Leaf morphology of progenies in *Q. suber*, *Q. ilex*, and their hybrids using multivariate and geometric morphometric analysis

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The genus *Quercus* is known for the occurrence of frequent hybridization events between species. Although this phenomenon is not common among holm oak (*Q. ilex*) and cork oak (*Q. suber*), these species can hybridize when they coexist in mixed stands. The result of hybridization is a viable hybrid progeny with very heterogeneous leaf morphology. Literature concerning the leaf morphology of *suber-ilex* hybrid seedlings is scarce, and non-existent from a quantitative point of view. In the case of the leaf morphology of hybrids and their progeny, it has been observed a high frequency of leaves with fluctuating asymmetry or developmental abnormalities, which can have a marked effect on fitness. In this work, we have characterized seedlings' leaf morphology corresponding to two- and four-year-old half-sib progenies of holm oak, cork oak and their hybrids. For this purpose, three to ten leaves of each individual were collected, and two methodologies were used for analysis. Firstly, we used a classic morphological analysis of twelve variables that were reduced using multivariate techniques. On the other hand, shape of the leaves was thoroughly analyzed by geometric morphometric analysis methods. The extent of fluctuating asymmetry and the presence of developmental abnormalities of seedlings were analyzed calculating an asymmetry index. The results indicate that thickness is the most discriminating trait between species, and that the hybrid progenies do not show a third different phenotype compared to the parental species. However, half-siblings tend to show similar leaf morphology between them, depending on the genetic adscription of the parents. While fluctuating asymmetry was found in half-sib progenies of the parental species and the hybrids, a significant proportion of hybrid half-sibs showed strong leaf asymmetry, probably due to modifications of the epigenetic systems that control leaf development at the shoot apical meristems and leaf primordia.

Keywords: Hybridization, Fluctuating Asymmetry, Leaf Morphology, Procrustes Analysis

Introduction

Quercus suber and *Q. ilex* are two Mediterranean oak species that are included into different subgeneric groups (Ilex and Cerris), and can be easily distinguished according to phenotypic traits of bark, cupules of the acorns, or leaves (Amaral Franco 1990). However, when both species occur in sympatric populations in the siliceous or decarbonated areas from the Western Mediterranean, they hybridize, and individuals with intermediate or mixed phenotypes can be found in natural populations (Laguna 1881).

These hybrids are able to produce viable acorns when pollinated by *Q. suber*, or by *Q. ilex* (López De Heredia et al. 2017). The seedling stage is crucial for plant population dynamics due to its higher vulnerability to environmental constraints in comparison with seed and adult stages (Harper 1977). Provided the low hybrid frequency found at the adult stage in natural populations (Burgarella et al. 2009), the question arises whether hybrid seedlings have different phenotypes to those of the parental species; and, if so, whether those different phenotypes may involve a more reduced fitness of hybrid seedlings.

The detection of hybrids in the field could be masked by the way the phenotypic characters segregate in the the first generation of offspring (F1), and subsequent generations. The phenotypic analysis of F1 hybrids in 46 plant taxa showed 44.6 % of intermediate characters, while 45.2 % of the characters in hybrids were similar to either parental species, and 10.2 % presented transgressive characters (Rieseberg & Ellstrand 1993). For the *Q. ilex - Q. suber* complex, a recent study has approached the molecular characterization of seedlings of *Q. ilex*, *Q. suber*, and *Q. ilex × suber* progenies grown in nursery conditions in three different years (López De Heredia et al.

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Tab. 1 - Number of analyzed plants by cohort, species and family.

Species	Family	Cohort 2012	Cohort 2014
Hybrids	LG1	21	38
	LG2	-	5
	IS	59	77
	ZLR	19	14
	Total	*99*	*134*
Q. ilex	I1	19	5
	I2	9	4
	I3	-	4
	I4	-	3
	Total	*28*	*16*
Q. suber	S1	8	2
	S2	11	4
	S3	-	4
	S4	-	4
	Total	*19*	*14*

2017). The quantitative analysis of phenotypes on these progenies, specifically of leaf morphological traits, may shed light on the way these characters segregate in relation to the specific category of the seedlings previously estimated.

Leaf morphology plays an important role in species adaptations related to photosynthetic ability of plants: heat dissipation, light interception and hydraulic resistance, among others, and is crucial in early stages of seedling growth (Valladares & Niinemets 2008). The relationship of leaf morphological traits and the specific categories of hybrid oaks determined with molecular markers has been widely studied in natural populations (Kremer et al. 2002). Leaf traits are extremely variable between and within populations, and even among branches within a tree (Bruschi et al. 2003). Moreover, significant ontogenetic (Kleinschmit et al. 1995) and seasonal (Blue & Jensen 1988) variation have also been reported. Therefore, in order to reduce bias and obtain sound results, leaf morphological approaches should be performed in controlled conditions on known pedigree individuals, or on controlled crosses or field trials (Gugerli et al. 2007). However, to our knowledge, the analysis of leaf morphology of hybrid seedlings of different age has not been addressed in the *Q. ilex - Q. suber* complex.

One of the undesired effects of hybridization on leaf morphology is the yielding of asymmetries in the lamina that may have an effect on seedling stability and fitness when they are of great magnitude (Parsons 1990). According to the intensity and causes of leaf distortions, two types of asymmetries have been described for oak leaves: (i) fluctuating asymmetry that consists of small random variations in the right and left sides of a bilateral trait that deviate from perfect symmetry (Graham et al. 2010), which is usually related to environmental variation in oaks (Hódar 2002, Puerta-Piñero et al. 2008, Viscosi 2015), but also

to genotypic variation (Albarrán-Lara et al. 2010); and (ii) strong asymmetries produced by cytonuclear inconsistence or (epi)genetic stress during leaf blade differentiation (Lodha et al. 2013). Both types of asymmetry may increase with hybridization and introgression (Wilsey et al. 1998, Albarrán-Lara et al. 2010).

Therefore, the specific aims of present study were: (i) to quantify developmental and fluctuating asymmetry in hybrid progenies; (ii) to characterize the leaf morphology of two and four year-old progenies from open pollinated *Q. ilex × Q. suber* mother trees; (iii) to identify the factors (cohort, species, family, genotype) accounting for variation in leaf morphological traits; and (iv) to inspect the correlation of morphological variables and previously inferred specific assignation of seedlings.

Material and methods

Experimental design

The sampling site is a mixed *dehesa*, *i.e.*, a highly anthropised open woodland, located in Central Spain (39° 59′ N, 5° 7′ W). Four mature hybrids were identified according to their phenotypes. The hybrids occur in three areas of contrasting density and specific composition: (1) "La Isla" (Is), where *Q. ilex* and *Q. suber* mix at a density of 38 trees ha^{-1}, being *Q. suber* slightly more abundant; (2) "La Laguna" (LG), a low density area (20 trees ha^{-1}) dominated by *Q. ilex*, where two hybrids co-occur; and (3) ZL, which is a denser area of 123.5 trees ha^{-1} (38.3 trees ha^{-1} for *Q. ilex* and 85.2 trees ha^{-1} for *Q. suber*).

To obtain open pollinated, half-sib progenies, acorns were collected from the canopy of the four hybrids, in November 2012, and 2014. In addition, acorns were also collected from two *Q. ilex*, and four *Q. suber* individuals in 2012, and from two *Q. ilex* and two *Q. suber* individuals in 2014, respectively. The sampling scheme is detailed in Tab. 1. The full sampling scheme and the procedure to obtain half-sib families of seedlings is detailed in López De Heredia et al. (2017).

In January 2016, three to ten (mean = 5.4; s.d. = 2.86) fully-developed leaves were collected from the surviving 2012 (1361 leaves) and 2014 (1631 leaves) seedlings. Leaves were collected in alternate position along the entire stem. Leaves were dried in a plant press and scanned for further analysis, always visualizing the abaxial side of the leaf. The specific category of each seedling was assessed by the value of the q_s parameter obtained with the Bayesian procedure implemented in the software Structure (Pritchard et al. 2010) for eight nuclear microsatellite genetic data as implemented in Burgarella et al. (2009) (López De Heredia et al. 2017).

Fluctuating and developmental leaf asymmetry

Fluctuating and developmental leaf asym-

metry were investigated using semi-automated measures of the leaf surface in the right and left sides that were performed with the software ImageJ (Abramoff et al. 2004). Further, an asymmetry index was computed for each leaf as follows (eqn. 1):

$$LSAI = \frac{|LS - RS|}{LS + RS} \tag{1}$$

where *LS* is the surface of the left side of the leaf lamina and *RS* is the surface of the right side of the leaf lamina. For *LSAI* < 0.05, leaves are symmetrical; for 0.05 < *LSAI* < 0.2, leaves show fluctuating asymmetry; and for *LSAI* > 0.2, leaves present strong asymmetries related to developmental anomalies.

The frequency of plants showing leaf fluctuating asymmetry was quantified by a fluctuating seedling asymmetry index defined as (eqn. 2):

$$FSAI_i = \frac{N'_i}{N_i} \tag{2}$$

where N'_i is the number of leaves of seedling *i* with a *LSAI* value ranging between 0.05 and 0.2, and N_i is the measured number of leaves of seedling *i*.

The frequency of plants showing strong asymmetries related to development was quantified by a developmental seedling asymmetry index defined as (eqn. 3):

$$DSAI_i = \frac{N''_i}{N_i} \tag{3}$$

where N''_i is the number of leaves of seedling *i* with a *LSAI* value higher than 0.2, and N_i is the measured number of leaves of seedling *i*.

Leaf morphological multivariate analysis

Twelve leaf morphological traits were measured for multivariate analysis. Dried leaves were weighed with a precision balance to estimate dry weight (DW). The thickness of the leaf lamina (T) was estimated as the mean value of four measurements at different points of the lamina. The number of teeth (NT) was manually counted and log-transformed to achieve normality. The remaining nine leaf traits were automatically obtained from scanned leaves with the image analysis system Win-Folia™ (Regent Instruments Inc., Canada), and were the following (Fig. 1a): leaf lamina perimeter (P); leaf lamina surface (S); leaf total length (L); leaf width at 50 % of leaf total length (W); leaf lamina length (LL); leaf lamina maximum width (WM); petiole length (PL); ratio between the leaf lamina maximum width and the leaf lamina length (WM/LL); a shape coefficient (SH) estimated as $4\prod S/P^2$. Measures on leaves that showed strong asymmetries, *i.e.*, leaves with LSAI > 0.2 were discarded from further analyses.

Median, mean, maximum/minimum val-

ues and standard deviation were scored for each variable at family, species and co-hort levels, respectively. A principal component analysis (PCA) was performed by scaling the original variables with the "prin-comp" command from the R 3.3.1 "stats" package (R Core Team 2016). The two principal components (PCs) explaining most of the total variance were selected. New variables were created from the scores of the components, thus simplifying further analysis. The mean scores by individual of the first two principal components were bi-plotted to graphically inspect differences between species, and one-way ANOVAs were performed for this factor. *Post-hoc* comparisons were performed in order to determine the differences between levels of the specific factor. Tukey's HSD (Honestly Significant Difference) test was performed with the "HSD.tsts" function from the "agricolae" library (Mendiburu & Simon 2015) of R package.

Leaf morphometric geometric analysis

Landmark-based morphometric geometric analysis provides a deeper insight on leaf shape variation than the analysis of the shape coefficient ($4\prod S/P^2$) and the ratio between the leaf lamina maximum width and the leaf lamina length (WM/LL) of the standard morphological multivariate analysis (Bonhomme et al. 2014). The geometric morphometric analysis was conducted using the software LeafAnalyser (Weight et al. 2008) upon the scanned leaves. To obtain 2D landmarks as X-Y coordinates it is necessary to define two reference points in the leaf (Fig. 1b): (i) a centroid that was automatically computed by the software using the mean of all the X-Y coordinates of all pixels within the leaf margin; and (ii) the leaf apex, so the leaf is oriented. Forty-nine additional equidistant points were defined along the leaf margin, for which their respective X-Y coordinates were scored.

Once the X-Y coordinates were scored, we used the "gpagen" function from the "geomorph" library (Adams & Otárola-Castillo 2013) in R 3.3.1 to perform Procrustes superimposition (Rohlf & Slice 1990). Generalized Procrustes Analysis translates all landmark configurations to a common location, re-scaling all to unit centroid size, and rotating all into an optimal least-squares alignment with an iteratively estimated mean reference shape. These transformations minimize the differences in location between compared landmark data.

A Principal Component Analysis was performed with the "plotTangentSpace" function and the first two principal components were bi-plotted to visualize differences between groups. The "morphol.disparity" function was used to estimate the morphological disparity between cohort, species and families, respectively, as the Procrustes distance, and the significance of differences among groups was estimated through a p-value.
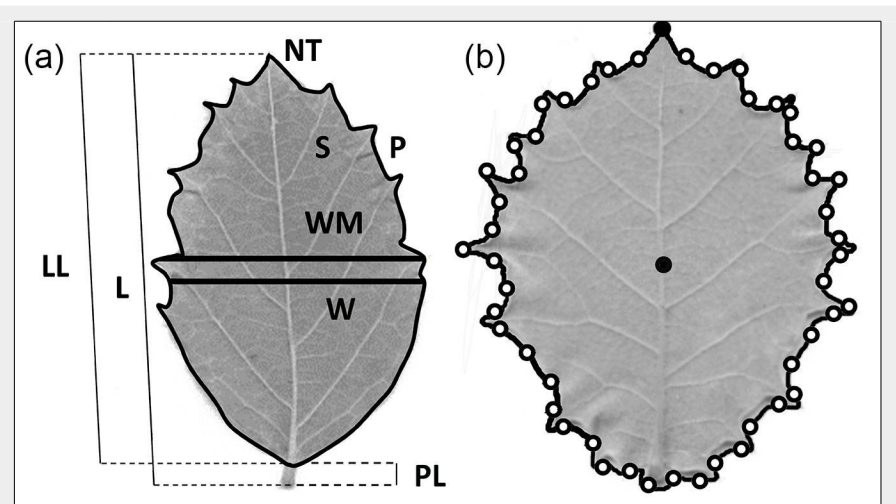

Fig. 1 - (a) Leaf variables measured for multivariate analysis with the image analysis software WinFolia™. (b) Schematic position of leaf apex and centroid (full black dots), and landmarks (empty black dots) for morphometric geometric analysis of shape determined with the software LeafAnalyser.

Mixed effects linear models and variance components estimations

Based upon the new variables (*i.e.*, principal components scores) that mostly contributed to the overall variation for both morphological and morphometric geometric analysis, mixed effects linear model considering the cohort as a fixed effect and species (*Q. suber, Q. ilex* and hybrids), family and plant as nested factors as random effects were built with the "lme" function of the "nlme" library (Pinheiro et al. 2017) of R package. The model was the following (eqn. 4):

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_{k(j)} + \delta_{l(k(j))} + \varepsilon_{ijklm} \quad (4)$$

where μ is the mean of the principal component, α_i is the effect of cohort i, β_j is the effect of species j, $\gamma_{k(j)}$ is the effect of the family k within species j, $\delta_{l(k(j))}$ is the effect of the plant l within family k within species j, and ε_{ijklm} is the terms the error (E).

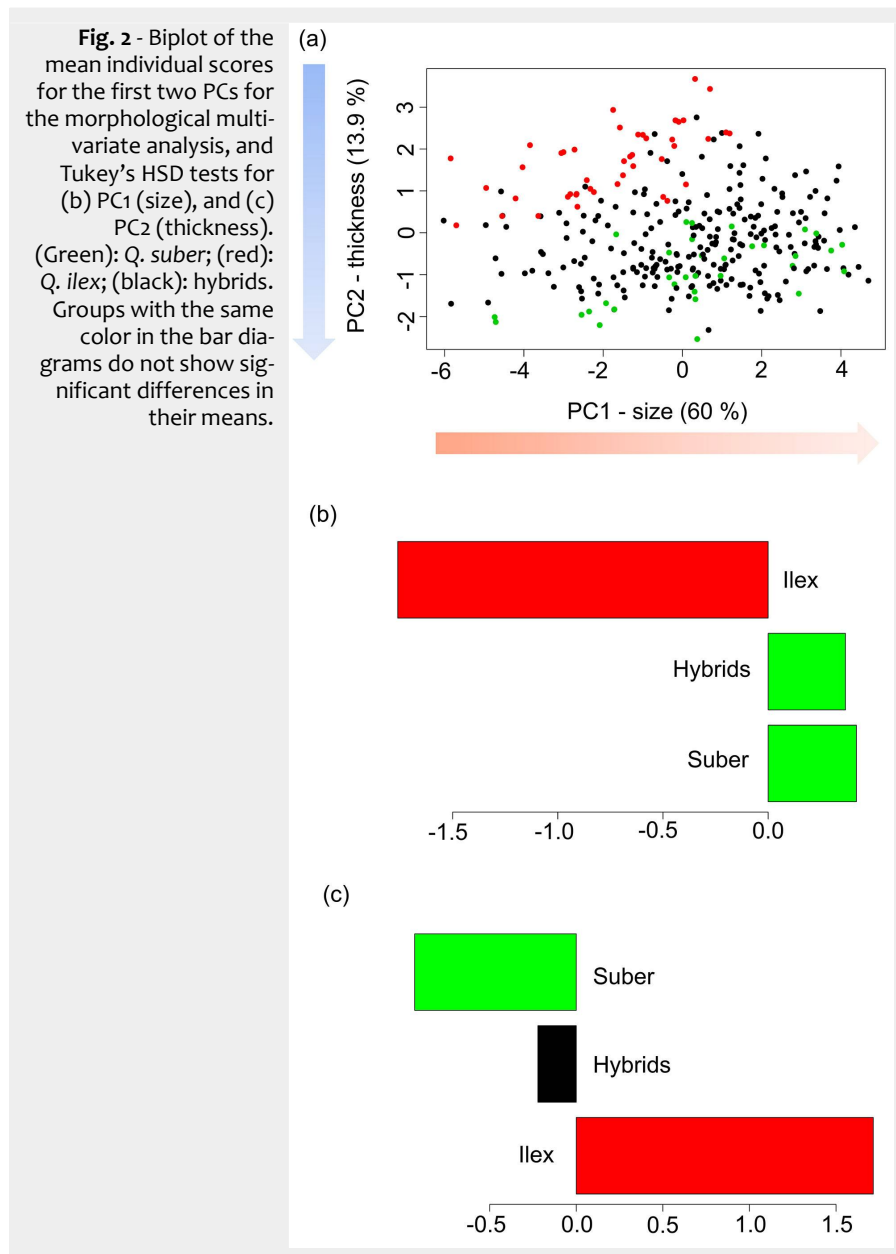All the random factors showed a normal distribution as follows: β_j: N(0; σ_S); $\gamma_{k(j)}$: N(0;

$\sigma_{S(F)}$); $\delta_{l(k(j))}$: N(0; $\sigma_{S(F(P))}$); and ε_{ijklm}: N(0; σ_E). The "varcomp" command was used to obtain estimators of the random terms of the model $\sigma_{(S)}$, $\sigma_{S(F)}$, $\sigma_{S(F(P))}$, and σ_E for PC1 (size), PC2 (thickness) and PC1.shape (shape) using the restricted maximum likelihood method to identify the factors that contribute significantly to variation in the principal components of the model above.

Correlation of leaf morphology with genetic specific category parameters

Correlations between the mean principal component scores obtained for morphological and morphometric geometric analyses and the specific category of hybrid seedlings and inferred fathers, estimated as the q values obtained from Bayesian analysis from microsatellite data (López De Heredia et al. 2017), were calculated with Pearson's r coefficient using the "cor" function of R. Linear models were fitted for the principal component scores that showed high correlation values.

Tab. 2 - Contribution of the measured variables to the first three principal components. (*): variables that contributed most to each PC.

Variable	PC1	PC2	PC3
NT	-0.54505911	0.57133737	0.06719112
DW	-0.94474033*	0.02529156	-0.0277579
T	0.00236017	0.83459101*	0.05697182
S	-0.95021391*	-0.2347433	-0.04317772
P	-0.96134823*	0.12877999	0.05231506
L	-0.9329851*	-0.23694868	0.09173586
W	-0.964661*	-0.05444766	-0.11786569
LL	-0.93677664*	-0.23051776	0.08068672
WM	-0.96422764*	-0.05474921	-0.11193033
PL	-0.58461261	-0.06033392	-0.02877686
WM_LL	-0.0613093	0.22895923	-0.95775912*
SH	0.51759531	-0.63655759	-0.22501442

Fig. 2 - Biplot of the mean individual scores for the first two PCs for the morphological multivariate analysis, and Tukey's HSD tests for (b) PC1 (size), and (c) PC2 (thickness). (Green): *Q. suber*; (red): *Q. ilex*; (black): hybrids. Groups with the same color in the bar diagrams do not show significant differences in their means.

Results

Leaf fluctuating and developmental asymmetry

Fluctuating asymmetry was detected for a significant percentage of the seedling progenies, irrespective of the parental specific category. According to the results of the $FSAI_i$ parameter, evidence of fluctuating asymmetry was obtained for leaves of *Q. suber*, *Q. ilex*, and hybrid progenies in very similar proportions (Fig. S1a in Supplementary material). A closer examination of the distribution of fluctuating asymmetry for the hybrids progenies revealed that there were slight differences between seedlings of different cohorts depending on the family to which the plants belong (Fig. S1b, Fig. S1c, Fig. S1d). Actually, there were no significant differences between cohorts for the Is progeny (F = 0.65, p-value = 0.421), and only slight, yet significant, differences were scored for the LG1 (F = 5.95, p-value = 0.0179), and ZL (F = 8.17, p-value = 0.0075) families.

Strong asymmetries of diverse nature (Fig. S2 in Supplementary material) related to developmental anomalies were detected for 29.9 % of all seedlings. This type of asymmetry was almost an exclusive character of the hybrid progenies, with only a single *Q. ilex* individual showing leaf asymmetry (Fig. S3a). In this case, however, strong differences in the frequency of asymmetric leaves were scored between two and four year-old seedlings of the hybrid progenies, specially for IS (F = 42.34, p-value < 0.0001 – Fig. S3b), and to a less extent for ZL (F = 2.184, p-value = 0.145 – Fig. S3d) families. For LG family, although the boxplots suggest that there were differences between cohorts (Fig. S3c), these differences were not significant (F = 6.542, p-value = 0.0156). In all three cases, higher proportion of asymmetric leaves was detected for two than for four year-old seedlings.

Leaf morphological multivariate analysis

After removing asymmetric leaves related to developmental anomalies, the first three principal components explained 82.2 % of total variance. The variables that mostly contributed to the two first principal components were related to size (DW, dry weight; S, surface; P, perimeter; L, total length; W, leaf width at 50 % of leaf total length; LL, lamina length; and WM, leaf lamina maximum width) for PC1, and to leaf thickness (T) for PC2 (Tab. 2). The variables that mostly contributed to PC3 were related to leaf shape (WM_LL, ratio between the leaf lamina maximum width and the leaf lamina length – Tab. 2). However, PC3 only explained 8.3 % of the total variance, so it was not considered in further analysis. Indeed, variation in shape is better captured using morphometric geometric analysis than with the WM_LL variable (see below).

The biplot of the mean individual scores for PC1 and PC2 showed significant differences between *Q. ilex* and *Q. suber* progenies (Fig. 2a). For PC1, negative values indicate bigger leaves (*Q. ilex*), while positive values point to leaves of smaller size (*Q. suber*). While seedlings of hybrid families showed high variability in leaf size, there were non-significant differences among means with leaves of *Q. suber* seedlings, at the same time that both were significantly different than leaves of *Q. ilex* progenies, according to the HSD Tukey's tests (Fig. 2b). For PC2, *Q. ilex* progenies showed positive values, and therefore thicker leaves, while *Q. suber* progenies presented negative values. Interestingly, for this character, hybrid progenies showed intermediate val-

Tab. 3 - ANOVA results for the fixed effects (cohort) of the linear mixed effects models (eqn. 4) fitted for the principal component scores related to leaf size (PC1), leaf thickness (PC2), and leaf shape (PC1.shape). (df): degrees of freedom; (SS): sum of squares; (MS): mean squares; (***): p< 0.001.

Scores	Effect	df	SS	MS	F-value
PC1 (size)	α_i (cohort)	1	2210	2209.7	342.2***
	Residuals	2819	18203	6.5	-
PC2 (thickness)	α_i (cohort)	1	1060	1059.6	817.3***
	Residuals	2819	3655	1.3	-
PC1.shape	α_i (cohort)	1	0.43	0.4301	78.98***
	Residuals	2755	15.00	0.0054	-

ues that were significantly different from both *Q. ilex* and *Q. suber* progenies.

Leaf morphometric geometric analysis

The principal component analysis of the landmark coordinates after Procrustes transformation showed that the two first components accounted for 56 % of overall variance. Actually, the first component (PC1.shape) explained 44.8 % of the overall variance. Although the mean scores for the three specific categories overlapped to some extent, the biplot of the mean individual scores for PC1.shape and PC2.shape, showed significant differences between the progenies of *Q. ilex* and *Q. suber* along the PC1.shape axis (Fig. 3). The hybrid families presented intermediate or similar values to *Q. suber* progenies for this component related to shape.

Mixed effects linear models and variance components estimations

The fitted models showed significant differences between two and four year-old seedlings for the three principal components related to size, thickness, and shape of the leaves (Tab. 3). However, these differences were more evident for size and thickness (Fig. S4a in Supplementary material) than for shape (Fig. S4b). The random terms of the models, however, showed different impact of each factor depending on the morphological trait, as estimated from the variance components (Fig. S5). The term species (β_i) explained high variance percentages for leaf thickness (51.1 %), and leaf shape (33.2 %), consistent with the results of the principal component analysis. For leaf size, however, less percentage of variance was found for species, while high variance percentages were scored due to variation within plants (ε_{ijklm}, 37.8 %), and be-
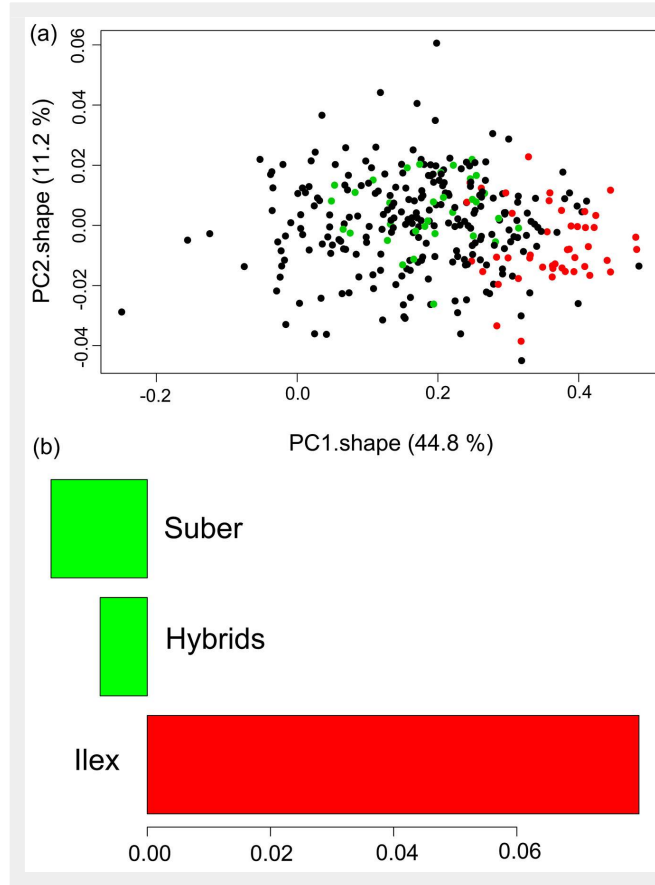

Fig. 3 - Biplot of the mean individual scores for the first two PCs for the morphometric geometric analysis, and Tukey's HSD tests for PC1.shape. (Green): *Q. suber*; (red): *Q. ilex*; (black): hybrids. Groups with the same color in the bar diagrams do not show significant differences in their means.

Fig. 4 - Linear models fitted for the q_s parameter obtained for the seedlings *vs.* the mean individual values of PC1 (size), PC2 (thickness) and PC1.shape (shape) for Is, LG1, and ZL families. (Black dots): 2012 seedlings; (red dots): 2014 seedlings.
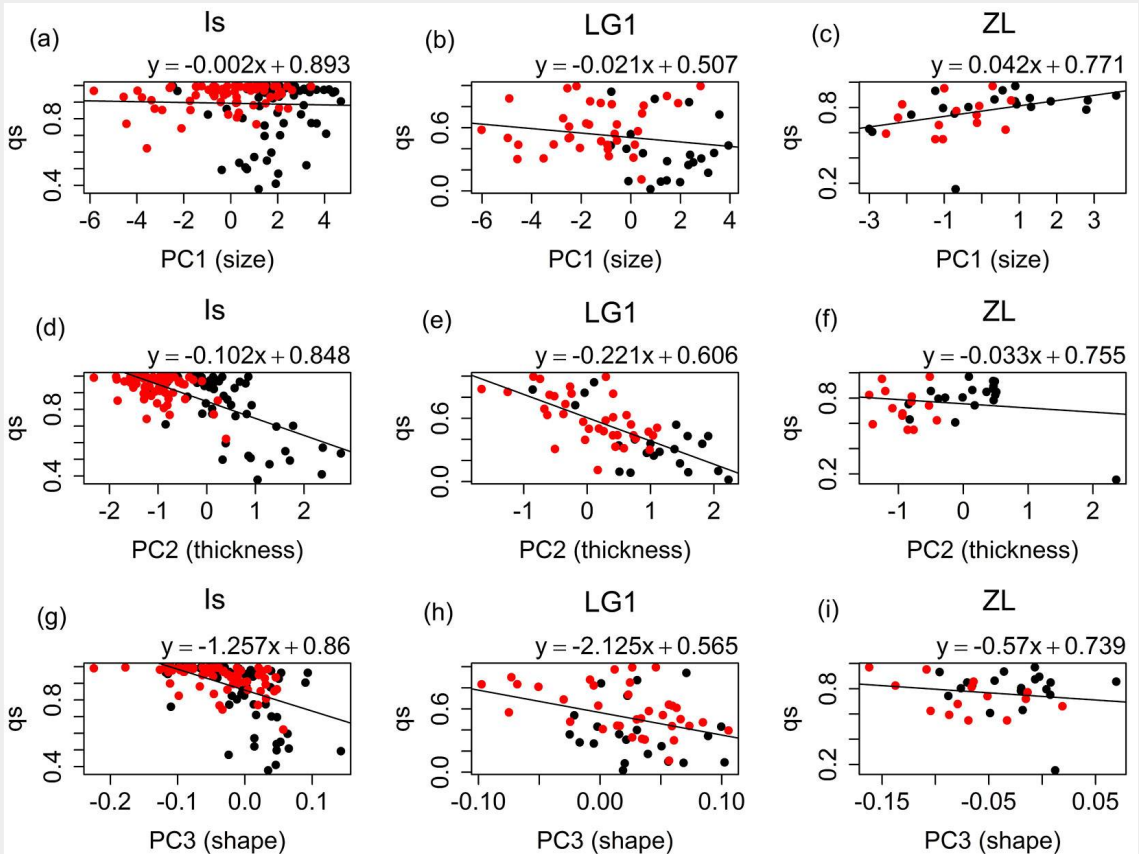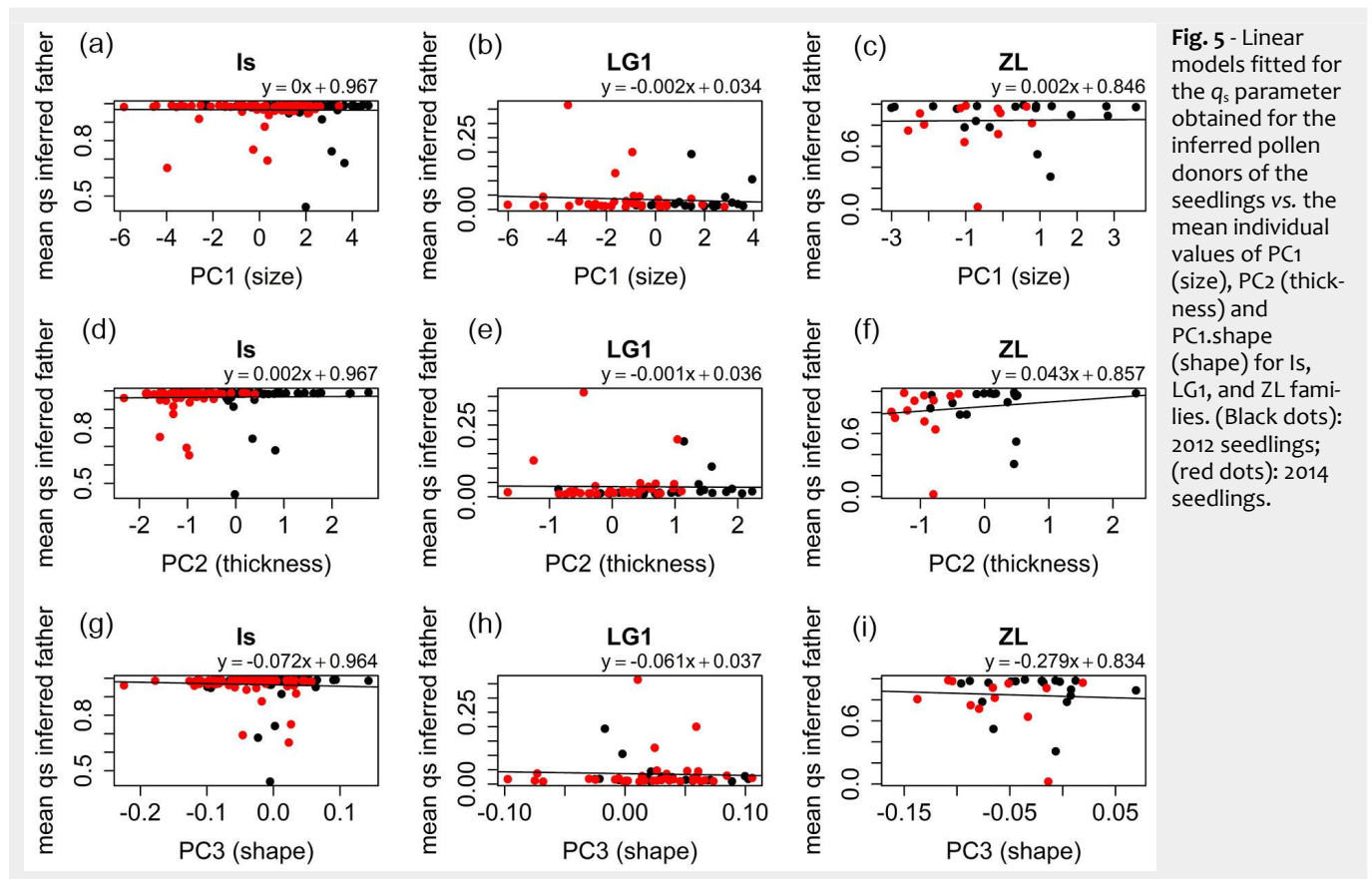
Fig. 5 - Linear models fitted for the q_s parameter obtained for the inferred pollen donors of the seedlings *vs.* the mean individual values of PC1 (size), PC2 (thickness) and PC1.shape (shape) for Is, LG1, and ZL families. (Black dots): 2012 seedlings; (red dots): 2014 seedlings.

tween plants within families within species ($\delta_{l(k(j))}$, 38.2 %). Interestingly, the percentage of variance explained by the term family within species ($\gamma_{k(j)}$) was small for the three principal components.

Correlation of leaf morphology with genetic specific category parameters

For the Is and LG1 families strong significant negative correlations were found between leaf thickness (PC2) and q_s values of the seedlings estimated with Structure (Is: r = -0.67, p-value < 0.001; LG1: r = -0.71, p-value < 0.001 – Fig. 4d, Fig. 4e), and between leaf shape (PC1.shape) and q_s values (Is: r = -0.51, p-value < 0.001; LG1: r = -0.36, p-value = 0.006 – Fig. 4g, Fig. 4h), respectively (López De Heredia et al. 2017). For leaf size (PC1), correlations were non-significant for the Is (r = -0.03, p-value = 0.705 – Fig. 4a) and LG1 (r = -0.18, p-value = 0.163 – Fig. 4b) families. For the ZL family, significant positive correlation was found between these two variables (r = 0.42, p-value = 0.015 – Fig. 4c). However, only a small part of the ZL samples q_s values below 0.5, and therefore, this correlation needs to be interpreted with caution. This circumstance produced also non-significant correlations for the ZL family between leaf thickness (r = -0.16, p-value = 0.371) and shape (r = -0.17, p-value = 0.334), and the specific category (Fig. 4f, Fig. 4i). Correlation of the morphological characters with the inferred q_s values of the potential pollen donors was very low for all the half-sib families and cohorts (Fig. 5).

Discussion

Leaf fluctuating and developmental asymmetry

A high proportion of leaves with fluctuating and developmental asymmetries in seedlings was found in this study. However, the frequency of fluctuating and developmental asymmetry was variable between cohorts, species and families, supporting the hypothesis of factors of different nature operating on each type of asymmetry (Milligan et al. 2008).

The proportion of seedlings with leaves showing fluctuating asymmetry was high for the hybrid progenies, but also for the pure species' families. Determining the causes of fluctuating asymmetry for the seedlings of this study is not straightforward. Fluctuating asymmetry induction by changes in biotic and abiotic factors has been documented for oak species (Puerta-Piñero et al. 2008, Viscosi 2015). Therefore an effect of small-scale heterogeneity in nursery conditions, both in water stress or in light-shade conditions, cannot be completely discarded. Large morphological and physiological variation has been acknowledged for *Q. ilex* leaves in relation to ecotypic variation at broad scales associated with water availability (Niinemets 2015, Peguero-Pina et al. 2014), and, specifically, fluctuating asymmetry in response to hydric stress was reported for *Q. ilex* (Hódar 2002). In addition, leaf structural plasticity in *Q. ilex* and *Q. suber* is a stronger determinant for leaf acclimation to incident photo-

synthetic photon flux density than biochemical and physiological plasticity (Vaz et al. 2011); thus fluctuating asymmetry could be also generated by the structural adjustment of leaf structures during acclimation to changes in light intensity.

From studies in natural populations, it has been argued that the genotypic composition of the populations (Wilsey et al. 1998), and hybridization (Albarrán-Lara et al. 2010) may produce fluctuating asymmetry. However, no significant differences in the frequency of fluctuating asymmetry between *Q. ilex*, *Q. suber*, and hybrid seedlings were scored with the *FSAI*$_i$ index in the present study. While fluctuating asymmetry is more likely due to environmental stress than to hybridization, the strong asymmetric patterns found only for hybrid progenies are associated to anomalies produced during leaf development.

Actually, leaf asymmetries are not the exclusive anomalies observed in hybrid progenies. Seedlings that lack leaves, seedlings with multiple stems or strong ramification patterns, and dwarf seedlings without apparent apical growth are also frequent. These types of malformations in hybrids have been described for other angiosperms (Takanashi & Marubashi 2017), and are related to genetic incompatibilities that modify the epigenetic system controlling the differentiation of the apical shoot meristem and the leaf primordia (Lodha et al. 2013). Networks of genes coding for small RNAs and transcription factor-like proteins determine the polarity specification of flat

bifacial leaves (Nicotra et al. 2011). Possibly, disruptions of regulatory networks due to interspecific genomic incompatibilities are responsible for producing abnormal phenotypes in *Q. ilex × Q. suber* progenies. As reported for the model species *Arabidopsis*, the repression of key genes that regulate the adaxial-abaxial polarity, such as ETT/ARF3, ARF4, and others that regulate the proximal-distal leaf length (class 1 KNOX homeobox genes – BP, KNAT2), produces asymmetric leaves (Matsumura et al. 2016).

The frequency of leaf asymmetries associated to development was significantly higher for two year-old seedlings than for four year-old seedlings. One possible explanation is that the modification of the epigenetic systems involved in the development of the foliar primordia produced by hybridization induces a higher degree of malformation in younger than in older plants. The few studies that have investigated the relationship between age and leaf anomalies suggest that leaf asymmetry decreased with age in leaves (Milligan et al. 2008). Alternatively, extremely asymmetric leaves may fall from the seedlings earlier, showing apparently shorter life-spans than normal leaves. However, for deciduous trees, leaf life span is independent of asymmetry (Kozlov 2003). Although not verified in field experiments, early expressed abnormality probably results in lower fitness of the hybrid seedlings, and in higher mortality rates than pure species. This could account for the low frequency of adult hybrid trees in mixed populations, no matter they produce high amount of viable acorns (López De Heredia et al. 2017).

Leaf morphological variation in Q. ilex, Q. suber seedlings

Both multivariate and morphometric geometric analyses allowed the leaf morphological characterization of the seedlings that did not present asymmetrical patterns. In our study, the best descriptors of leaf morphology for seedlings of *Q. ilex* and *Q. suber* are lamina size, thickness, and shape, being leaf thickness the most discriminant character between *Q. ilex* and *Q. suber*. This result is consistent with the higher tolerance of *Q. ilex* seedlings to high values of light irradiance as compared with *Q. suber* seedlings (Quero et al. 2006). Castro-Díez et al. (2000) showed, for a wide range of species, that the thicker mesophyll of evergreen seedling leaves was not due to cell wall thickening and sclerification, but a consequence of a larger number of cell layers to increase the photosynthetic capacity per area unit.

The significantly thicker leaves found for four year-old seedlings of both species from our study, matches with the increasing in thickness and leaf mass dry per unit area between cohorts reported for seedlings of the same species (Mediavilla et al. 2011). Furthermore, species with a long average leaf life span, *Q. ilex* in this case (Me-

diavilla & Escudero 2003), tend to have high leaf thickness and mass per unit area, low nutrient concentrations and show maximum photosynthetic rates (Poorter et al. 2009).

Quercus ilex presented also leaves of higher size than *Q. suber* seedlings. Leaf area is known to be a character of great plasticity in deciduous temperate trees (Sack et al. 2006, and references therein). This great plasticity was also described for evergreen species, such as *Q. ilex* (Mediavilla et al. 2012), and *Q. suber* (Cardillo & Bernal 2006). Interestingly, leaf size from adult trees is traditionally described as following the opposite pattern, *i.e.*, adult *Q. ilex* has smaller leaves than adult *Q. suber* (Amaral Franco 1990). Actually, two year-old seedlings presented bigger leaves than four year-old seedlings. This seems to be an exception for evergreen species, because although leaf size varies across ontogenetic stages (Poorter 2007), linear allometric relationships during ontogenetic development are expected (Cornelissen 1999). Other authors, however, have reported that the increments in leaf mass per unit area as the seedling increases, seem to be achieved only through increased thickness, with no associated changes in density (Mediavilla et al. 2012), and therefore, no impact on leaf surface.

The morphometric analysis showed significant differences between the leaf shape of *Q. ilex* and *Q. suber* seedlings. Leaves of *Q. ilex* seedlings show suborbicular shape, while *Q. suber* seedlings present ovate leaves (Amaral Franco 1990). Leaf shape is highly dependent on general regulators of shoot apical meristem and cell cycling in the initial stages of leaf development (Barkoulas et al. 2007), and, during the last stages, is adjusted by the environmental conditions (Tsukaya 2005). In this case, leaf shape showed very slight differences between seedlings of both cohorts, suggesting that it is a more stable character than size or thickness. Indeed, leaf shape is less variable across environments than size (McDonald et al. 2003).

Segregation of morphological traits of hybrid progenies

Hybrid seedlings presented very diverse phenotypes that may resemble the phenotypes of pure species, or present transgressive traits possibly due to epistasis, heterosis and/or developmental instability (Rieseberg & Ellstrand 1993). Leaf traits related to size, thickness, and shape showed independent segregation patterns in hybrid progenies. The correlation between the mean scores of the principal components related to size, thickness and shape, and the genetic category (q_s) of the hybrid offspring is rather high (Fig. 4), particularly for those hybrid families that presented a wide spectrum of q_s values (Is, LG1). On the contrary, correlation with the q_s of the potential fathers is rather poor (Fig. 5), suggesting a maternal effect in the leaf characters

of the seedlings. However, the mixed model did not detect a significant effect of the maternal genotype for any trait.

Leaf growth traits often show intermediate values in hybrids, as reported by Durković et al. 2012 for *Sorbus*. In *Quercus*, Himrane et al. (2004) reported a wide spectrum of foliar phenotypes for *Q. faginea × Q. pubescens* hybrids, including those very close to the parental ones and other intermediate, although these latter ones were very scarce. In our case, only thickness, which is a highly discriminant trait between the parental species, showed an intermediate class between leaves of *Q. suber* and *Q. ilex* progenies. Conversely, leaf size and shape of hybrid families in our study were more similar to *Q. suber* than to *Q. ilex*, and only the family LG1, mostly pollinated by *Q. ilex*, presented intermediate values for these traits. This result point to differential segregation of genes that regulate leaf size, thickness and shape, and is not surprising, considering that most leaf traits are under polygenic control in oaks (Scotti-Saintagne et al. 2004), but also that some of these genes may be pleiotropic (Gailing 2008).

Conclusion

While fluctuating asymmetry can be detected equally for *Q. suber, Q. ilex* and hybrid seedlings, a significantly higher proportion of developmental leaf asymmetry is found in hybrid offspring, which would be likely related to higher mortality of the seedlings, accounting for the low frequency of adult hybrid trees in mixed populations. Among the normally developed leaves, thickness was the most discriminant character between species, followed by size and shape. Hybrid offspring leaves show intermediate values, related to the individual genetic composition (q_s), although maternal effects cannot be discarded.

Acknowledgments

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Supplementary Material

Fig. S1 - Fluctuating seedling asymmetry across cohorts and families.

Fig. S2 - Normal and asymmetric leaves.

Fig. S3 - Developmental seedling asymmetry across cohorts and families.

Fig. S4 - Principal component variation between cohorts.

Fig. S5 - Variance components for the random terms of the models.

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