes. The same populations were characterized genetically with amplified fragment length polymorphisms (AFLP) genomic scans. With these data, we investigated the extent to which leaf phenotypes change with plant size, genetic processes and in response to environmental conditions inside and outside the canopy.

3. The size of trees measured in the field was clearly associated with the phenotype of sun but not to that of shade leaves. The phenotype of sun leaves depended on both direct and diffuse light, while that of shade leaves was found to correlate only with diffuse radiation. Additionally, light availability inside the canopy was conditioned by the shape of external leaves, and increasing elongation of sun leaves led to higher radiation in the inner canopy.

4. The field phenotypes of both inner and outer canopy leaves were correlated with genetic variation among populations. Conversely, in the common garden, the different genotypes expressed a homogeneous sun phenotype, while phenotypic differences among populations remained apparent in shade leaves.

5. We conclude that, in agreement with our working hypothesis, canopy plasticity is both cause and consequence of the environment experienced by the plant and might lead to the differential expression of genetic polymorphisms among leaves. Furthermore, we propose that it can contribute to buffer abiotic stress and to the partition of light use within the tree crown.

**Key-words:** allometry, genomic selection, molecular ecology, niche construction, olive tree, permutation ANOVA, plant behaviour, standing variation

## Introduction

Leaf morphology and physiology in tree canopies is modulated in response to within-crown gradients in environmental conditions (Zwieniecki, Boyce & Holbrook 2004; Sack &

Holbrook 2006; Niinemets 2010). Simultaneously, leaf phenotype is also the product of physiological and ontogenetic limitations such as biomechanical constraints and the allocation of finite resources (Yang & Midmore 2005; Sack *et al.* 2006; Niklas & Cobb 2010). These two mechanisms of leaf phenotypic variation are not mutually exclusive. In fact, it has been suggested that the environmental heterogeneity

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within canopies could foster phenotypic specialization among branches in a manner analogous to that of the ramets of clonal plants (Rubio De Casas *et al.* 2007).

Across the growing season, outer layers of the canopy are more exposed and thus face more drastic changes in the abiotic conditions than inner layers (Niinemets 2007). Some findings on photosynthesis partitioning seem to indicate that outer layers play a dual role, photosynthesizing when conditions external to the canopy are optimal while simultaneously buffering the environment for inner canopy layers (Howell, Kelly & Turnbull 2002). Internal layers of the canopy encounter a more stable environment, at least in dense canopies where sunflecks are short and of low light intensity (Gratani, Covone & Larcher 2006; Diaz-Espejo, Nicolas & Fernandez 2007; Brantley & Young 2009).

The working hypothesis of the present paper is that canopy plasticity can induce leaves in the inner and outer canopy to change differently in response to the environment. Consequently, the overall canopy phenotype is a composite of individual module phenotypes that vary across populations because of genetic and environmental constraints. This hypothesis is based on the fact that accommodation to environmental conditions at the canopy scale leads to specialization at smaller scales (module or leaf, Niinemets & Valladares 2004). This specialization derives in part from the aforementioned buffering of within-crown environmental variation by external layers of the canopy (cf. Laland, Odling-Smee & Feldman 1999). Furthermore, we assume that selection operates upon modular responses and interactions because variation in module phenotype can result in fitness differences at the organismal level (De Kroon *et al.* 2005).

To test this hypothesis, we combined field observations and common garden experiments and measured how outer and inner canopy leaf phenotypes changed in response to the environment and to what extent changes in leaf phenotypes reflect allometric growth and genetic variation. The plant system used was the wild olive tree (*Olea europaea* L.), an emblematic Mediterranean sclerophyll. Recent research by our group showed that canopy plasticity in *O. europaea* entails the simultaneous and divergent modification of both external and internal canopy modules. In fact, under abiotic stress and between distinct environments, external canopy layers vary more drastically than inner ones (García-Verdugo *et al.* 2009a). The following questions were asked:

1. Are changes in the outer layers of the canopy consistent with a buffering role?

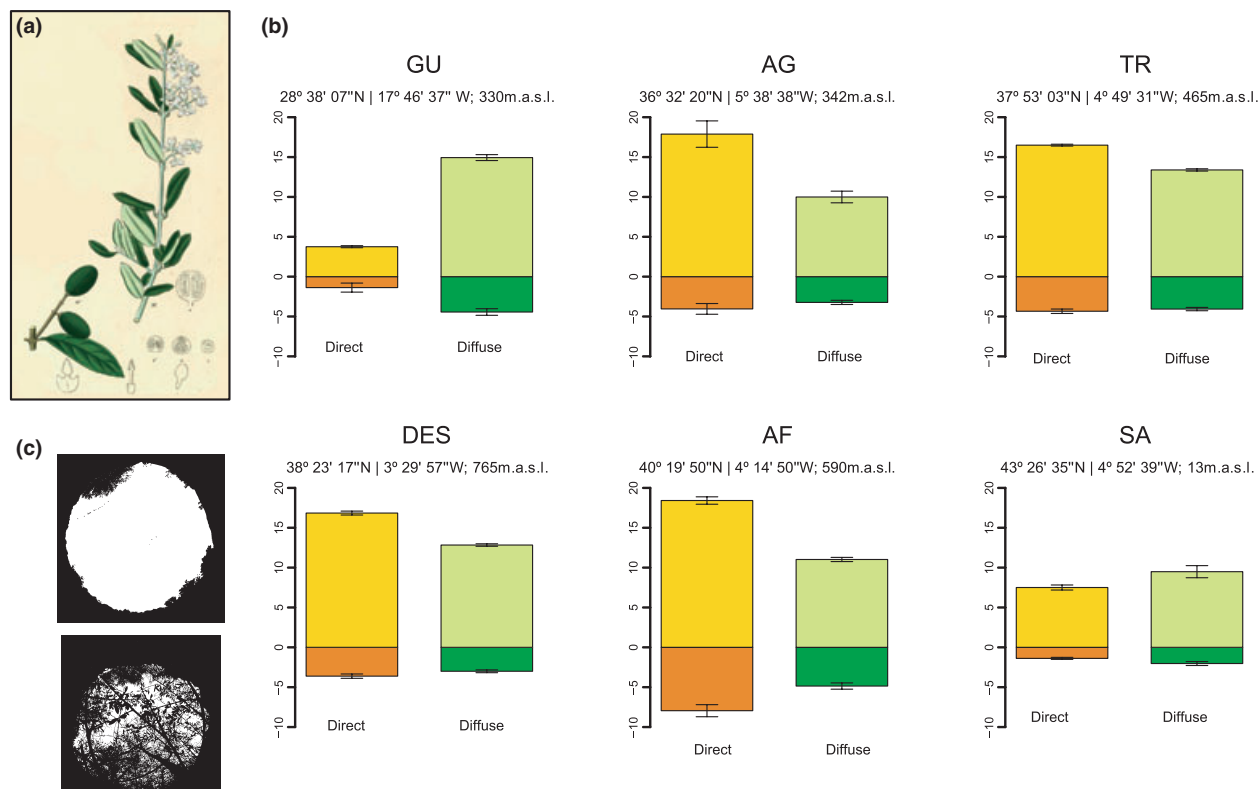
If leaves in the outer canopy are shielding those in the inner canopy from environmental stress, then phenotypic shifts in outer canopy layers should be associated with the emergence of a more stable environment in the inner canopy. Moreover, a similar sun leaf phenotype is expected in plants growing under common conditions because the homogenous environment would force all genotypes to express a convergent sun phenotype leading to similar inner canopy environments.

2. Is the influence of allometric growth constant across the canopy? The effect of allometry might vary between modules of the same plant, confounding phenotypic differences at the population scale, e.g. the leaves of modules of a given size might produce similar leaves regardless of their local environment.
3. Do genetic differences between populations correlate with differences in leaf phenotypes? If so, is the manifestation of genetic differentiation the same in sun and shade leaves? Leaf phenotypes might be the product of acclimation to local conditions. If that is the case, variation among populations would reflect only environmental differences. Conversely, neutral drift or natural selection might limit the potential phenotypes. Also, as different modules encounter different environments (i.e. sun vs shade), their phenotypes might not reflect equally the genetic divergence among populations.

## Materials and methods

*Olea europaea* occupies a wide range of environments from arid maquis to subtropical fog forests. Six populations along an approximately 2000-km-long gradient from the Canary Islands to Northern Iberia were chosen so as to encompass wide genetic diversity, represent the main western Mediterranean formations where *O. europaea* is prevalent (Costa, Morla & Sainz 1997) and maximize latitudinal and climatic differences. Five *O. europaea* populations spread across the Iberian Peninsula and one population from La Palma island (Canary Islands) were sampled (Fig. 1). Three 25 × 25 m plots were marked out in each population and five individuals per plot randomly chosen. In order to gather comparable data, all samplings were conducted in midsummer (July–August 2003), when plants are assumed to be in drought-induced aestivation. Only plants of more than 1.5 m of height bearing fruit were included in the study to avoid juveniles. We took the individual crown as the functional unit. Tree height and the two widest canopy diameters were measured for each plant to estimate plant biovolume as basal area × height. Measurements and samples were taken on current-year foliage in two exposures: south-facing, fully exposed upper canopy (SUN) and north-facing, inner canopy (SHADE) leaves. In a preliminary field survey, we found that the percentage of branching (i.e. proportion of developed buds along 15 cm from the shoot apex) was three times higher in the outer than in the inner layers of the tree crown. Sun leaves were, then, mostly produced by short shoots and shade leaves by long shoots, a pattern previously reported for deciduous tree species (Niklas & Cobb 2010).

In the field, phenotypic variation often correlates with variables such as latitude or climatic conditions. These variables are likely a proxy of the actual factors that exert selective pressures on populations (Roff & Mousseau 2005). Solar radiation was employed as a surrogate of the microhabitat conditions in each canopy. Radiation levels result from the interaction of many factors (cloudiness, site openness) that condition other variables such as temperature and water vapour pressure. Moreover, solar radiation determines the photosynthetic environment and thus is highly influential on the leaf phenotype. The solar radiation exposure of each individual was quantified with hemispheric photographs. Two photographs from each tree, one directly above a leaf in SUN and one in SHADE were taken using a Nikon Coolpix 4500 camera with an 8-mm fisheye lens. To represent the light environment within the canopy, shade photo-



**Fig. 1.** (a) Depiction of leaves and reproductive organs of *Olea europaea* (taken from Reichenbach 1854–1855); (b) Solar radiation levels (PPFD) in the *O. europaea* populations studied in mol photons m<sup>-2</sup> day<sup>-1</sup>. Values were calculated by analysing two hemispheric photographs of every individual with Gap Light Analyzer (GLA V2.0; Frazer, Canham & Lertzman 1999; Frazer *et al.* 2001). In every plot, darker colours and negative PPFD values are used to indicate the shade conditions, while lighter colours and positive values indicate sun. The shade values are given as negative for the sake of representation. *O. europaea* subsp. *europaea* populations. AF, Aldea del Fresno; AG, Alcalá de los Gazules; DES, Despeñaperros; SA, San Antolín; TR, Trassierra; *O. europaea* subsp. *guanchica*. GU, La Palma. (c) Example of hemispheric photographs taken in the SUN and SHADE exposure of the same individual.

graphs were taken over the portion of the inner canopy where shade leaves were previously sampled, avoiding leaves or stems that were too close to the lens and that could underestimate the proportion of incident light. Gap Light Analyzer (GLA) Version 2.0 (Frazer, Canham & Lertzman 1999; Frazer *et al.* 2001) was used to process and analyse the digital images and to compute total, direct and diffuse solar radiation for each plant and canopy exposure following previous studies (García-Verdugo *et al.* 2009b). Estimates of direct, indirect and total radiation were obtained from the Spanish Meteorological Agency from stations that were less than 25 km away from each population. These estimates were then corrected for topography (i.e. elevation, slope and orientation) to calculate site-specific cloudiness and incorporated into the GLA models to improve accuracy. GLA software also records the number and duration of sunfleck events based on a user-defined interval of change in solar position throughout the growing season (set to 2 min and from January 1 to December 31, respectively).

#### MORPHOLOGICAL AND PIGMENT DATA

Leaf angle, mean leaf area, specific leaf area (SLA) and leaf index (leaf length divided by leaf width) were calculated for ten leaves from each of the two exposures of the 90 individuals (15 plants from six populations). Leaf angle to horizontal was measured in the field using a protractor on ten mature leaves per exposure. Three leaves per exposure

were taken at noon from the first three individuals of each plot and immediately stored in liquid nitrogen until their pigments were analysed (Rubio De Casas *et al.* 2007). Briefly, chlorophylls and carotenoids were separated by HPLC (Waters Corp., Milford, MA, USA), following pigment extraction in cool acetone. The peaks were identified and quantified with pure commercial standards (VKI, Hørsholm, Denmark). Eight characters representing leaf form and function were considered to characterize the leaf phenotype: foliar area, leaf angle, SLA, leaf index, total xanthophyll pool (violaxanthin + antheraxanthin + zeaxanthin, VAZ) on a chlorophyll basis, chlorophyll a/b ratio, total chlorophyll content and  $\beta$ -carotene content on a chlorophyll basis. All analyses of the phenotype and its comparison with genomic and environmental data were conducted separately for sun and shade values, calculated as the means per individual and exposure of each of the eight characters. These individual values were then summarized with a principal component analysis (PCA), of which the axes with eigenvalues  $\geq 1$  were retained and used to represent the phenotypic values in all posterior analyses. Differences in phenotype between canopy exposures within and across populations were investigated using a split-plot mixed effects linear model of the form:

$$\text{Trait} \sim \text{Population} \times \text{Exposure} + \text{Plant size}$$

where populations were treated as blocks of three plots and canopy exposure as the within-plot treatment with two levels, sun and

shade, with exposure treated as fixed factors and population and plot as random. Plant size, estimated as  $\log(\text{biovolume})$ , was included in all analyses to account for allometric differences. Biovolume was used as a canopy size proxy because the total aerial size of *O. europaea* plants can be well approximated with an ellipsoid – of – revolution: they tend to have a shrub habit, are multistemmed and have leaves near the ground. Linear models were fit using maximum likelihood with the 'lme' function in the nlme R package (Pinheiro *et al.* 2009). To determine significant differences between populations, models with and without the 'population' random factor were run and their fit to the data compared using the 'anova' function in the stats R package. The difference in the likelihood ratio between the models with and without 'population' provided an estimate of the factor's effect (R development core team, 2009; see Crawley 2009, p. 653 for details). Differences between exposures (i.e. because of canopy plasticity) and because of allometric growth were estimated by the main effects of the linear model, while the interaction term 'Population  $\times$  Exposure' provided an estimate of the differences in canopy plasticity between different populations. Differences among populations were then determined separately for the phenotypes of the two canopy exposures. The axes resulting from the PCA of phenotypic values were regressed against the total, diffuse and direct radiation values obtained with GLA across populations for a characterization of how the phenotype of individual trees responds to the local environment. Regressions were conducted independently for sun and shade leaves. Shade PCA phenotypic values were also regressed against sun radiation values to determine their dependence on extra-canopy radiation. Additionally, the influence of outer canopy layers on inner canopy light environment was investigated by regressing the ratio SHADE radiation/SUN radiation (i.e. the proportion of the external radiation that reaches the inner canopy) against the morphological values of sun leaves (leaf angle, index, and area and SLA) of the same plants. In all analyses, data were transformed as needed to conform to normality assumptions.

#### COMMON GARDEN EXPERIMENT

Ten shoots exhibiting signs of recent growth (green, non-lignified stems) were cut in the field from the outer canopy of the individuals previously sampled in each population. Shoots were transported to a greenhouse under refrigerated and humid conditions. Here, the shoots were cut and only the apical 10 cm were retained. To ensure that all leaves would develop under common conditions, all leaves except the apical pair were removed from the cuttings. The base of the cuttings was then treated with commercial rooting hormones (0.06% indolebutyric acid and 0.08%  $\alpha$ -naphthyl acetamide). On November 2003, the cuttings were planted in 1-L pots containing commercial rooting medium and taken to a cold greenhouse. In June 2004, they were planted in 50-L pots containing a 50% mixture of commercial lightweight potting soil and peat moss (v/v), and grown under full sun. Pots were distributed randomly in a single  $5 \times 10 \text{ m}^2$  block. Water potential levels in the pots were controlled with two Watermark WEM-II soil moisture sensors placed in different pots and coupled to a Watermark datalogger (Irrometer, Riverside, CA, USA). Drought stress was avoided and optimal conditions ensured by automatically watering the pots when water potential reached -10 KPa (García-Verdugo *et al.* 2009a,b). Plants were supplemented twice a year with commercial fertilizer (NPK; 7:5:6). In the summer of 2005, the cuttings that survived winter (30% of the total) had grown enough to flower. Watering was withheld between July 15 and September 1

every summer, so as to induce water stress aestivation and thus mimic field conditions. We included all the populations that had surviving cuttings from at least three different genotypes in posterior analyses. In 2005, 1 week after watering was stopped, we took the same measurements and conducted the same sampling approach that had been used in the field on plants from AF (four genotypes), AG (four genotypes), SA (three genotypes) and TR (four genotypes). The resulting values were ordained using PCA, the components of which with eigenvalues  $\geq 1$  were used for further analyses. Phenotypic differences between exposures and populations were investigated using linear models with the same main and interaction effects (Plant Size, Population, Exposure and Population  $\times$  Exposure) as those used for the analysis of field data. In these models, all effects were considered fixed and no level below population was included. Given the small sample size, permutation tests instead of *F* values were used to estimate significance levels and therefore avoid assumptions of normality (Anderson 2001; Anderson & Ter Braak 2003) using the 'lmp' function of the lmpPerm R package with *P*-values calculated with 5000 iterations or with enough iterations so that the standard deviation of the estimated *P*-value fell below 0.005 (Wheeler 2010). The influence of outer canopy layers on inner canopy light environment was investigated by regressing the radiation levels recorded in shade of the common garden plants against the morphological values of sun leaves (leaf angle, index, and area and SLA) of the same plants.

#### GENETIC SAMPLING AND ANALYSES

Leaf material was collected in the field from the same *O. europaea* individuals that were sampled for pigment analysis. These individuals were genotyped using the AFLP technique (Vos *et al.* 1995) as described in (Rubio De Casas *et al.* 2006). All fragments smaller than 140 bp or present in  $< 5\%$  or absent in more than 95% of the individuals were eliminated from the matrix to eliminate biases because of fragment homoplasy (Caballero, Quesada & Rolan-Alvarez 2008) or ascertainment (Foll, Beaumont & Gaggiotti 2008a). The resulting data set was subjected to analyses of molecular variance (AMOVA) with ARLEQUIN, v 2.00 (Schneider, Roessli & Excoffier 2000) to determine genetic structure and to compute population pairwise *F*<sub>st</sub> values (Excoffier, Smouse & Quattro 1992). The AFLP fragments were then analysed with BayeScan (Foll & Gaggiotti 2008b) to distinguish putative loci subject to selection from those following a neutral pattern of differentiation. As this software tends to inflate selection estimates (Foll & Gaggiotti 2008b), we considered loci to diverge following a neutral pattern when their Bayes factor was below 3 ( $\log_{10}(\text{BF}) \leq 0.5$ ) and to be under selection only when their Bayes factor was above 10 ( $\log_{10}(\text{BF}) \geq 1$ ). All intermediate values, considered by the BayeScan manual to be under 'substantial' selection, were treated as ambiguous. Matrices of pairwise genetic distances between individuals were computed separately for outlier and neutral loci using Dice's index of similarity as formulated in Nei & Li (1979). Then, the correlation of each of the genetic distances matrices with the first three axes of the PCA of phenotypic variables was assessed with the multiple regression-based method proposed by Nievergelt, Libiger & Schork (2007) with the web-based GAMOVA tool (available at <http://polymorphism.scripps.edu/~cabney/cgi-bin/mmr.cgi>). This method is particularly suited to identify and characterize the strength of relationships and genetic variations between individuals and variables collected on the same individuals (e.g. phenotypic traits, population-level features, environmental parameters; Herrera & Bazaga 2008). GAMOVA regressions were computed independently for SUN and SHADE values, to



determine whether phenotypic similarities between individuals in any of the canopy positions are dependent on genetic differences.

## Results

### ENVIRONMENTAL CONDITIONS AND LEAF PHENOTYPES IN THE FIELD

Plant size varied significantly among populations ( $F_{5,84} = 9.282$ ,  $P < 0.0001$ ), with two of them (AG and DES) having trees much bigger than the rest (Plant biovolume in  $\text{m}^3$ : GU  $435.7 \pm 131.6$ ; AG  $1150.0 \pm 99.2$ ; DES  $1866.3 \pm 396.4$ ; AF  $153.1 \pm 26.2$ ; SA  $281.0 \pm 184.6$ ). Solar radiation, the variable chosen to represent local environmental conditions, is shown in Fig. 1. Light availability was also significantly different among populations (total radiation:  $F_{5,174} = 5.358$ ,  $P < 0.001$ ; direct radiation:  $F_{5,174} = 15.055$ ,  $P < 0.0001$ ; diffuse radiation:  $F_{5,174} = 2.867$ ,  $P < 0.05$ ). Differences in solar radiation were not only quantitative but qualitative: two of the populations (GU and SA) had higher levels of diffuse radiation, while in all other populations, the direct component was predominant (Fig. 1). The first three axes of the PCA of phenotypic data had eigenvalues  $\geq 1$ , accounting for 68.45% of the variance, and were thus retained for further analyses. Mean values per population and canopy exposure for each of the phenotypic traits considered and for the first three axes of the PCA are included in Table S1 in Supporting Information. Plant size was found to significantly affect the overall phenotype, as defined by the three PCA axes, although the only individual trait significantly associated with plant size was the angle of sun leaves (Table 1). Interestingly, allometric effects on the phenotype appeared to be primarily because of the influence of plant size on sun phenotypes (Table 1). The comparison of phenotypic values across populations revealed that all the studied characters (except leaf angle) as well as the PCA were significantly differ-

ent across populations. Canopy plasticity, measured as the main effect of exposure in the linear model, appeared to be significant in every case. However, differences in plasticity between populations were only clearly significant for morphological traits: the interaction effect between population and exposure was not significant for three of the pigment traits and two of the PCA axes (Table 1). The comparisons of the components of the phenotypic analysis with local radiation conditions showed that sun values of the first two PCA axes were strongly associated with both diffuse (PC1  $F_{1,51} = 15.19$ ;  $P < 0.001$ , PC2  $F_{1,51} = 5.64$ ;  $P = 0.02$ ) and direct radiation (PC1  $F_{1,51} = 26.61$ ;  $P < 0.0001$ , PC2  $F_{1,51} = 79.53$ ;  $P < 0.0001$ ), while changes in shade phenotypes appeared to reflect changes mostly in diffuse radiation. This type of radiation was found to have a significant association with both PC1 and PC2 of SHADE values ( $F_{1,51} = 8.22$ ;  $P < 0.01$  and  $F_{1,51} = 5.37$ ;  $P = 0.02$  respectively), while direct radiation was only found to be significantly associated, albeit highly so, with PC2 for the same leaves ( $F_{1,51} = 40.34$ ;  $P < 0.0001$ ). No significant relationship was found between PC3 and either radiation type. The relationship between each radiation type and PC1 for both exposures is shown in Fig. 2. Light availability within the canopy appeared to be strongly influenced by the morphology of sun leaves. Leaf index (log transformed) exhibited a significant association with inner canopy radiation ( $F_{1,88} = 8.790$ ,  $P < 0.01$ ,  $R^2 = 0.09$ ; Fig. 3a), leaf angle and SLA had a marginally significant influence ( $F_{1,88} = 4.318$ ,  $P = 0.04$ ;  $F_{1,88} = 4.865$ ,  $P = 0.04$ , respectively), while leaf area of sun leaves was not found to be associated with the in-crown environment.

### CANOPY PHENOTYPIC VARIATION IN THE COMMON GARDEN

Plants grown in the common garden were several orders of magnitude smaller than those measured in the field. No

**Table 1.** Comparison of phenotypes across canopy exposures and populations. The effect of canopy position and population of origin was determined using split-plot mixed models with exposure and plant size as fixed-effects factors and population as a random factor. Errors were calculated at the plot level. The significance of differences among populations was calculated by comparing the relative fit of models with and without the population factor. Numbers indicate  $F$ -values except for 'Population', where they indicate the likelihood ratio (LR) of the models with (i.e. full) and without the factor

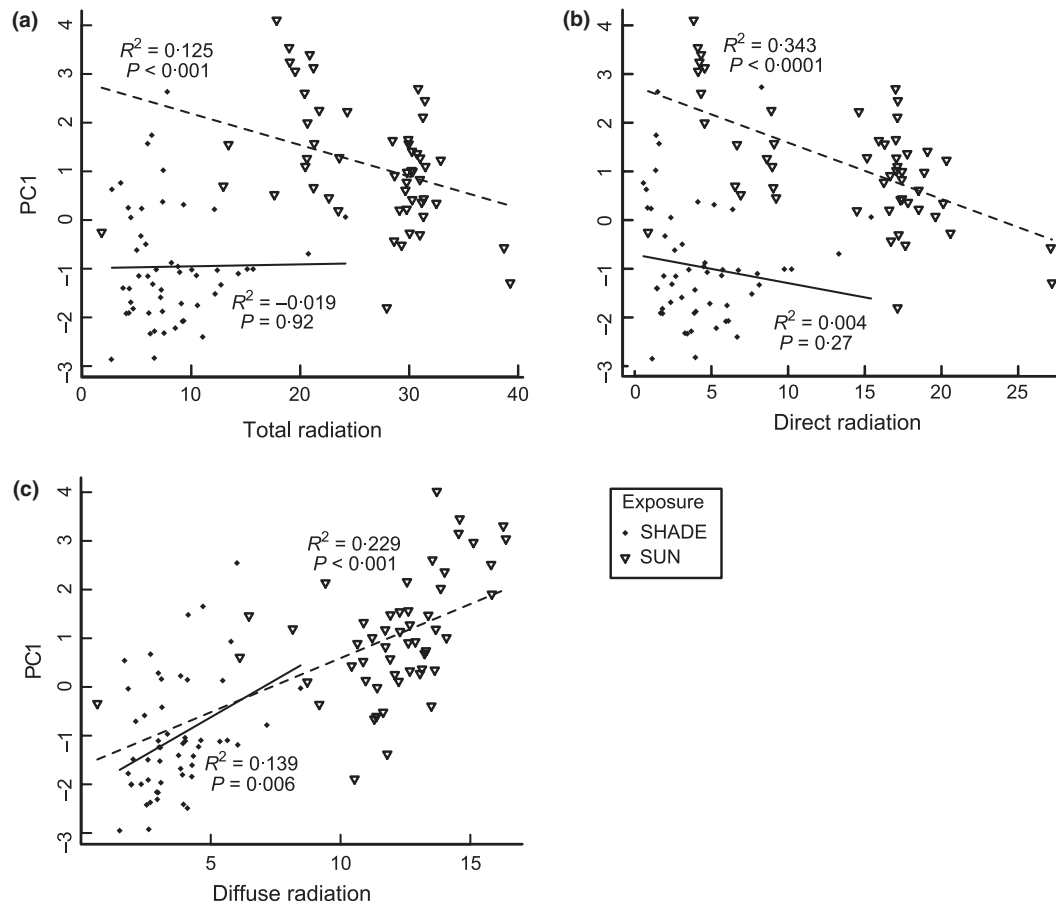
	Area <sup>†</sup>	SLA <sup>†</sup>	LI <sup>†</sup>	Ang <sup>†</sup>	Chla + b <sup>‡</sup>	Chla/b <sup>‡</sup>	Bcar <sup>‡</sup>	VAZ <sup>‡</sup>	PC1 <sup>‡</sup>	PC2 <sup>‡</sup>	PC3 <sup>‡</sup>
Plant size	1.98	5.00	0.02	0.00	2.17	0.26	0.41	2.69	15.34**	19.43***	14.02**
Exposure	60.88***	7.31*	602.11***	848.78***	11.47***	10.19**	39.75***	94.89***	215.66***	144.63***	25.53**
Population	350.85***	496.41***	603.97***	52.17***	40.61***	68.52***	47.05***	53.16***	95.69***	85.83***	54.79***
Pop. × Exp.	19.55***	88.04***	48.30***	9.53***	0.70	5.44**	1.03	1.30	1.02	3.883*	2.37
SUN											
Plant Size	0.00	0.12	0.00	4.84*	0.00	0.30	0.85	1.04	13.83***	29.78***	8.49*
Population	221.45***	238.09***	325.24***	22.95***	28.82***	63.31***	24.78***	35.28***	40.81***	65.04***	33.66***
SHADE											
Plant Size	3.39	1.32	0.02	0.06	3.95	0.10	0.03	1.72	3.61	0.01	5.95*
Population	137.08***	273.99***	264.79***	28.22***	17.20**	25.62***	19.19**	20.91**	32.07***	42.43***	30.64***

Population: d.f. = 5; Exposure: d.f. = 1; Pop × Exp: d.f. = 5.

<sup>†</sup> $n = 180$  Total, 90 Exposure specific.

<sup>‡</sup> $n = 108$  Total, 54 Exposure specific; SLA, specific leaf area.

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



**Fig. 2.** Relationship between the leaf phenotype and solar radiation ( $n = 54$ ). The graphs represent linear regression of SUN (open triangles) and SHADE (closed circles) phenotypes against total (a), direct (b) and diffuse (c) radiation ( $\text{mol photons m}^{-2} \text{ day}^{-1}$ ). Phenotypes are represented by the first axis of the principal PCA. Radiation was measured using hemispheric photographs.

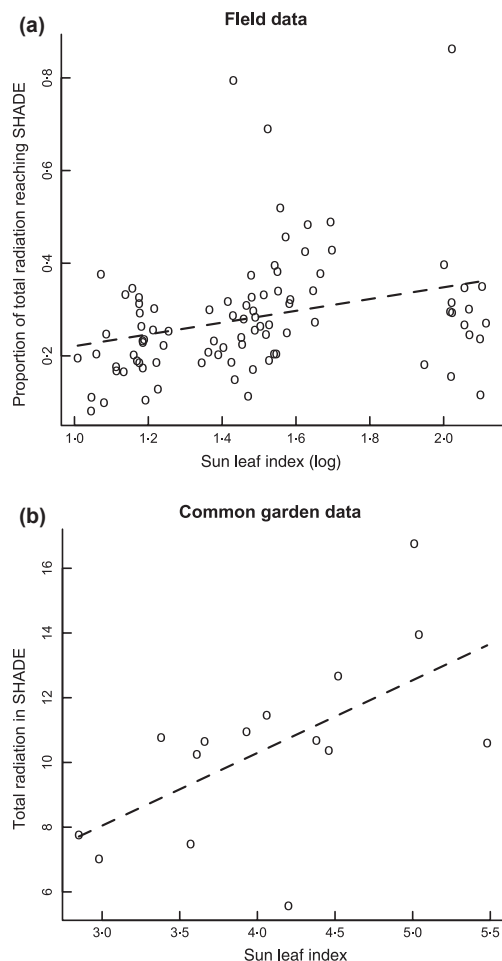
population had a mean biovolume  $> 1 \text{ m}^3$  in the common garden (Table S2 in Supporting Information). Still, canopies were large enough to have differentiated sun and shade phenotypes. Results of the permutation ANOVA of common garden measurements are shown in Table 2. Three morphological characters (SLA, leaf area and angle) differed significantly between exposures, while three characters (SLA, leaf index and VAZ) differed significantly between populations. In two of these (SLA and leaf index), those differences appeared to be the result of differences between shade leaves, as sun leaves were not significantly different between populations in any case. The first three axes resulting from the PCA conducted to summarize the multidimensional phenotypes had eigenvalues  $> 1$  and accounted for 74.46% of the total variation (Table 2). The first two components differed significantly between canopy exposures, and the second one also among populations. These population differences appeared to be as a result of variability in inner canopy leaves, as both PC1 and PC2 of shade leaves differed among populations, while no significant differences were found in sun PCA values among populations (Table 2). Differences in plasticity between populations, measured by the interaction factor of the linear model, were only found to be significant for total chlorophyll content and for none of the PCA axes (Table 2).

Plant size had a significant effect on three characters (leaf area and index and VAZ) and the second and third PCA axes. These allometric effects relied on differences between shade leaves in all cases except leaf area (Table 2).

The influence of the shape of outer canopy leaves, as defined by the leaf index, on the intracanalopy light environment was also found to be significant in the common garden ( $F_{1,13} = 7.643$ ,  $P = 0.02$ ,  $R^2 = 0.37$ ; Fig. 3b). None of the other three morphological traits of outer canopy leaves considered (leaf angle and area and SLA) was found to have a significant relationship with the light levels of the inner canopy.

#### POPULATION GENETIC DIFFERENTIATION AND VARIATION IN LEAF PHENOTYPES

A total of 285 AFLP fragments (loci) were used to characterize genetic differences among populations, 23 of which appeared to be under substantial selection and six exhibited signs of strong selection (Fig. 4). Genetic differences between populations were significant according to AMOVA in all three cases (non-selected loci, loci exhibiting a substantial selective signal and loci under strong selection, Table S3 in Supporting Information). Population-level analyses of genetic diversity (Table S3) revealed that the population with the highest



**Fig. 3.** Influence of leaf shape, [measured as the leaf index (leaf length/leaf width)], of leaves from the outer canopy (SUN) on the solar radiation in the inner canopy. (a) Relationship between the leaf index of sun leaves (log transformed) and the proportion of the radiation received in the outer canopy that reaches the inner canopy in field-measured trees.  $N = 90$ ; (b) Association between shade radiation levels and leaf index of exposed leaves in the common garden plants ( $n = 15$ ). Dashed lines indicate least squares linear regression fits. See text for details.

genetic differentiation (as measured by the population-specific  $F_{ST}$ ) was SA, both in neutral and selective divergence patterns. Inbreeding estimates were highest in the island population (GU). GAMOVA results for the association between genetic and phenotypic differentiation can be found in Table 3. According to these analyses, phenotypic values were clearly influenced by genetic variation, although no clear connection with genetic markers potentially under selection was observed. A significant association at the individual plant level was observed between phenotypic values (estimated with PCA values) for both SUN and SHADE and Nei's genetic distances computed with genomic markers that were not under strong selection.

## Discussion

Inner and outer canopy layers of wild olive trees differ in their leaf phenotypes. This phenotypic variation correlates with

neutral genetic divergence and light availability at the population level. Results from the field and the common garden experiments presented here indicate that phenotypic changes in outer canopy layers are mostly driven by external environmental conditions and that the emerging sun phenotypes modify the environment experienced inside the canopy. The phenotype of leaves of the inner canopy was found to be strongly influenced by diffuse radiation and to reflect genetic differences among populations to a bigger extent than that of more exposed leaves. Conversely, the leaf phenotype of exposed canopy layers of adult trees appeared to be more dependent on allometric variation.

## CANOPY PLASTICITY, PLANT SIZE AND LIGHT AVAILABILITY

Differentiation between inner and outer canopy layers was significant for every trait (Table 1). The leaves in the two exposures appeared to reflect plant size and environmental conditions differently. The phenotype of inner canopy leaves (summarized with a PCA) had a strong association with diffuse radiation (Fig. 2). Radiation inside the canopy depends on local radiation conditions external to the tree, but our results showed that it is also a direct outcome of the phenotype expressed by sun leaves, namely of their shape (Fig. 3). Total solar radiation inside the canopy increased as *O. europaea* leaves became more elongated (i.e. larger leaf index). Incoming light is thus dependent on the flux deflected by more external canopy structures, which in the case of *O. europaea* have leaf surfaces that maximize the diffuse component inside the canopy (Combes *et al.* 2007). This is in agreement with theoretical models (Cai *et al.* 2009; although see Brodersen *et al.* 2008). Distribution of direct sunlight inside canopies varies stochastically in space and time, while diffuse radiation is more homogeneous (Cai *et al.* 2009). Consequently, phenotypic changes in the outer canopy can potentially create a more homogeneous radiation environment within the canopy. In fact, sun leaves measured in the field appeared to change their phenotype allometrically, while no significant changes associated with plant size were observed in shade leaves. This is congruent with a modular behaviour where the phenotype of the uppermost canopy layers changes progressively, while the inner canopy environment remains stable. Some caution is necessary in the interpretation of the results, though. Only a few characters have been analysed and the PCA left over 30% of the phenotypic variation unexplained. Therefore, our phenotypic estimates are inevitably limited and there might well be variation that has not been accounted for. However, it is clear that significant differences in response to light and plant size were found between sun and shade canopy exposures and that these differences appear to be concomitant to changes in the phenotype of sun leaves.

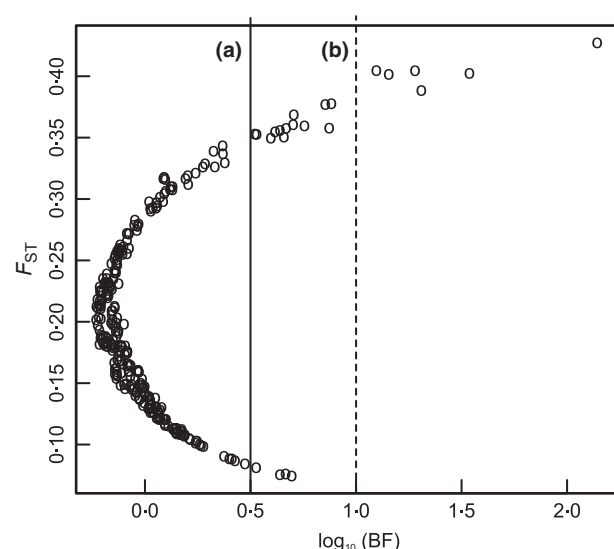
Other authors have described that modules in the outer canopy differ more drastically than those in the inner canopy (Ishihara & Kikuzawa 2009). This phenotypic differentiation between canopy modules is a phenomenon intrinsic to can-

**Table 2.** Phenotypic differences between populations and exposures in the common garden. The data are the results of permutation ANOVA on pigment and morphological traits and on the three first axes of a PCA of phenotypic values identical to the one performed on field data (values in brackets represent % variation explained by each axis) measured on plants from four populations grown in a common garden. The first value in every cell is the corresponding MS and the second the number of iterations used

	Area	SLA	LI	Ang	Chla + b	Chla/b	Bcar	VAZ	PC1 (31·15)	PC2 (23·14)	PC3 (20·17)
Plant size	59 250**	0·1	3·0**	633·3	2377·0	0·03	0·36	0·01***	5·22	5·327*	17·450***
	5000	51	5000	1295	1883	51	424	5000	1621	4294	5000
Exposure	30 334*	35·0**	2·0*	3779·3***	933·0	0·26	1·03	$2 \times 10^{-2}$	7·592*	23·510***	0·39
	3438	5000	5000	5000	51	472	1312	575	5000	5000	96
Population	9361	14·7**	1·0*	97·6	368·2	0·22	0·58	$2 \times 10^{-2}$ *	4·24	2·8429*	1·68
	1692	5000	5000	228	320	709	1681	4291	5000	5000	988
Pop. × Exp.	2365	4·2	0·1	90·4	2696·8**	0·17	0·83	$6 \times 10^{-3}$	1·78	1·89	2·7
	305	2282	88	364	5000	772	1191	491	1201	2513	5000
Residuals	5667	2·4	0·3	193·7	509·00	0·75	0·57	$7 \times 10^{-3}$	1·54	0·9	1·02
SUN											
Plant Size	15 197·1	2·0	1·5	1752·2*	244·6	0·14	0·73	$5 \times 10^{-2}$	7·48	1·08	6·03
	1282	181	969	5000	142	217	96	1562	531	149	1090
Population	1917	1·6	0·7	26·1	11 404·3	0·25	1·16	$3 \times 10^{-2}$	4·4	0·63	2·12
	136	525	622	51	1222	672	1115	1238	729	110	436
Residuals	2969·1	1·1	0·6	206·9	477·4	0·3	1·16	0	2·69	1·13	1·42
SHADE											
Plant Size	48 824*	0·9	1·6**	39·3	2841·9*	0·02	$1·7 \times 10^{-5}$	$7 \times 10^{-2}$ **	0·25	4·951*	11·922**
	3049	51	5000	85	2723	103	53	5000	136	3852	5000
Population	11 232	18·2*	0·4*	213·9	973·5	0·1	$2·9 \times 10^{-4}$	$3 \times 10^{-3}$	1·407*	4·323*	1·54
	704	5000	5000	1544	1697	2068	557	565	4251	5000	889
Residuals	8454	3·7	0·1	84	520·6	0·05	$1·5 \times 10^{-4}$	$4 \times 10^{-4}$	0·29	0·69	0·66

Full data set analysis  $n = 30$ ; by exposure analyses  $n = 15$ . Plant size and exposure d.f. = 1, Population d.f. = 3, Residuals d.f. = 21/10; SLA, specific leaf area.

\* $P < 0·05$ ; \*\* $P < 0·01$ ; \*\*\* $P < 0·001$



**Fig. 4.** Results of the genomic selection analysis. Plot of  $F_{ST}$  values for each individual band (loci) against the  $\log_{10}$  of the Bayes factor, which represents the ratio of the posterior probability of the selected and neutral models. For each locus, neutral differentiation can be assumed if  $\log_{10}(\text{BF}) \leq 0·5$ , denoted by the dotted line (a). Loci with a  $\log_{10}(\text{BF}) \geq 1$  are considered to be under selection (solid line); (b). The differentiation pattern for loci between (a) and (b) is ambiguous.

opy development and is likely to depend on canopy size (Sack *et al.* 2006). Indeed, we recorded less pronounced differences between outer and inner canopy and a higher

dependence of traits and PCA components on plant size in the common garden where plants were much smaller. Moreover, allometric effects in the common garden appeared to be more significant in shade leaves. This was not unexpected, as modularity and changes in leaf phenotypes vary as canopy size increases over the lifetime of a tree (Niklas & Cobb 2010).

The simultaneous change of sun and shade leaves might generate phenotypes able to exploit resources complementarily, generating for instance an inner canopy environment of high diffuse radiation and the phenotype best adapted to capture it. Such a scenario is congruent with models of foraging behaviour described for clonal plants (De Kroon *et al.* 2009); in this case, the resources used by the different modules are the different types of radiation. Previous works have established the role in differential radiation capture and photosynthetic performance derived from the segregation of several phenotypes in tree canopies (Rubio De Casas *et al.* 2007; Niinemets 2010). The emergence of leaf phenotypes of high SLA, and therefore a comparatively low number of palisade layers, is congruent with optimization theories that predict a lower amount of resources to be allocated to leaves in the inner canopy (Hirose & Werger 1987). A reduction in the number of palisade layers might reduce the capacity of efficiently capturing direct light while not affecting the use of diffuse radiation (Vogelmann & Martin 1993). In the case of *O. europaea*, the adaptation of shade leaves to harvest diffuse radiation can prove particularly beneficial. In Mediterranean



**Table 3.** Results of the generalized analysis of molecular variance (GAMOVA) investigating relationships between genetic distances and phenotypic similarities between individuals. Results represent  $F$ -ratios for the multiple regressions of genetic distances (estimated using Nei's index) and the axes of the PCA of phenotypic values. Independent GAMOVA was run for each of the three data sets derived from the BayeScan (Foll & Gaggiotti 2008b) analysis of AFLP data. Loci are classified according to the deviation of their respective  $F_{ST}$  values from neutrality: loci that follow a neutral pattern of differentiation (Neutral), loci that exhibit a strong selective signal according to BayeScan (under selection) and loci in an intermediate situation (ambiguous)

	Neutral loci ( $n = 265$ )	Ambiguous ( $n = 23$ )	Under selection ( $n = 6$ )
PC1 SUN	6.577***	16.947***	0.65
PC2 SUN	6.545***	15.182***	0.251
PC3 SUN	1.856*	3.636*	-0.389
PC1 SHADE	6.276***	13.229***	1.705
PC2 SHADE	3.932**	9.595**	-0.490
PC3 SHADE	3.216**	9.643**	0.6445

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

environments, sunlight is rarely a limiting factor and intense direct radiation can even be damaging because of low temperatures or water stress (Werner, Correia & Beyschlag 1999; Martínez-Ferri *et al.* 2004). Sofo *et al.* (2009) proved that photosynthetic performance of *O. europaea* increases under shade conditions during water stress. Moreover, the intracanalopy environment is not only more dimly illuminated than the outer crown, it is also more humid and more thermally stable (Gratani, Covone & Larcher 2006). The diverging phenotypes of modules in different positions of the canopy might thus maximize overall performance, with sun leaves most active when conditions for light capture are optimal (i.e. during spring, and early morning and late afternoon in summer without drought (Díaz-Espejo, Nicolas & Fernandez 2007) and shade leaves ensuring a stable photosynthetic performance throughout the year.

#### PHENOTYPIC DIFFERENTIATION BETWEEN POPULATIONS

All characters and PCA axes differed significantly between populations. In particular, the population of the Canary Islands (GU) exhibited remarkable differences compared with the rest. It had bigger, more elongated leaves with steeper angles in SHADE than any other population. Wild olive populations of the Canary Islands have been purported to form a differentiated taxon, *O. europaea* subsp. *guanchica*, and some of the differences observed (i.e. greater leaf elongation) are congruent with systematic descriptions of this taxon (Green 2002). However, some characters could be the outcome of acclimation to the particular environmental conditions of the Macaronesian cloud forests. For instance, the SLA of GU was relatively high, similar to that of shade leaves of other populations (Table S1). This could be a result of the light environment of the cloud forest where the

radiation is predominantly diffuse. A similar pattern was found in the leaves of SA, the Iberian population with the highest ambient humidity (Rubio De Casas *et al.* 2002), so it is well possible that this is an adaptation to environments of high relative humidity and a concomitant lack of directionality in solar radiation, as has been proposed elsewhere (García-Verdugo *et al.* 2010). Unfortunately, although plants of SA maintained higher SLA values when grown in the common garden, we cannot confirm their convergence with Canarian individuals because all the GU plants planted were killed by winter frosts before any measurements could be taken. Another trait that seemed to indicate a convergence of these two populations was the ratio of the light-harvesting apparatus (Chl a/b). In both populations, the proportion of chlorophyll a relative to b was very high. This might well be a common response in habitats where diffuse radiation is prevalent. The high proportion of energy-dissipating pigments could be a mechanism to cope with temporal heterogeneity in the light environment of cloudy sites as has been described for tropical cloud forests (Krause *et al.* 2003).

Differences between exposures indicate an important role for canopy plasticity in the phenotypic divergence between sun and shade leaves. However, differences in plasticity among populations, as measured by the interaction term of the linear models, were not always significant. They appeared to be so for morphological traits, but only for one of the pigment traits and the second PCA axis (23.14% of the total variation). These results could indicate that the phenotypic divergence of modules is an integrated canopy-level response (Sprugel 2002; Kazuhiko 2007) in which a new phenotype in one exposure entails a modification of the phenotype in other canopy positions such that the multiple independent modular changes result in functionally equivalent solutions at the canopy level (Kennedy 2010). Common garden results were compatible with this hypothesis. In a controlled environment, the different genotypes appeared to converge towards a common SUN leaf phenotype. Conversely, shade leaves exhibited significant differences among populations in two of the three components used to summarize the phenotype. We cannot establish unambiguously at this point to what extent the fact that this modular response (i.e. sun convergence and shade differentiation across populations in the common garden) is because of a 'purposeful behavior' (*sensu* Trewavas 2009) aimed at optimizing foraging or to the manifestation of standing genetic variation under the less selective environment of the inner canopy. These explanations are not mutually exclusive, and further research is needed to clarify the relative role of each in the phenotypic differentiation of canopy modules.

#### GENETIC DIFFERENTIATION AND CANOPY PLASTICITY

Population genetic analyses found significant differences in the three genetic data sets considered (Table S3): neutrally diverging loci, loci under substantial selection and loci under strong selection. These results seemed to prove that wild olive

tree populations have undergone genetic differentiation because of both neutral processes and natural selection. However, some caution is needed in the interpretation of the selection estimates. Thresholds of selection in BayeScan (Foll, Beaumont & Gaggiotti 2008a) only reflect deviations from a probability distribution that is assumed to reflect neutral divergence (Fig. 4). This approach tends to overestimate the number of loci under selection (type I errors, Bonin, Ehrich & Manel 2007; Caballero, Quesada & Rolan-Alvarez 2008). In the light of the biogeographic history of *O. europaea*, the degree of differentiation of some populations is likely to have been influenced by other causes (Rubio De Casas *et al.* 2006; Besnard, Rubio De Casas & Vargas 2009). Isolation by distance, for instance, is likely to bias the results of genetic differentiation of the population from the Canary Islands when comparing it with the rest (García-Verdugo *et al.* 2009b). The delimitation of non-selected loci and loci under strong selection is fairly conservative, but loci considered by BayeScan to be under 'substantial' selection are in an uncertain situation and it is likely that their frequency deviated from neutrality because of forces other than selection (Foll, Beaumont & Gaggiotti 2008a).

Phenotypic differences in the individual phenotypic sun and shade values correlated strongly with genetic distances between individuals (Table 3), suggesting that genetic differentiation entails phenotypic differentiation in the leaves of *O. europaea*. The effect of the local environment on external leaves was confirmed by common garden experiments. Plants grown under the same conditions expressed a convergent phenotype in the outer canopy, but inner canopy leaves remained different between populations (Table 2). As mentioned previously, some of the differences could result from adaptation to particular environments because differences in photosynthetic conditions are expected to have some evolutionary impact (Givnish, Montgomery & Goldstein 2004). However, no correlation was found between phenotypic values and genomic selection, and other conclusive evidence for the role of selective pressures in the conformation of leaf phenotypes is lacking.

## Conclusions

Leaf phenotypes in outer layers and inner layers vary differentially with environmental conditions, allometry and genetic differentiation. The modular phenotype shifts could be part of an integrated response at the level of the canopy to maximize resource use, although further research is needed to clarify the evolutionary and ecological role of canopy plasticity.

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## Supporting Information

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

**Table S1.** Phenotypic values of field populations.

**Table S2.** Phenotypic values in the common garden.

**Table S3.** Results of the analyses of overall molecular variance (AMOVA) and population-specific estimates of mean genetic inbreeding ( $F_{IS}$ ) and differentiation ( $F_{ST}$ ).

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