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Molecular evidence of bidirectional introgression between *Quercus suber* and *Quercus ilex*

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Cork oak and holm oak share a large part of their natural range, and are known to hybridize in mixed stands. This hybridization is supposed to have played a relevant role in the past history of cork oak. Previous research has reported that F_1 hybrids are produced with holm oak acting as pollen recipient, therefore carrying holm oak chloroplast. Additionally, F_1 hybrids have been assumed to be pollinated mostly by cork oak. Continued backcrossing of F_1 hybrids with cork oak (supported by flowering phenology) could have created the organellar introgression patterns observed nowadays in Eastern Spain and Southern France cork oak populations. On the contrary, no organellar introgression has been detected in holm oak and multiple generation backcross individuals to holm oak have not been reported so far. In this work, we examined whether hybrids preferentially backcross with cork oak or with holm oak. To reach this goal, we genotyped by using eight microsatellite loci the progeny of four cork and four holm oak trees (33 and 44 half-siblings, respectively), and of four hybrids (468 half-siblings) collected over three years from a natural mixed population. We used the STRUCTURE software to estimate the proportion of the genotype of each seedling inherited from cork oak (q_s) or from holm oak (q_i). The ratio of the offspring q value over the mother q value helped determine the source of pollen that originated each acorn. Our results show for the first time that hybrid trees can be effectively pollinated by both parental species. Additionally, each hybrid tree was predominantly pollinated by the most abundant oak species in its vicinity. These results confirm the occurrence of bidirectional introgression, previously suggested for adult hybrid trees in the field, and point out the pattern of introgression in the seedlings could be most affected by the abundance of the parental species.

Keywords: Cork Oak, Holm Oak, Hybridization, Introgression, Microsatellites

Introduction

Hybridization between cork oak (*Quercus suber* L.) and holm oak (*Q. ilex* L.) has been known for a long time. The first written references to putative hybrid individuals date back to the mid-19th century (Colmeiro &

Boutelou 1854, Laguna 1881, Borzi 1881). The ranges of cork and holm oak overlap in the western Mediterranean basin, where they form mixed stands on acid and decarbonated soils, the preferred ones for cork oak. Morphologically intermediate, presumably hybrid individuals, can be found in these stands, and are easily identifiable by their bark, and by some leaf intermediate traits, such as leaf thickness, which show characteristics of both parental species (Laguna 1881). These putative hybrid individuals occur at low frequency and are scattered among the pure cork and holm oaks. Introgression of genes from one species to another can have important adaptive and evolutionary consequences (Excoffier et al. 2009, Petit et al. 2004). In the case of holm and cork oaks, it has been proposed that such process may have helped cork oak thrive in adverse environmental conditions (e.g., during the glacial pulses of the Pleistocene – López de Heredia et al. 2007).

Molecular markers have confirmed the hybrid nature of phenotypically intermediate individuals and were used to assess the

extent of hybridization between *Q. suber* and *Q. ilex* in natural mixed populations. Analysis of chloroplast DNA (cpDNA), which is maternally inherited in oaks, detected *Q. suber* populations in Southern France and Eastern Spain with a typical *Q. ilex* lineage cpDNA (Jiménez et al. 2004, López de Heredia et al. 2005, Lumaret et al. 2005). Phenology of both species as well as post pollination barriers as those reported by Boavida et al. (2001) favor hybridization with *Q. suber* acting as pollen donor, so that hybrids carry the *ilex* chloroplast. Chloroplast introgression pattern suggests hybrid trees carrying the *ilex* chloroplast currently backcrossed with *Q. suber* as pollen donor. Using isozymes (Elena-Roselló et al. 1992, Oliveira et al. 2003) or nuclear microsatellites (Soto et al. 2003, Burgarella et al. 2009), previous studies confirmed the hybrid character of morphologically intermediate oak trees, and showed the current existence of hybridization. Burgarella et al. (2009) estimated a low rate of ongoing introgression in cork oak over its range (~2%), a result confirmed by Lumaret & Jabbour-Zahab (2009 – ~4%). These stud-

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ies suggested that introgression could occur bidirectionally. However, if the flowering phenology of first generation hybrids (F_1 hybrids) is more similar to *Q. suber*, as suggested by Perea García-Calvo (2006), they would preferentially backcross with *Q. suber*, while mating with *Q. ilex*, especially when the latter species acts as pollen donor, would be much less frequent.

In the current work, we genotyped seedlings of morphologically putative hybrid trees, to identify backcrossing events with either *Q. ilex* or *Q. suber* as pollen donors, and to quantify the frequency of introgression towards each of the parental species. Our study provides a deeper insight in the hybridization and introgression processes which could be currently taking place between these two species.

Material and methods

Sampling site and acorn collection

The sampling site is located in Central Spain, (39° 59' N, 05° 07' W) in a mixed "dehesa", i.e., highly anthropized open woodland. *Quercus suber* and *Q. ilex* are the dominant species, while there are some stands with comparatively few *Q. faginea* individuals. Four mature hybrid trees were identified by their phenotypes. These hybrid trees occurred in three distinct areas (IS, LG, and ZL) which differed in tree density and species composition. One hybrid was identified in IS, where *Q. ilex* and *Q. suber* occur at a density of 38 trees ha⁻¹ and where *Q. suber* is slightly more abundant. Two hybrids were found in LG, approximately 25 m apart and 740 m away from the IS hybrid. At LG, tree density was low (20 trees ha⁻¹) and dominated by *Q. ilex*. The fourth hybrid tree was found in ZL, where tree density was 123.5 trees/ha, with more *Q. suber* trees (38.3 trees ha⁻¹ for *Q. ilex* and 85.2 trees ha⁻¹ for *Q. suber*), and approximately 2780 m away from the two LG hybrids and 3140 m away from the IS hybrid.

Acorns were collected in 2011, 2012 and

2014 on each of the four hybrid trees and also from four randomly selected cork oak and four holm oak trees located in IS (Tab. 1). Each year, all acorns from LG2 and ZL hybrid trees were collected, as well as approximately 1/3 of the acorns on the LG1 and IS hybrids.

All collected acorns were sown in peat-perlite substrate (3:1 proportion) in 3L containers in a greenhouse room kept at 22 °C, with a relative humidity of 50-60%. Seedlings were watered daily for 8 min. and five months after sowing, germination rate was estimated and leaf tissue collected from the surviving seedlings for DNA extraction.

Genotyping

DNA extraction was performed with a modification of the protocol by Doyle & Doyle (1990 – Dumolin et al. 1995). We used eight polymorphic nuclear microsatellites (nSSRs) for genotyping: MSQ4, MSQ13 (Dow et al. 1995), QpZAG9, QpZAG15, QpZAG36, (Steinkellner et al. 1997), QrZAG7, QrZAG11 and QrZAG20 (Kampfer et al. 1998). Microsatellites were amplified under standard PCR conditions (Soto et al. 2003, 2007). Fluorescence labelled PCR products were analyzed in a 4300 LI-COR® automated sequencer (LI-COR Biosciences, Lincoln, NE, USA). Allele sizes were determined with the SAGA Microsatellite Analysis Software (LI-COR Biosciences, Lincoln, NE, USA). The presence of null alleles was examined using ML-Null (Kalinowski & Taper 2006).

Nuclear admixture analyses

We carried out admixture analyses using the Bayesian clustering approach implemented in the program STRUCTURE ver. 2.3.4 (Pritchard et al. 2000). In this model, the posterior probability (q) provides an estimation of the proportion of an individual genome originating from each of K differentiated genetic pools. Values of K ranging from 1 to 5 were tested in order to find the optimal K value. A value of $K=2$ was identified as the optimal K value following the

methodology described by Evanno et al. (2005) and was used in all subsequent analyses. In order to improve q estimations (Burgarella et al. 2009), we included in the STRUCTURE analyses the genotypes of approximately 95 *Q. ilex* and 96 *Q. suber* parental trees from the same stand, previously analyzed by Soto et al. (2007), along with 59,671 virtual individuals simulated with the software SimHyb (Soto et al. 2018). These simulated individuals included a majority of parental trees with some F_1 hybrids and backcrosses (Tab. S1 in Supplementary material).

Calculations were carried out under the admixture model assuming independent allele frequencies, as it has been done previously (Burgarella et al. 2009). A burn-in of 50,000 steps followed by 100,000 iterations were used, after verifying that results did not vary significantly across runs or with longer burn-in/iteration cycles. Assignment of seedlings to specific categories based on the q_s values (estimation of the proportion of the genome coming from *Q. suber*) used the following thresholds: $q_s \geq 0.85$: *suber*; $0.85 < q_s \leq 0.65$: *suberoid*; $0.65 \leq q_s < 0.35$: *hybrid*; $0.35 \leq q_s < 0.15$: *ilicoid*; $q_s \leq 0.15$: *ilex*. The software Colony (Jones & Wang 2010) was used to infer selfing rates in the progeny using the adult trees as potential fathers and allowing up to one mismatch per seedling, and to detect potential crosses between hybrid trees.

The software R (R Core Team 2013) was used to draw graphics, linear regressions between putative paternal q_s and q_{sol}/q_{sm} (the ratio between the offspring and the maternal q_s), and to prepare a script to obtain all the possible paternal contributions for each offspring.

Results and discussion

Germination rates

A total of 671 acorns were collected from hybrid trees, and 53 and 49 acorns from *Q. ilex* and *Q. suber*, respectively (Tab. 1). Hybrid trees from LG2 and ZL produced fewer acorns than hybrid trees from LG1 and IS, and their germination rates were also lower (24% and 46%, respectively), while germination rates were above 70% for hybrids from LG1 and IS, as well as for the parental species trees (Fig. S1 in Supplementary material).

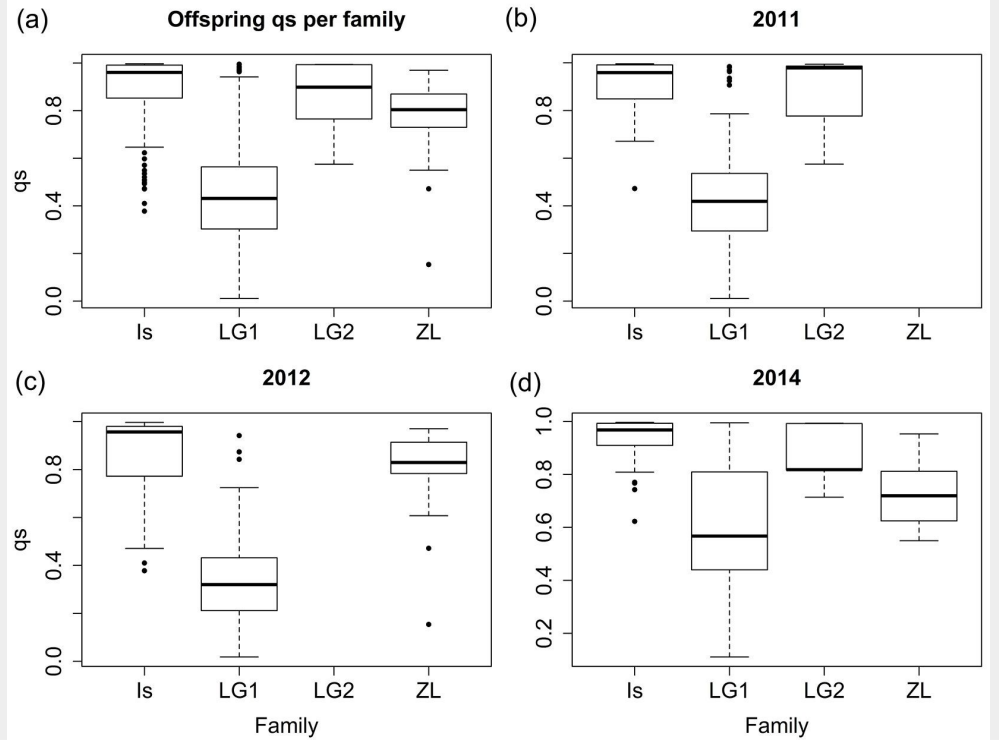
Assignment to specific categories

We did not detect null alleles for any locus. Autofecundation was ruled out, as the estimated selfing probability was <0.001 for every hybrid seedling. Moreover, mating between hybrid trees was also discarded after inspection of potential parents for each offspring estimated by Colony. The most probable number of differentiated genetic pools in the population was $K = 2$ (Fig. S2). The q_s values (estimation of the proportion of the genome coming from *Q. suber*) obtained for the hybrid trees were 0.8763 for IS, 0.5988 for LG1,

Tab. 1 - Number of acorns sowed, germinated, and genotyped (sowed-germinated-genotyped) categorized by mother tree (family), taxa and cohort.

Taxa	Genotype	2011	2012	2014
Hybrids	LG1	184 - 155 - 148	36 - 24 - 21	59 - 39 - 38
	LG2	19 - 3 - 3	-	14 - 5 - 5
	IS	106 - 85 - 84	76 - 61 - 59	95 - 77 - 77
	ZL	12 - 0 - 0	36 - 24 - 19	34 - 14 - 14
	Total	321 - 243 - 235	148 - 109 - 99	202 - 135 - 134
<i>Q. ilex</i>	I1	-	22 - 21 - 19	5 - 5 - 5
	I2	-	12 - 11 - 9	5 - 4 - 4
	I3	-	-	4 - 4 - 4
	I4	-	-	5 - 3 - 3
	Total	-	34 - 32 - 28	19 - 16 - 16
<i>Q. suber</i>	S1	-	12 - 8 - 8	4 - 2 - 2
	S2	-	18 - 12 - 11	5 - 4 - 4
	S3	-	-	5 - 4 - 4
	S4	-	-	5 - 4 - 4
	Total	-	30 - 20 - 19	19 - 14 - 14

Fig. 1 - Boxplots of the q_s parameter across families for (a) all years combined and separately for (b) 2011, (c) 2012, and (d) 2014. Sample sizes are presented in Tab. 1.



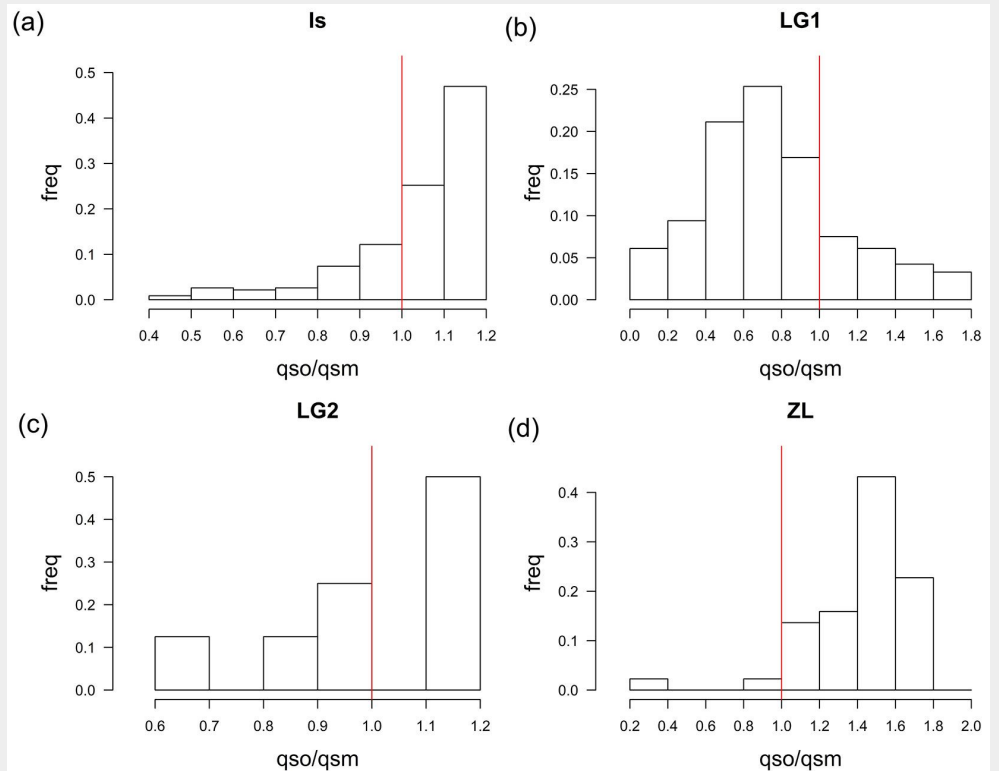
0.8680 for LG2 and 0.5489 for ZL. These results would classify the hybrid trees at LG1 and ZL as most likely first generation hybrids (F₁), and hybrid trees at IS and LG2 as more “advanced introgressed” individuals, at least second-generation backcrosses with *Q. suber*. These results are consistent with preferential backcrossing with *Q. suber*.

Then, q_s values were also estimated for

the progenies of the hybrids (Fig. 1). The high q_s values obtained for most of the hybrid offspring, particularly for IS, LG2 and ZL, indicate a high contribution of *Q. suber* to their genomes and therefore previous works could have classified these seedlings as backcrosses to *Q. suber*. Conversely, the offspring of LG1 shows a broader range of q_s , with an average value of 0.4512, closer to *Q. ilex*. However, assignment to specific

classes based exclusively on q_s may be biased using this set of markers, especially for advanced introgressed individuals (Soto et al. 2018). Actually, more than 30% of the virtual introgressed individuals included in the simulation as control would be misassigned according to q_s . Following Soto et al. (2018), we used the ratio q_{sol}/q_{sm} to determine the most likely genetic pool of the pollen donor for each offspring (Fig.

Fig. 2 - Histograms of the relative frequencies of the q_{sol}/q_{sm} ratio between the offspring and the maternal q_s for Is (a), LG1 (b), LG2 (c), and ZL (d) families. The bars to the right of the vertical red line indicate pollination by individuals with a higher percentage of *suber* genomes. The bars to the left of the vertical red line indicate pollination by individuals with a higher percentage of *ilex* genomes.



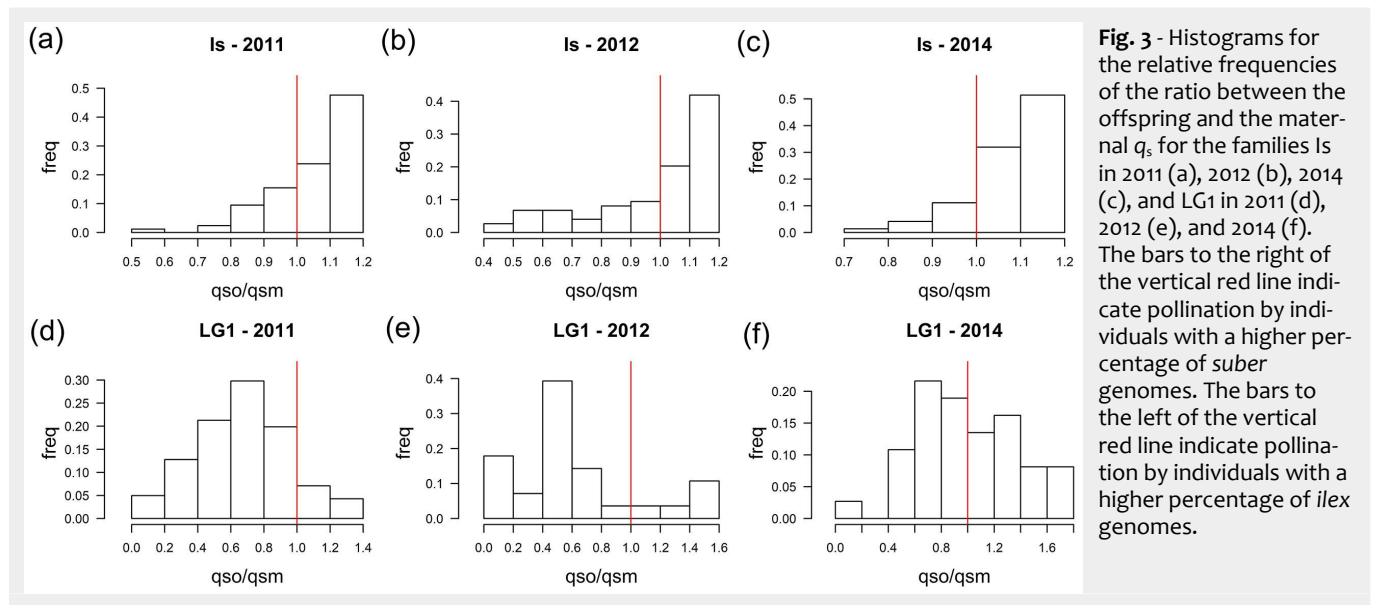


Fig. 3 - Histograms for the relative frequencies of the ratio between the offspring and the maternal q_s for the families IS in 2011 (a), 2012 (b), 2014 (c), and LG1 in 2011 (d), 2012 (e), and 2014 (f). The bars to the right of the vertical red line indicate pollination by individuals with a higher percentage of *suber* genomes. The bars to the left of the vertical red line indicate pollination by individuals with a higher percentage of *ilex* genomes.

2). These data support backcrossing with both parental species with many of the LG1 offspring having *Q. ilex* as father, and many offspring of the IS and ZL hybrid trees having *Q. suber* as father. Offspring of the LG2 hybrid tree had either *Q. suber* or *Q. ilex* as father. Fig. 3 shows the distribution of the q_s ratio throughout the studied years for IS and LG1, the trees that produced more acorns. Although the general trends are kept for the whole period, some differences among years can be noticed, particularly for LG1: in 2011, probably a masting year with high acorn production, the vast majority of the seedlings came from *Q. ilex*

pollen (84.5%), while in 2014, with a much lower acorn production, the proportion of effective pollinations from *Q. ilex* decreased to 54.1%.

To get additional support for the results on pollination direction we prepared an R script to compare each individual offspring and its mother's genotypes, and to obtain all the possible paternal genetic contributions (haplotypes). We then estimated their q_s value using STRUCTURE. Fig. 4a shows a boxplot of the distribution of the q_s values assigned to all the possible paternal haplotypes for each mother tree, and Fig. 4b and Fig. 4c show these distribution

through the studied years for IS and LG1.

Finally, Fig. 5 shows the significant correlations above 0.80 for the estimated paternal q_s and the ratio q_{so}/q_{sm} , especially for IS (Fig. 5a) and LG1 (Fig. 5b), the trees with larger progenies. This result confirms that using the ratio q_{so}/q_{sm} is a good approach to identify the genetic pool of the pollen donor, provided the mother tree is known.

These results support bidirectional introgression, where hybrid trees backcross with both parental species. Bidirectional introgression was also suggested by genotypic data collected by Burgarella et al. (2009) using the same set of nuclear microsatellite markers, on adult trees phenotypically classified as *Q. suber* and as *Q. ilex* sampled all across the overlapping distribution ranges of these two species. Lumaret & Jabbour-Zahab (2009), using the same set of microsatellite markers, also reported the possibility of bidirectional introgression.

Each adult hybrid receives pollen from both parental species but is preferentially pollinated by either *Q. ilex* or *Q. suber* (Fig. 2). The major pollen donor for each hybrid tree seems to reflect the abundance of parental tree species in the area. For example, the hybrid tree LG1, located in La Laguna, is mostly pollinated by *Q. ilex*, the dominant species in that area (the number of seedlings from LG2 is too low to be conclusive); on the other hand, IS and ZL hybrids produced more seedlings from *Q. suber* pollen, the more abundant species in these areas. Therefore, the frequency of backcrossing with a parental species could be at least partially driven by pollen availability. Simulation studies using the oak complex *Q. robur* × *Q. petraea* have shown that the direction of introgression can be influenced by the composition of the pollen pool on individuals or stigmas which is itself affected by the spatial configuration of the parental species (Lagache et al. 2013, Klein et al. 2017). The direction of introgres-

Fig. 4 - Boxplots of the q_s parameter of the inferred putative fathers across families (a), and across cohorts for IS (b) and LG1 (c) families.

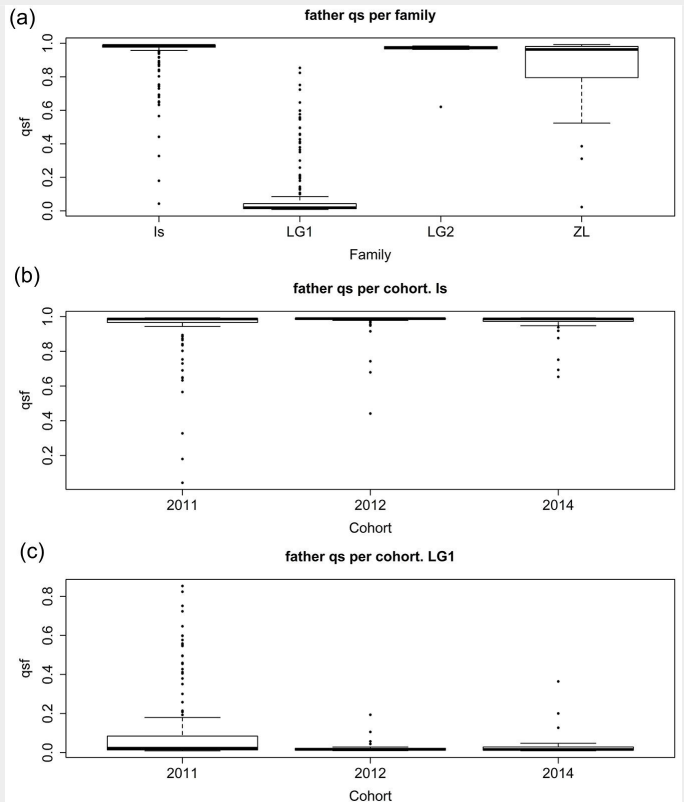
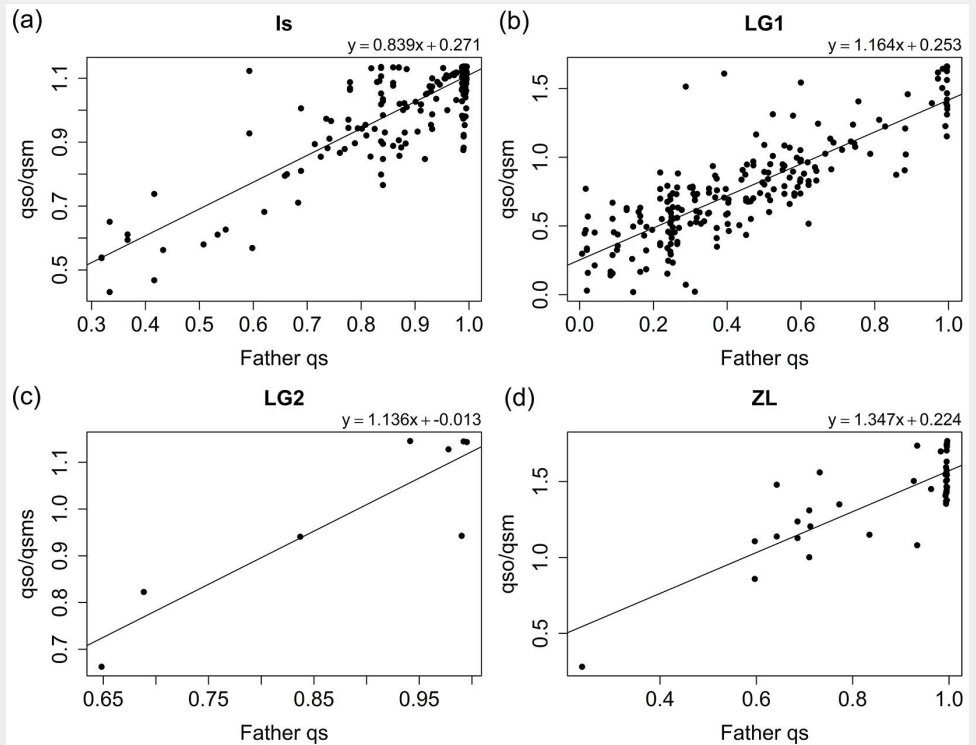


Fig. 5 - Correlation of the ratio between the offspring and the maternal q_s and the q_s parameter of the inferred putative fathers for Is (a), LG1 (b), LG2 (c), and ZL (d) families. Fitted linear model for each family are indicated.



sion depending on the abundance of the parental species in an area would be consistent with the findings in *Eucalyptus* (Field et al. 2008), *Senecio* (Prentis et al. 2007) and some mangroves (Zhou et al. 2008).

Based on differential growth rate of pollen tubes, Boavida et al. (2001) reported significantly higher success rate for the interspecific crosses with *Q. suber* acting as pollen donor rather than as female parent. However, the high number of hybrid offspring having *Q. ilex* as pollen donor, supports at least the partial breakage of this prezygotic incompatibility between introgressed hybrids and *Q. ilex* pollen. Notwithstanding, a potential effect of post-zygotic barriers on the observed gene flow directionality cannot be discarded. If some type of incompatibility existed between the two parental species, a high proportion of viable embryos could be expected for families of parent trees that have similar specific categories (estimated in our case through q_s). Interestingly, the majority of seedlings of the IS parent, classified as an “advanced introgressed” cork oak tree ($q_s = 0.8763$), had *Q. suber* as pollen donor. However, although both ZL and LG1 hybrids show intermediate q_s values (0.5489 and 0.5988, respectively), they show contrasting proportions of effective pollinations from *Q. suber* and *Q. ilex*. As previously stated, the *Q. ilex* neighborhood of LG1 could account for the abundance of LG1 \times *Q. ilex* backcrosses. In the case of ZL an overabundance of backcrosses to *Q. suber* was scored, considering the specific composition of the surrounding trees. Although the number of seedlings from this tree is still low to draw definitive conclu-

sions, this fact could be due to a phenological synchrony between this tree’s female flowers and pollination of the surrounding *Q. suber* (Perea García-Calvo 2006), or to post-pollination processes (Varela et al. 2008), such as genomic incompatibilities between this hybrid and surrounding *Q. ilex* individuals. As shown for European white oaks, first-generation hybrids will also more likely mate with the more abundant species, leading to potential asymmetric introgression (Lepais et al. 2009). Asymmetric introgression based on the abundance of the parental species has been described in other Mediterranean (Neophytou et al. 2011) and non-Mediterranean tree species (Bacilieri et al. 1996, Curtu et al. 2007, Eaton et al. 2015).

Conclusions

These results support current bidirectional introgression with *Q. suber* and *Q. ilex*. Analysis of the progenies of four open pollinated hybrid trees from a natural mixed population reveals that each mother tree is predominantly fertilized by one of the two parental species, usually the most abundant in its vicinity. The frequency of backcrossing could at least be partially driven by the availability of pollen when female flowers are receptive. However, other post-pollination processes, such as genetic incompatibilities cannot be excluded. To clarify this point, controlled pollinations and monitoring of pollen tube development and abortion of embryos should be performed. Additionally, further investigations should take advantage of modern high-throughput sequencing methodologies that will allow the examination of larger proportions of the species’

genomes. Moreover, using these methodologies, it may be possible to identify the genome portions that determine hybridizing capability, and/or reproductive isolation in the *Q. ilex* - *Q. suber* complex.

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AS and ULH conceived the idea, designed the experiments, collected the plant material and drafted the manuscript; HS and ULH genotyped the seedlings and adult trees; ULH, HS and AS performed the admixture and statistical analyses. All the authors have contributed to the final manuscript.

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Supplementary Material

Fig. S1 - Germination rates of the progenies of the hybrids, *Q. ilex* and *Q. suber* for 2011, 2012 and 2014.

Fig S2 - Most probable number of genetic groups (K) inferred with the test of Evanno et al. (2005).

Fig S3 - Barplots showing the relative contribution of *Q. ilex* and *Q. suber* to each offspring as the proportion of q_i and q_s , estimated with STRUCTURE.

Tab. S1 - Number of virtual individuals by the specific categories defined according to q_s intervals generated with SIMHYB (Soto et al. 2017).

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