Bioversity International

Quercus suber
Recent genetic research

Gösta Eriksson

EUFORGEN

European Forest Genetic Resources Programme (EUFORGEN)

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Foreword

This report was prepared as a supplement to the Technical Bulletin for the genetic conservation of cork oak. As such, it should not be considered a strictly scientific report, but rather based on scientific data. Only information available to the scientific community is quoted herein and investigations relevant to the genetic conservation of cork oak are included.

1. Genetic variation among and within populations

1.1 Traits related to adaptation

The first example is taken from a study involving an international series of provenance trials with populations from the entire distribution area of cork oak (Varela *et al.* 2015). Figure 1.1 reveals a large variation in relative height growth among the studied populations. The minimum relative growth in this Portuguese trial, ≈80%, was recorded for a Spanish population, while the highest growth originated from a Moroccan population, ≈124%. Generally, the Moroccan populations showed a positive growth trend. Figure 1.2 reveals a large variation in mortality among the populations, 23-59%. Six of the nine Portuguese populations had lower mortalities than the trial mean mortality.

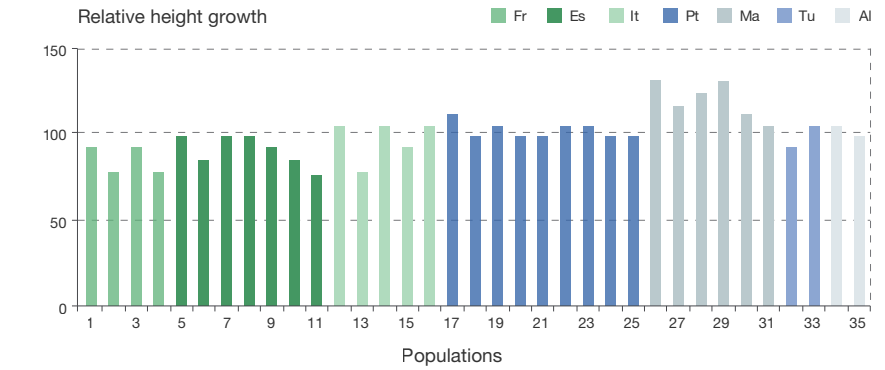

Figure 1.1. The relative height growth of 35 populations in the Portuguese Monte Fava trial, belonging to the international series of cork oak provenance trials. Trial mean height = 195 cm at age 9 (Varela *et al.* 2015).

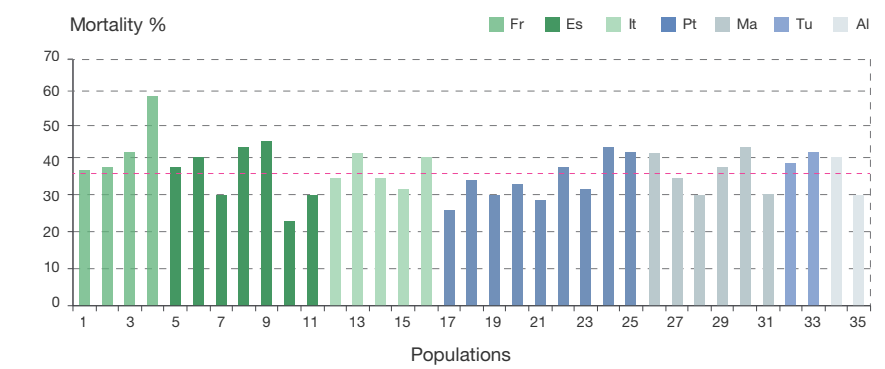

Figure 1.2. The mortality at age 9 in 35 populations studied in a Portuguese trial at Monte Fava, belonging to the international series of provenance trials with cork oak. The mean mortality was estimated at 37.5% (Varela *et al.* 2015).

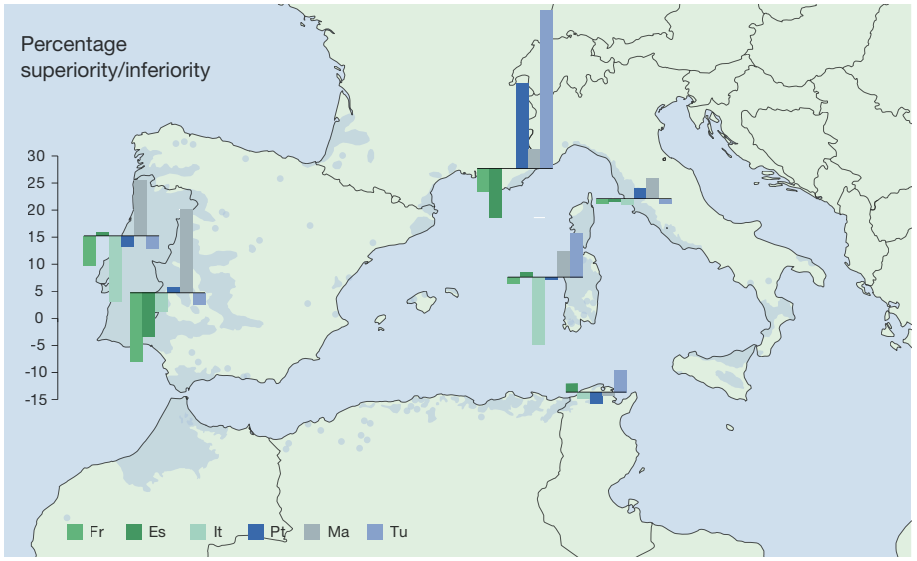

Figure 1.3. The average superiority or inferiority in tree height in percentage of populations from France (4 pops.), Italy (5 pops.), Morocco (6 pops.), Portugal (9 pops.), Spain (7 pops.) and Tunisia (2 pops.) in six provenance trials in France, Italy, Portugal and Tunisia. Measurements were carried out at various ages; 9 -14 years. The number of populations from the individual countries varied among trials (Varela *et al.* 2015).

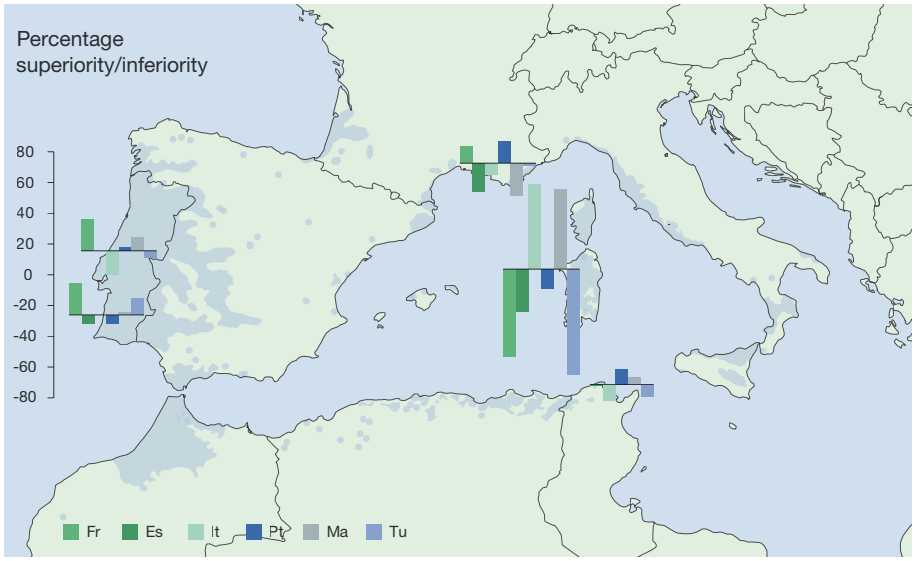

Figure 1.4. The average superiority or inferiority in survival of populations from France (4 pops.), Italy (5 pops.), Morocco (6 pops.), Portugal (9 pops.), Spain (7 pops.), and Tunisia (2 pops.) in five provenance trials in France, Italy, Portugal and Tunisia. Recordings were carried out at various ages; 9 - 14 years. The number of populations from the individual countries varied among trials (Varela *et al.* 2015).

In Figure 1.3 and Figure 1.4, the trends in populations' performance for select trials are illustrated. With the exception of the Tunisian trial, the Moroccan populations performed above the mean value within each trial. Moreover, the southern transfer of French populations resulted in a reduction in tree growth. In the French trial, the two Tunisian populations showed superior growth. The deviations from the mean mortality were limited, with the exception of the Sardinian trial. Large deviations in the Sardinian trial might be attributed to the low percentage of mortality.

Twenty-six of the populations in the above referenced international trials were also included in the Tunisian trial, in which assessments were carried out at age six (Gandour *et al.* 2007). The variation among populations with respect to growth and morphological traits was estimated by Q_{ST}, a corresponding estimate to F_{ST} estimates for marker traits (Figure 1.5). These estimates varied in the range of 0.13 to 0.25, with significant population differences. Significant positive correlations between longitude and the following traits were obtained: tree height, diameter at stem base, vigour, form and survival. A strong correlation between genetic and geographic distances was also noted. Two main geographic groups were revealed: the first comprising Morocco, Portugal and Spain; the second comprising Algeria, Italy and Tunisia.

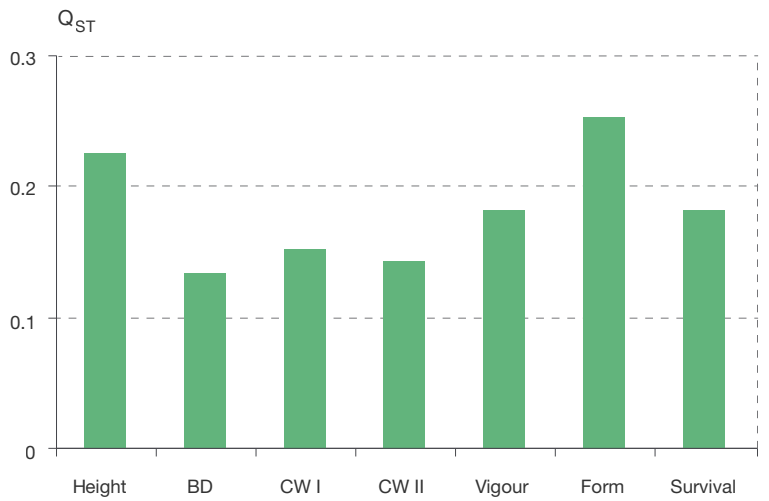

Figure 1.5. Population differentiation estimated by Q_{ST} for growth and morphological traits in a Tunisian field trial with 26 *Q. suber* populations. Assessments were carried out at age 6. BD = basal diameter; CW I and CW II = crown width at two perpendicular directions; form and vigour were classified in three classes (Gandour *et al.* 2007).

The relationship between alleles in six microsatellite loci and five traits assessed at age nine for 13 Spanish populations were reported by Ramirez-Valiente *et al.* (2010a). The field trial was located at latitude 39.85°N, longitude 6.17°W and altitude 375 masl. Figure 1.6 reveals a fairly strong relationship between the frequency of allele 188 and height and leaf size. Negative relationships with the two other alleles were found to be weaker. In addition, the trees homozygous for allele 188 were both the tallest and had the largest leaf size. It is encouraging that relationships between allele frequency in a microsatellite locus and adaptive traits were identified.

In the same trial, Ramirez-Valiente *et al.* (2010b) studied leaf size, specific leaf area (SLA), carbon isotope discrimination, nitrogen content and growth under two years, with contrasting summer precipitation at the trial site. The populations were grouped into five classes, according to climatic conditions at population origin. Significant differences among climatic groups were noted for all traits except for nitrogen content. Of particular interest is the differential performance of the populations under the two water availability conditions. In terms of shoot growth, Figure 1.7 reveals populations from the hottest conditions benefitted most from increased water availability. This means that there was a genetic variation in phenotypic plasticity for shoot growth among these populations. Analogous results were obtained for leaf size.

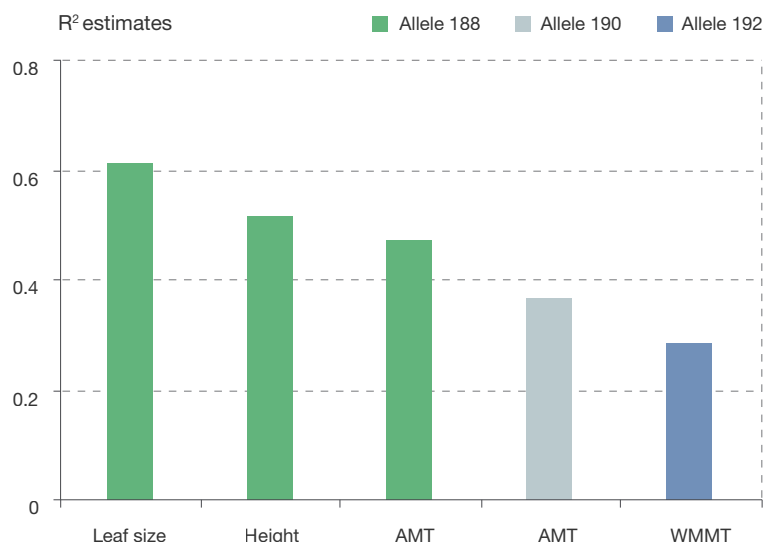

Figure 1.6. The strength of relationships between an allele in a microsatellite locus and growth traits or climatic variables. AMT = annual mean temperature, WMMT = winter mean minimum temperature. The relationships with alleles 190 and 192 are negative (Ramirez-Valiente *et al.* 2010a).

Photoinhibition[1] was studied in ten populations under drought conditions after the establishment phase passed (Aranda *et al.* 2005). The ratio between variable and maximum fluorescence was recorded over a three year period from November to November, revealing that the ratio difference among populations was significant during winter months. Photoinhibition was significantly related to annual mean temperature at population origin, $R^2 = 0.73$. No significance was noted for the corresponding relationship with annual precipitation.

Bulitta *et al.* (2011) reported on cork quality in 24 stands across eight regions of Sardinia, Italy. Two to three trees per stand were analysed in this pioneering work, comprising 26 traits related to cork quality. A selection of the strongest correlations identified include:

- **Cork volume mass** - cork resistance to 50% compression in radial, axial and tangential directions; positive correlations
- **Cork moisture** - cork resistance to 50% compression in radial, axial and tangential directions; positive correlations

1 Photoinhibition is the inhibition of photosynthesis due to high levels of light.

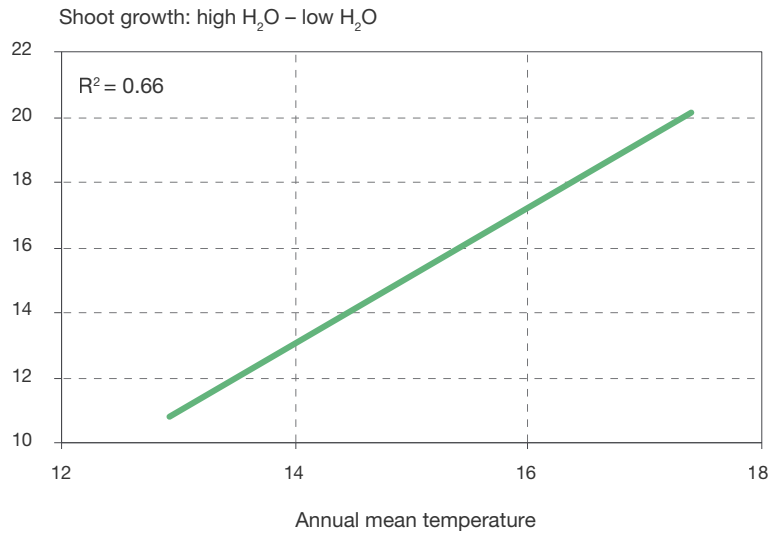

Figure 1.7. The relationship between annual mean temperature at population origin and the difference in shoot growth during a ”wet” and ”dry” summer, 27.5 and 79.6 mm precipitation (Ramirez-Valiente *et al.* 2010b).

- **Elevation** - % dimensional recovery of cork after 50% compression in the radial direction; positive correlation
- **Elevation** - cork moisture; negative correlation
- **Soil pH** - class of cork; positive correlation
- **Mg** - % dimensional recovery of cork after 50% compression in the radial direction; negative correlation.

The slower growth rate at high elevation promoted development of uniformity of ring width and thereby a good cork quality.

The survival of 13 Spanish cork oak populations was monitored over four years in a field trial at latitude 38.36°N, longitude 3.85°W and altitude 560 masl (Ramirez-Valiente *et al.* 2009). A xeric index (considering the difference between the monthly means of the coldest and hottest month and the monthly precipitation) for populations at the trial site was calculated. Figure 1.8 reveals a considerable and significant difference among populations in terms of survival after four years at the site with a high xeric index, 111.8. In theory, populations from xeric sites are expected to have a better survival rate than populations originating from less xeric sites. However, Figure 1.8 shows such a relationship is far from perfect or predictable. Populations with big acorns had a better survival rate than populations with small acorns.

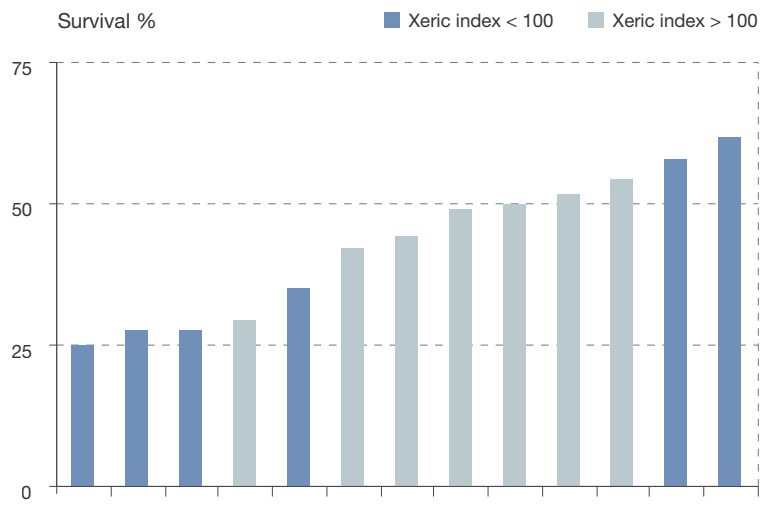

Figure 1.8. The percentage survival of 13 Spanish populations after four years in a field trial at latitude 38.36°N, longitude 3.85°W, and altitude 560 masl in southern Spain. Populations with xeric indices below and above 100 are shown with different colors (Ramirez-Valiente *et al.* 2009).

Variation in eight quantitative traits and microsatellite genotypes was studied in a Spanish field trial with three populations from Morocco, Portugal and Spain (Ramirez-Valiente *et al.* 2014b). Each population was represented by 15 open-pollinated families. Significant population differences were noted for flushing and two leaf traits, while three leaf traits displayed significant family differences. Carbon isotope discrimination and nitrogen content did not show any significant differences at the population or family levels.

The mean F_{ST} for microsatellite variation was estimated at 0.023. One microsatellite locus contributed most to this difference, $F_{ST} \approx 0.10$. The among-family F_{ST} for microsatellites was somewhat higher, 0.071. There was a noteworthy differentiation between two families within one population, amounting to almost 30%. A number of significant correlations were noted for relationships between microsatellite genotypes and leaf traits, but these relationships explained less than 40% of the observed variation.

In an earlier publication, Ramirez-Valiente *et al.* (2011) presented heritabilities for the same traits in the individual populations (Figure 1.9). It is somewhat disturbing that little consistency in the heritability estimates across populations was evident. Only the heritability for carbon isotope discrimination showed a level of consistency among the populations. It is likely that this trait is of adaptive significance.

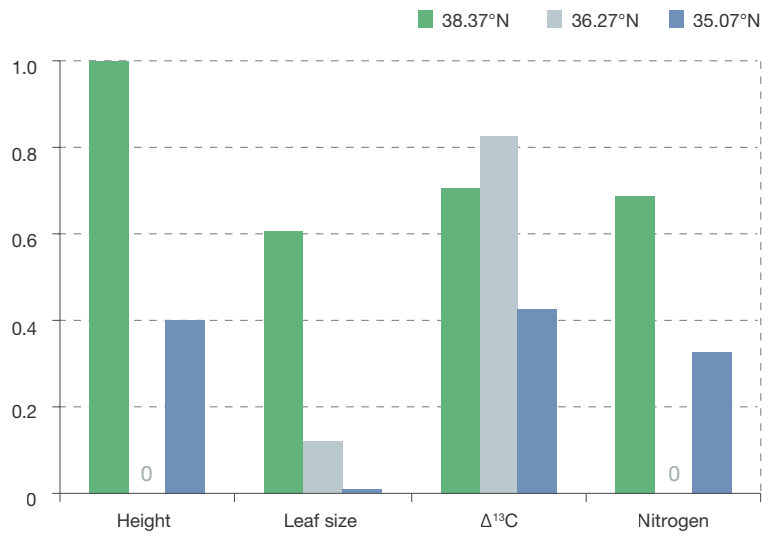

Figure 1.9. Heritabilities of four traits in three cork oak populations from Portugal, Spain and Morocco growing in a field trial at 38.35°N and 3.85°W (Ramirez-Valiente *et al.* 2011).

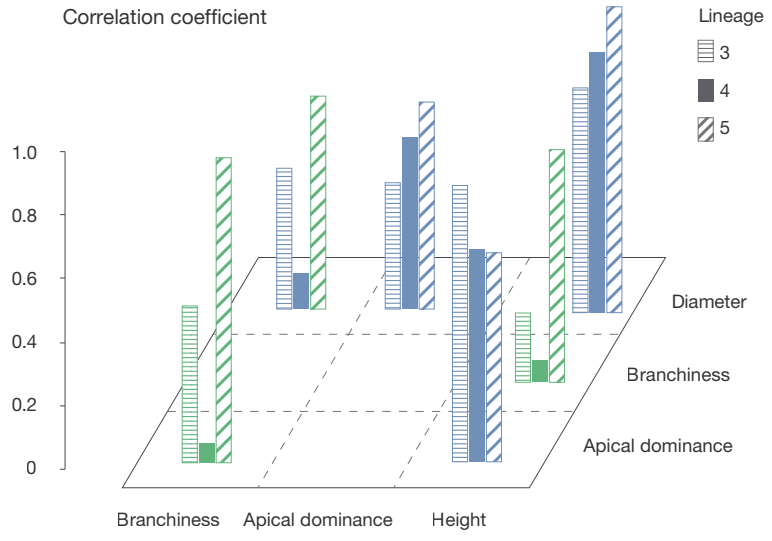

Figure 1.10. Pearson correlation coefficients between four traits in a Spanish provenance trial at latitude 39.85°N, longitude 6.02°W, altitude 275 masl. Separate correlations for three different chloroplast lineages were estimated. Columns in green are negative coefficients and in blue are positive coefficients (Ramirez-Valiente *et al.* 2014a).

In a field trial of 31 populations covering the entire cork oak distribution area at latitude 39.85°N, longitude 6.02°W and altitude 375 masl, Ramirez-Valiente *et al.* (2014a) reported significant population differences at age seven for tree height, diameter, apical dominance and branchiness. The effects of three chloroplast DNA (cpDNA) lineages on these four traits were investigated. Height and diameter lineage were non-significant, while branchiness and apical dominance were significantly related to lineage.

The relationships between the two latter traits and maximum temperature at the hottest month or minimum temperature during the coldest month were calculated. The Pearson correlation coefficients for these relationships were only higher than 0.60 twice. Generally, the degree of explanation of these relationships was low. The pairwise relationships among the four traits were estimated separately for the three lineages (Figure 1.10). As expected, the relationship between tree height and diameter resulted in all three correlation coefficients above 0.70 (0.71 – 0.96). Similarly, the correlation coefficients for the relationship between height and apical dominance were all above 0.60 (0.66 – 0.87). The relationships between branchiness and apical dominance or height were all negative, with varying degrees of strength. It would have been interesting to analyse relationships between the four traits and geographic or ambient factors, independent of the cpDNA lineage.

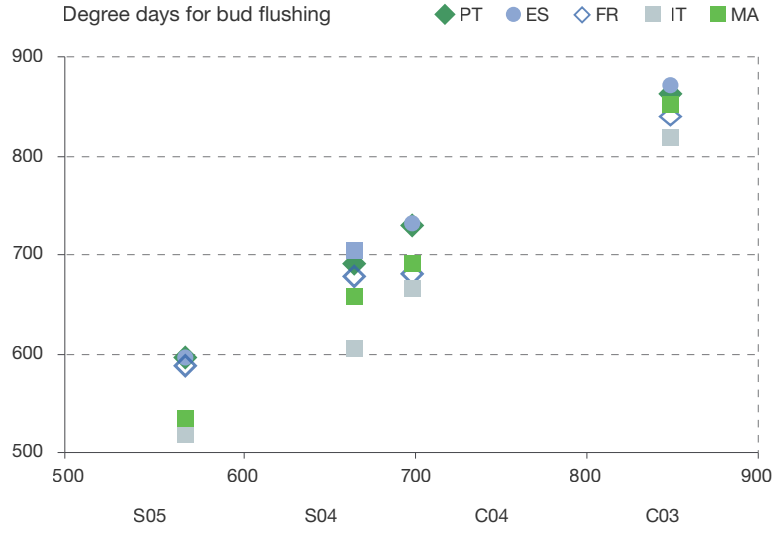

Figure 1.11. Mean degree days for bud flushing for French (4), Italian (5), Moroccan (6), Portuguese (9) and Spanish populations studied in two Portuguese provenance trials during two years. S and C stand for southern (lat. 38.00°N and long. 8.70°W) and central (lat. 39.05°N and long. 8.59°W) Portugal. 03 – 05 stand for years of assessment, 2003 – 2005 (Sampaio *et al.* 2016).

In two Portuguese trials belonging to the international series of provenance trials, bud flushing was recorded (Sampaio *et al.* 2016). A four-degree scale was used for flushing. Over two years, the trees were monitored weekly from February to May, at ages 5-6 and 6-7 in the two field trials, respectively. When two of four buds examined per tree had *very young green leaves* (= stage four), it was defined as bud flushing. After expansion of new leaves, leaf pest damage at ages six and seven was recorded in the southern trial, using the same trees as those studied for bud flushing. In addition, eleven nuclear microsatellites were studied to characterize the populations.

The joint analysis of bud flushing for the two trials revealed strongly significant population differences. Similarly, the effect of year was strongly significant, while the population x year interaction was non-significant. Eastern populations had a significantly earlier bud flushing than western populations. In Figure 1.11, it is apparent that the mean values for degree days to flushing were greater for Portuguese and Spanish populations than for Italian populations, at all recordings. French and Moroccan populations took an intermediate position. The separation of one western and one eastern group of populations was confirmed by the analysis of nuclear microsatellites.

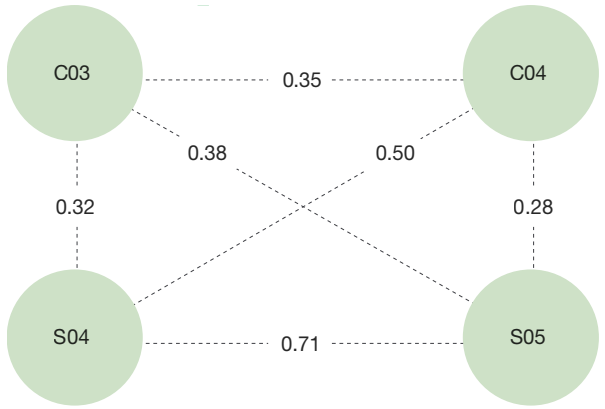

Figure 1.12. The R^2 estimates for the relationships between bud flushing dates of 35 populations recorded over two years in two Portuguese provenance trials. C and S stand for southern (lat. 38.00°N and long. 8.70°W) and central (lat. 39.05°N and long. 8.59°W) Portugal. 03 – 05 stand for years of assessment, 2003 – 2005 (Sampaio *et al*. 2016).

Based on Pearson correlations, the stability over trials and years was not strong, with the exception of bud flushing dates during two years in the southern trial (Figure 1.12). All other relationships had a degree of explanation of 50% or less. The repeatability for bud flushing was strong in both field trials and study years, 0.79 – 0.94. The population effect was regarded as fixed, meaning no variance component for the population effect was estimated. This also means narrow-sense heritability was not estimated. The relationship between bud flushing and geographic variables (latitude, longitude and altitude) or climatic variables (annual mean temperature and precipitation) for the population origins were tested for each trial and study year. The four relationships with longitude were all negative and significant. Similarly, three of the relationships with mean temperature were negative and significant. However, the degree of explanation was low in all cases, < 30%. Significant effects of bud flushing on percentage of damaged leaves were noted, but the degrees of explanation for these relationships were low, 19 and 22% for the two years of observation.

1.2 Markers

1.2.1 Isozymes

As with many other tree species, isozyme variation has been studied in cork oak populations. In Table 1.1, the main results from four investigations have been compiled. From a genetic conservation perspective, the differentiation among populations is of largest significance. The F_{IS} or G_{IS} estimates for isozyme variability were larger than 0.10 in two investigations, which are among the largest estimates for a wind-pollinated tree species. Also, the F_{IS} estimates were high in two of the investigations, >0.10. This suggests a substantial inbreeding in select populations.

Table 1.1. Genetic parameters of cork oak populations in different studies

Populations	Number of polymorphic loci	H_o	F_{ST} / G_{ST}	F_{IS}	Observations	Reference
7 - Spanish	10	0.262	0.169	0.173	No linkage with geography	Elena-Rosello (1996)
40 - Entire distribution area	7	0.283	0.11	-0.001	Two geographically distinct groups	Tuomi-Lumaret (1998)
18 - Mainly Spanish	14	0.145	0.033	0.118	Significantly lower diversity in marginal populations	Jimenez *et al.* (1999)
10 - Entire distribution area	7	Not reported	0.08	-0.04	Focus on relationship with other oak species	Tuomi-Lumaret (2001)

The high estimate of F_{IS} in the paper by Jimenez *et al.* (1999) might be attributed to the inclusion of marginal populations. These populations had lower genetic diversity than central populations and contributed most to population differentiation.

In spite of the low G_{ST} estimate, Toumi and Lumaret (1998) distinguished two large groups of populations. One group included 18 populations from the Iberian Peninsula and two western French populations (Landes and Roussillon), referred to as the "western group" below. The second group comprised 16 populations from North Africa, Italy, Provence and the islands of Corsica and Sardinia, referred to as the "eastern group" below. The genetic differentiation among populations was observed to be lower in the eastern group than that of the western. The western group was characterized by a higher percentage of polymorphic loci and higher number of alleles per locus. According to the authors, this difference may be due to a genetic bottleneck related to founder effects and genetic drift, which eliminated the low frequency alleles in the geographical area corresponding to the eastern group. Alternatively, postglacial re-colonization from southern to northern regions may have occurred independently in the western and the eastern parts of cork oak distribution from distinct refugia.

The absence of any geographic differentiation among the seven populations studied by Elena-Rossello and Cabrero (1996) might be attributed to the low number of populations and the relatively narrow origin of the populations.

Jimenez *et al.* (1999) noted a difference between Spanish populations located in central and marginal areas of cork oak, with a significant component of the total genetic diversity contributed by marginal populations. The difference in level of inbreeding estimated with F_{IS} did not differ among the types of populations. According to a specific bottleneck test, two isolated Spanish populations were found to have passed a bottleneck in population number.

As regards isozyme variation, Toumi and Lumaret (2001) concluded that *Q. suber* deviated strongly from *Q. ilex*.

In these studies, the average number of alleles per locus varied in the range from 2.0 to 2.5, which is close to the highest estimates for oak species (Kremer and Petit 1993). Similarly, high values of observed heterozygosity (from 0.16 to 0.28, according to the set of loci analysed) and of polymorphic loci (64% to 80%) were observed.

1.2.2 Nuclear microsatellite variation

Hornero *et al.* (2001) investigated whether microsatellites developed for other oak species might also be used in cork oak. A low percentage, 7.5%, was polymorphic, with a maximum number of 19 alleles in one locus. The most remarkable result was the high F_{IS} estimates, 0.27 and 0.34, for two loci originally developed for *Q. robur*. These estimates suggest substantial inbreeding.

Similarly, Soto *et al.* (2003) tested microsatellites developed for *Q. macrocarpa* and *Q. petraea* for use in cork oak and holm oak. Crosses were carried out between holm oak and cork oak trees in a mixed stand containing the two species. Six microsatellites could be used.

Three microsatellites were used for identification of twelve trees from two populations in Spain (Gomez *et al.* 2001). The strong polymorphism in these three loci enabled the identification of ten of the twelve trees. Embryo cultures of 24 embryos originating from one tree revealed all of the tree's alleles were found in its progeny, verifying the usefulness of these markers.

Soto *et al.* (2007) analysed the spatial relationship in an 11.2-hectare mixed stand of cork oak and holm oak in Central Spain. Ninety-five and 96 adult trees of each species were genotyped, with the aid of nine microsatellites. In addition, 12 and 18 young trees were genotyped. Beyond 100 meters, there was no genetic structure. Up to this distance, there was a weak and negative relationship between distance and kinship. For the young trees, no clustering was detected. However, far reaching conclusions were advised against, given the limited number of trees included in the study. In addition, the parentage

analysis suggested occurrence of high gene flow from trees outside the study plot. No signs of inbreeding were noted.

The analysis of variation among and within 24 cork oak stands from Sardinia, Italy by RAPD[2], revealed 21% of the variation was attributed to among-population variation, while 79% was due to variation within the populations (Bulitta *et al.* 2011). The RAPD grouping agreed with the geographic origin of the populations.

In a study of gene flow between cork oak and holm oak, Lumaret and Jabbor-Zahab (2009) analysed ten microsatellite loci in 13 pure species populations of cork oak and 13 mixed populations of cork oak and holm oak. Five of the pure species populations occurred in the sympatric distribution area of the two species. There was a geographic structuring of the populations, with one western and one eastern group. The G_{ST} and R_{ST} for the eight allopatric populations were 0.060 and 0.092, respectively. The corresponding estimates for all cork oak populations were 0.075 and 0.093. For both groups of populations, the population differentiation estimates were in the range of differentiation noted for isozymes. The mean F_{IS} for the allopatric populations was low (0.03), but with considerable variation among the loci, ranging from -0.13 to 0.20. The F_{IS} estimates for all populations did not differ greatly from those of the allopatric populations; the mean value was 0.02 and the range of estimates for individual loci was more or less identical.

Fifty-three elite trees were genotyped to test the possibility to identify individual trees with ISSR[3] and SSR[4] markers (López-Aljorna *et al.* 2007). More than 90% of the trees could be identified. No relationship between the genotypes of the elite trees was observed.

1.2.3 Cytoplasmic DNA variation

Mitochondrial and chloroplast DNA (cpDNA) have both been shown to be maternally inherited in oaks (Dumolin *et al.* 1995). Chlorotypes[5] were used in several studies on the phylogeography of evergreen Mediterranean oaks. Three groups of chlorotypes were identified and designated as *suber, ilex-coccifera I,* and *ilex-coccifera II*.

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2 'Random Amplification of Polymorphic DNA

3 Inter-simple Sequence Repeat

4 Simple-sequence Repeat

5 A chlorotype is defined as a combination of SNP located on the chloroplast, i.e. a haplotype based on chloroplast SNP.

One mitochondrial and two cpDNA primer pairs were studied in 73 cork oak populations in Morocco (Belahbib *et al.* 2001). Thirty-one of these populations were mixed populations with *Q. ilex*. The G_{ST} estimate for *Q. suber* was 0.84, while it was much lower for *Q. ilex*, 0.33.

Chloroplast DNA variation was studied in numerous populations from the whole distribution areas of cork oak, holm oak and holly oak, using a RFLP[6] method on the whole cpDNA molecule after chloroplast isolation (Lumaret *et al.* 2002). It was found that cork oak possesses much lower cytoplasmic DNA variation than holm oak, especially for cpDNA.

In the Spanish cork oak populations, the highest haplotype diversity was observed in the Sierra Morena Mountains and in the two largest Balearic Islands, where 7 distinct *Q. suber* haplotypes were observed in nine small populations (Lopez de Heredia *et al.* 2005). The four haplotypes from Majorca did not occur on Minorca, where three other haplotypes were found. In conclusion, the Balearic Islands must be regarded as a refuge area for cork oak and measures should be taken to conserve Balearic cork oak populations. Moreover, holm oak haplotypes were observed predominantly in trees morphologically intermediate between the two species, whereas cork oak haplotypes were found very occasionally in those intermediate individuals (Lumaret *et al.* 2002).

Focusing mainly on the phylogeography of *Q. suber,* Lumaret *et al.* (2005) analysed 91 populations from the entire range of distribution with respect to chlorotypes. Western populations, including Portugal, Morocco and Spain had *suber* chlorotypes, while most other populations had *ilex-coccifera* chlorotypes.

Five hundred and eighty-seven *Q. suber* trees from 60 populations in the Iberian Peninsula were analysed in terms of chlorotypes (Lopez de Heredia *et al.* 2007a). Most cork oak populations had a *suber* lineage of chlorotypes, while populations in Central and North-eastern Spain had the *ilex-coccifera I* lineage.

Jimenez *et al.* (2004) studied 90 *Q. suber* populations and reported that 40% had the *ilex-coccifera* lineage. This lineage dominated the western part of the distribution area of cork oak.

Partitioning of the variation of three species - *Q. coccifera, Q. ilex*, and *Q. suber* - after AFLP[7] analysis showed 65.5% was attributed to differences among the species; 8.2% to clusters within the species; and 26.3% to variation within clusters (Lopez de Heredia 2007b). Three clusters (≈ geographic regions) were identified for *Q. suber*. In the western Iberian Peninsula and in Morocco, one chlorotype from *suber* lineage occurs widely, while other chlorotypes of this lineage occur in the eastern part of *Q. suber* distribution area, in which high cpDNA diversity exists.

6 Restriction Fragment Length Polymorphism

7 Amplified Fragment Length Polymorphism

Five different haplotypes were detected in 110 *Q. suber* populations from the entire range of the distribution (Magri *et al.* 2007). Extremely high G_{ST} and R_{ST} estimates were noted, both 0.96. A low number of trees per population and monomorphism in 105 populations explain these high estimates. Monomorphism for different haplotypes leads to high estimates of population differentiation. Five clearly distinguished geographic regions were noted:

- Portugal and neighbouring regions in Spain, Western Morocco and South-western France;
- The rest of Spain, including the Balearic Islands, and Eastern Morocco;
- Provence, Liguria Corsica, Sardinia, Algeria and Tunisia;
- Central and Southern Italy;
- North-Western coastal range of Italy.

Eleven chlorotypes were identified in the study of gene flow by Lumaret and Jabbour-Zahab (2009). Three lineage *suber* chlorotypes and four lineage *ilex* were detected in cork oak populations. One *ilex* chlorotype was found in one tree in one mixed population. Three other *ilex* chlorotypes were found in all trees in one population from Morocco, and in Spanish and French Catalonia populations. Thus, there is a total substitution of the *suber* lineage chlorotypes by *ilex* chlorotypes in certain regions of cork oak distribution. The population differentiation was large, amounting to 0.948. As stated elsewhere, such high estimates for population differentiation must be attributed to monomorphism for different haplotypes in different populations.

1.2.4 Ribosomal DNA variation

Bellarosa *et al.* (2005) studied the nuclear ribosomal DNA sequences encoding the 5.8S RNA in two *Q. suber* populations, as well as in 14 other populations from several oak species and possible interspecific hybrids. The study focused on the phylogeny of the Italian oak species. For this purpose, two internal transcribed spacers (ITS1 and ITS2) were analysed in the study populations. Cork oak was the first species possessing two ITS, one long and one short. The results confirmed the existing subdivision of genus *Quercus* based on morphological data.

1.2.5 Seed storage protein variation in cork oak

Seed storage protein markers might be used in studies of phylogeny of Mediterranean oaks (Bellarosa pers. comm.).

1.2.6 Conclusions as regards genetic variation

Substantial population differentiation was noted for several traits related to adaptedness. Haplotypes showed extremely large population differentiation in some cases, which must be attributed to the high percentage of monomorphic populations and with monomorphism for different haplotypes. The differentiation is limited when isozymes are used for studies of among-population variation, while microsatellites or RAPDs show a larger differentiation. Several studies reported a separation of eastern and western populations. Further subdivisions were also noted in some reports. One typical example for marker differentiation is given in Figure 1.13, based on a study of 19 Portuguese populations of cork oak with AFLP markers (Coelho *et al.* 2006).

1.3 Fruiting and regeneration studies

The flowering in six Portuguese populations was reported by Varela (2015). In the Quinta da Serra population, 20 trees were followed on annual basis from 1993 to 2014, with the exception of 2005. Three scoring classes were used: 1 = no or limited acorn production; 2 = half of the productive part of the crown produces acorns; and 3 = large quantity of acorns. Figure 1.14 reveals there was fluctuation in flowering score values between 1.5 and 2.5, without any clear pattern. As in many other cases, different trees contributed in varying degree to the acorn crop over this time period. Trees were ranked with acorn production in groups of five. Figure 1.15 reveals a considerable difference between the five most abundant flowering trees and the group of poorest flowering trees.

In one stand, both *Q. suber* and *Q. ilex* occurred. During the year of observation, the acorn scoring was estimated at 1.29 for *Q. suber* and 2.17 for *Q. ilex*.

Regeneration in one of the smallest existing *Q. suber* populations was studied by Pausas *et al.* (2006), who reported that virtually no regeneration took place in the shrubland area, but did occur both in *Q. suber* and *Pinus pinaster* parts. However, for satisfactory regeneration, shrub clearing is necessary to promote regeneration in the shrubland area, as is logging of pines to promote *Q. suber* seedling growth.

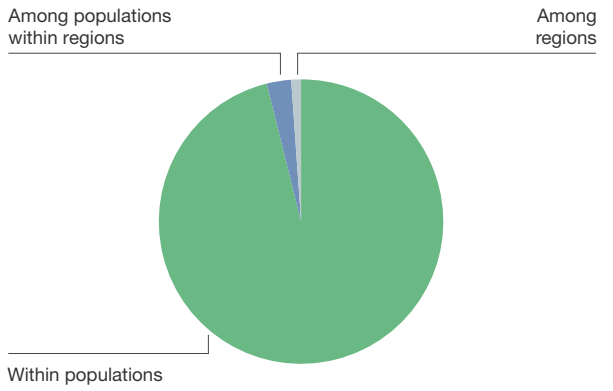

Figure 1.13.
The separation of the variation within populations, among populations within regions, and among three climatic regions in Portugal. The analysis was carried out by AFLPs (Coelho *et al.* 2006).

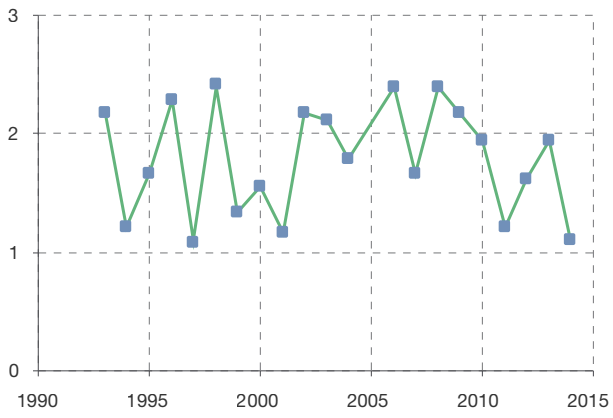

Figure 1.14.
The acorn crop scores from 1993 to 2014 in a cork oak stand at Quinta da Serra, Portugal. Three classes were used; 1 = no or limited acorn production, 2 = half of the productive part of the crown produces acorns, 3 = large quantity of acorns (Varela 2015).

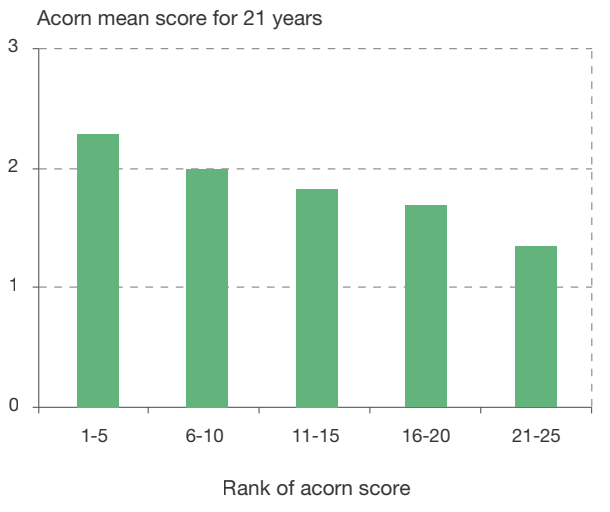

Figure 1.15.
The mean acorn score for cork oak trees in Quinta da Serra, Portugal based on scoring during 21 years, 1993 – 2014. The 25 trees were grouped into five classes dependent on acorn score. Three classes of scoring were used; 1 = no or limited acorn production, 2 = half of the productive part of the crown produces acorns, 3 = large quantity of acorns (Varela 2015).

Regeneration in three marginal cork oak populations of varying sizes in Spain was studied by Pons and Pausas (2006). A selection of the results are summarised in Figure 1.16, which reveals that type of plant community greatly impacts success in regeneration. Most problematic was regeneration in shrublands, as the three forest communities revealed the highest number of seedlings. However, the number of young trees was fewer than in open field communities. Evidently, seedlings suffer from competition with existing trees in forest communities.

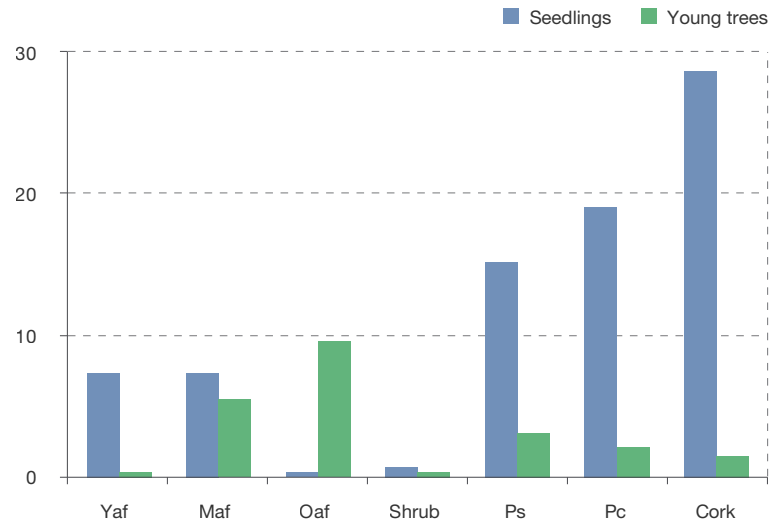

Figure 1.16. Number of seedlings and young trees in different plant communities in marginal populations in eastern Spain. Yaf = young abandoned field, Oaf = old abandoned field, Maf = intermediate to the two former, Shrub = shrublands, Ps = pine forest with shrubby understory, Pc = Pine forest with low understory; Cork = cork oak forest (Pons and Pausas 2006).

2. Hybridization

Cork oak is known to hybridize with other oak species. *Q. crenata* and *Q. afarès* are well-known hybrids with *Q. cerris* and *Q. canariensis,* respectively. Also, back-crosses with parental species occur, which may result in recovering part of the genome of one parental species in the other parental species.

Elena-Rosello (1992) reported that alleles in three isozyme loci (*AcPH1, Est1* and *LAP1*) discriminated between cork oak and holm oak. In a stand with 35 juvenile trees, eight adult holm oak trees and three adult cork oak trees were genotyped. Based on its genotype, one of the 35 juvenile trees was classified as an inter-specific hybrid tree. The authors reported the two species' flowering periods did not usually overlap. During exceptionally cold summers, an overlap was observed, indicating there was a potential for hybridization in such years.

Information from the *Pgi1* locus was used by Toumi and Lumaret (1998) to identify hybrids between cork oak and holm oak. In the 14 cork oak populations isolated from holm oak, and in 18 of the 26 cork oak populations from mixed stands, Toumi and Lumaret (1998) note, *specific alleles not observed in any other evergreen oak species of the Mediterranean area were found exclusively at the diagnostic locus* Pgi1. This suggests there was no introgression in these 32 populations of cork oak. Contrary to this, eight other cork oak populations in contact with holm oak had ten alleles which frequently occur in holm oak, suggesting introgression had taken place in these eight populations. It was suggested that introgression most frequently occurs from cork oak to holm oak. Support for this hypothesis is the protandry of the two species and a much earlier flowering in holm oak than in cork oak. This means late receptive female flowers of holm oak might be pollinated by early male flowering cork oak trees. It was noted that the interspecific hybridizations took place independently in different stands. The presence of local "holm oak alleles" in different cork oak populations supports this hypothesis. Alleles specific to holm oak were recovered in cork oak populations, mostly in areas where holm oak is predominant or was predominant in the past (*e.g.* in eastern and southern Spain, in Roussillon and in the mountains of Morocco).

In a study of introgression between cork oak and holm oak, Belahbib *et al.* (2001) collected material from 97 cork oak populations across seven regions in Morocco. Thirty-one of these populations were mixed populations with *Q. ilex*. In a first step, which included 24 populations, five pairs of chloroplast primers and two pairs of mitochondrial primers were used. In a second stage, including 73 populations, three chloroplast and one mitochondrial pair of primers were used.

Based on the results from the 31 mixed populations, an introgression ratio was calculated. The ratio is equal to 1.0 when the genetic variation is species-independent and zero when the two species are fully differentiated. The introgression index is estimated at 0.63 - higher than the expected introgression ratio of 0.33 - assuming an independent distribution of the

haplotypes in the two species. This suggests introgression between cork oak and holm oak did occur. The results of this investigation indicate gene flow is mainly from cork to holm oak. Belhabib *et al.* (2001) suggest *hybridization and introgression play some significant adaptive role*.

One of the most striking results in the study of Lumaret *et al.* (2002), was the detection of cork oak populations showing predominantly, or even exclusively, haplotypes belonging to holm oak lineage in the northeast and southern areas of Morocco, the eastern Iberian Peninsula, Balearic Islands and in French Catalonia. This is considered to be due to cork oak cytoplasmic introgression by holm oak. In a majority of mixed populations, distinct holm oak haplotypes were observed in the two species, suggesting the occurrence of ancient exchanges followed by differential migrations and divergence. At other select sites, the same haplotype was shared by both species, possibly as the result of more recent exchanges.

Boavida et al. (2001) carried out matings between cork oak or holm oak trees with pollen from the same species or from another species. Selfings were also carried out. Generally, the seed set was poor in most matings. It turned out that the *Q. ilex* x *Q. suber* mating resulted in a seed set of 25%, while the reciprocal cross did not result in any acorn production (Table 2.1). This was attributed to inability of the pollen tubes of *Q. ilex* to penetrate the stigmatic surface after germination. The *Q. suber* matings with *Q. faginea* and *Q. coccifera* resulted in low seed sets. These results suggest introgression into *Q. suber* by other oak species does not occur to any great extent. The selfing percentage was astonishingly high, 13.9% in *Q. ilex*.

Two of the six microsatellites mentioned above (Soto *et al.* 2007), *QpZAG9* and *MSQ13*, proved to be diagnostic between *Q. ilex* and *Q. suber* and allowed identification of interspecific hybrids, thus avoiding parentage analysis (Soto *et al.* 2003). Artificial species crosses *Q. ilex* x *Q. suber* were carried out with four parents of each species. Few acorns and seedlings were obtained. Of the 16 seedlings obtained from these crosses, three were identified as species hybrids.

Burgarella *et al.* (2009) used eight microsatellite loci for identification of pure *Q. suber* and *Q. ilex* trees among 1,487 trees in mixed stands of the two species across the range of distribution of cork oak. Dependent on the statistical evaluation technique, only 17 or 5 of these trees were genotyped as non-pure species. These trees were F_1 or back-crosses with either of the parental species. It was concluded

Table 2.1. Percentage seed set following intra- and interspecific crosses between *Q. suber* and other oak species. Results from selfings are also given.

	Male				
Female	*Q. suber*	*Q. ilex*	*Q. faginea*	*Q. coccifera*	Selfing
Q. suber	3	0	2	3	1
Q. ilex	25	35	No crosses carried out		14

that spontaneous hybridization between these two oak species occurs at a low frequency in nature. Contrary to earlier results, no asymmetry in the introgression between cork oak and holm oak was noted. The introgression rate was estimated at 2%.

Genotyping of 362 randomly sampled cork oak trees and 187 holm oak trees at 33 sites in French Catalonia and 13 sites in Provence was carried out by Mir *et al.* (2009). The morphology of 33 of the sampled trees showed characteristics of both species, suggesting hybrid origin. Three isozyme loci were studied, *PGI1, AcPh* and *LAP1.* The first two were fully diagnostic, and the third semi-diagnostic, as one allele was shared between the two species. In addition, five chloroplast haplotypes were analysed. Of the 33 morphologically intermediate trees, 23 turned out to be hybrids, with all growing in mixed stands. A hybrid index was calculated such that a value of zero represented pure *Q. ilex,* while 1.0 represented the index for pure *Q. suber*.

The 12 hybrids from French Catalonia had a mean hybrid index of 0.42; the corresponding estimate for the 11 hybrids in Provence was 0.48. The hybrid indices for holm oak populations and cork populations were close to the pure species values, i.e. <0.07 in holm oak and >0.95 in cork oak.

The chlorotype frequencies differed considerably in the two species, with chlorotype 21 dominating in holm oak (Figure 2.1). It was assumed that crosses

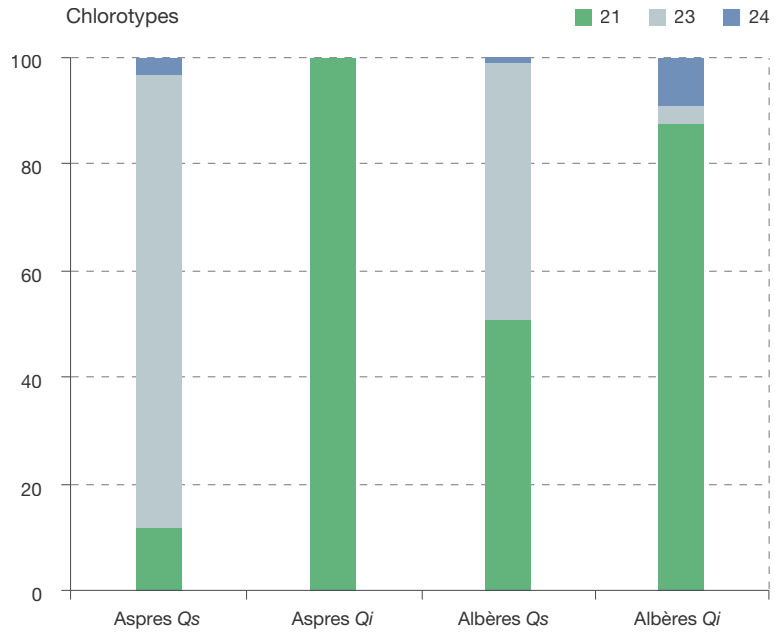

Figure 2.1. The frequency of three chlorotypes in cork oak and holm oak in two regions of French Catalonia. *Qs* and *Qi* are *Quercus suber* and *Q. ilex*, respectively (Mir *et al.* 2009).

among hybrids were rare and mainly involved back-crosses with parental species. The types of chloroplasts present in the hybrids suggests those in French Catalonia resulted from the cross of female holm oak with male cork oak, while two individuals in Provence suggest a reciprocal cross. This study revealed there was no dependence between nuclear and cytoplasmic introgression.

Isozyme genotypes were used to estimate the type and frequency of back-crosses in progenies from three *Q. ilex* x *Q. suber* hybrid trees in mixed forests (Oliveira *et al.* 2003). The estimates of back-crosses with *Q. suber* were 38%, 76% and 77%, respectively. Another important result of this study was the low occurrence of interspecific hybrids, 1%.

Information on hybridization between *Q. ilex* and *Q. suber* is available in the report by Staudt *et al.* (2004), focusing on species differences in emissions of chemical compounds. The study focused on two French localities, one in French Catalonia and the other on an island in Provence. In the French Catalonian population, 21 trees of each species were genotyped. All *Q. suber* trees had *ilex* chlorotypes, while the 21 *Q. ilex* trees all had *ilex* chlorotypes. Seven trees from Provence and 12 trees from Catalonia had molecular markers from both species in one or three loci, suggesting they are hybrids. The Catalonian trees had alleles from the opposite species in one locus, while the trees from Provence had alleles in two or three loci from the opposite species, suggesting hybridization was more recent at the Provence locality than that of French Catalonia.

Lumaret and Jabbour-Zahab (2009) studied gene flow between *Q. ilex* and *Q. suber* in eight allopatric and 13 sympatric populations of *Q. suber* and *Q. ilex*. Eight microsatellite loci were used to study the introgression. Bidirectional introgression was observed and estimated at 5%. A fraction (1.2%) were F_1 individuals. As expected, introgression primarily took place in mixed forests with the two species. The greater overlap in the two species' flowering times in the eastern section of the distribution area increased the probability for introgression. Open-pollinated acorns were collected from two morphologically *Q. ilex* trees and two potential hybrids, all four trees possessing an *ilex* chlorotype. The mean mortality after seven years in a field experiment was higher in the two hybrid trees' progeny than in the *Q. ilex* progenies, 76.9% and 33.3%, respectively. It was noted that all progeny trees resulted from outcrossing as alleles not occurring in the mother trees were found in the progeny. For three of the mother trees, molecular markers suggested all progeny individuals originated from crosses with *Q. ilex* trees. Two individuals from the fourth mother tree, one of which was a dwarf, were likely the result of a cross with *Q. suber*. In conclusion, the current rates of gene flow between the two species were found to be low and did not vary between geographic regions.

Phenological observations of receptivity and pollen dispersal are also useful for understanding of the potential for hybridization between *Q. suber* and *Q. ilex*, especially in mixed stands. This issue was investigated by Varela and Valdiviesso (1996) and Varela *et al.* (2008), who reported flowering usually occurs earlier in *Q. ilex* than in *Q. suber*, reducing the possibility for hybridization. However, under certain weather conditions, the flowering overlap might be more than two weeks.

2.1 Conclusions concerning interspecific hybridization with cork oak

Hybrids between cork oak and holm oak occur spontaneously in nature. However, the frequency of hybridization is low according to most reports. Conflicting results concerning asymmetric or non-asymmetric introgression have been reported. In case of asymmetry, cork oak is the pollinator of holm oak, according to some reports. This is attributed to the protandry in both species enabling early flowering cork oaks to pollinate late flowering holm oak trees. It is likely that the phenological stages of flowering are influenced by weather conditions in individual years, meaning that the overlap in flowering which occurs enables an interspecific hybridization. Poor vigour of F_1 species hybrids was reported in one instance.

3. Lessons for the genetic conservation of cork oak

The large variation among populations in fitness-contributing traits such as growth and survival indicates that genetic conservation should encompass populations from the entire range of abiotic and biotic conditions in the distribution area of the species. Marginal populations were included in some studies, which showed specific genetic constitutions. Thus, marginal populations deserve to be conserved.

The low frequency of introgression between holm oak and cork oak, as well as the poor vigour of F_1 interspecific hybrids, suggest concerns about introgression should not be too alarming. However, reports focusing on introgression between holm oak and cork oak clearly show that introgression between these two species has occurred in the past. Therefore, a safe distance between genetic conservation populations of cork oak and holm oak populations is justified.

The relatively large inbreeding in some reports calls for large genetic resource populations to avoid any inbreeding.

To guarantee a satisfactory regeneration in genetic resource populations, silvicultural measures might be needed.

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