HYBRIDS BETWEEN CORK OAK AND HOLM OAK: ISOENZYME ANALYSIS

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ABSTRACT

Hybrids between cork oak and holm oak occur in a large range of overlap between these two species in Portugal, and the subsequent crosspollination involving the hybrids and cork oak has been postulated to result in some of the worst cork-producing trees in Portugal. A methodological framework was set for the routine large scale detection of hybrid genotypes using isoenzyme analysis. The effectiveness of isoenzyme markers enabling the discrimination of the hybrid trees from both parent species was probed on the nursery-grown progenies derived from reference "pure" populations, selected mixed stands and hybrid individuals. No spontaneous hybrid was detected among 1141 seedlings originating in the mixed stands, thus suggesting that the incidence of hybridization is quite low. The seedlings derived from 3 hybrids confirmed that the discriminant isoenzyme loci recombined and segregated the parental types, and a strong preference for mating with cork oak in two of these hybrids was suggested. A substantial increase of the fixation index *F* within a single generation for the polymorphic cork oak phosphoglucose isomerase locus may indicate that a significant proportion of seedlings derive from short-range pollen, a factor that might weigh on the formation of hybrids between these two species.

Keywords: *Quercus suber* L.; *Quercus ilex* L. ssp. *rotundifolia* (Lam.) Tab. Morais; cork oak; cork quality; oak hybridization; isoenzyme analysis

INTRODUCTION

Cork oak (*Quercus suber* L.) is the only source for industrial cork, a raw material on high demand for the production of wine bottle stoppers and as an insulating material for construction. Most of its supply comes from more or less managed forests within its natural range, the Western Mediterranean, of which the 7×10^5 hectares in Portugal represent the most important share in a single country (DSPE 2001). The massive use of propagation materials in recent years for afforestation of agricultural areas, and the rising need to provide conditions for the natural regeneration in old stands, are factors that give a strong relevance to the question of how prevalent is the natural hybridization of this species with other oaks, with implications for the certification of propagation materials and the judgement on the proper management of mixed stands.

Within the genus *Quercus*, the formation of fertile interspecific hybrids is widely recorded, especially within subgenera or sections (SCHWARZ 1936-7, NIXON 1993, FRANCO 1990, TUTIN 1964, DUCOUSSO *et al.* 1993). Several combinations involving the cork oak have been characterized in Portugal (NATIVIDADE 1950), but the large extent of overlap of its geographic range with that of the sweet acorn holm oak *Q. ilex* L. ssp. *rotundifolia* (Lam.) Tab. Morais, estimated to be over 2×10^5 hectares of mixed stands dominated by at least one of these two species, is expected to make the hybridization with the latter the most likely (VASCONCELOS & FRANCO 1954) in spite of the fact that the parent species are considered to belong in separate subgenera (FRANCO 1990, TUTIN 1964, TOUMI & LUMARET 1998, BELLAROSA 2003). The *Quercus ilex* × *suber* P. Cout. (= *Q.* × *mixta* Villalobos *ex* Colmeiro [FRANCO 1990]) hybrids were once thought to be a separate taxon under the name of *Q. hispanica* Colm. et Bout. 1854 (reviewed by NATIVIDADE 1936), but their sporadic occurrence, invariably associated with the presumed parent species (COUTINHO 1888), and the intermediate features of the bark microanatomy (NATIVIDADE 1936), has established their hybrid character[1].

Assuming that these hybrids are able to backcross with normal cork oaks and thus generate introgressed "cork" oak progenies, the quality of cork in the mixed stands might be affected (NATIVIDADE 1936). It is well-known that cork quality is very sensitive to the growth conditions of the trees, mainly in connection with the overall maintenance of the stands where they grow, but an intraspecies genotypic component might be important as well,

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[1] The *hispanica* epithet is currently accepted only for the hybrids of cork oak with *Q. faginea* Lam. but widely used for the *Q.* × *pseudosuber* Santi (= *Q. crenata* Lam.) hybrids with *Q. cerris* L. (NATIVIDADE 1934, FRANCO 1990, BELLAROSA 2003), which are even commercialized as ornamentals under that invalid designation.

which can be exploited for breeding purposes (NATIVIDADE 1934, 1950, 1954, VARELA & ERIKSSON 1995). However, the knowledge on the genetic determination of cork quality remains in its infancy, for Portuguese provenances and elsewhere (CATALÁN *et al.* 1997). The occurrence of hybrids that can backcross with cork oak introduces an interspecies genotypic component that, depending on the prevalence of introgression, can be a very complicating factor for the management of *Quercus suber* germplasm. In spite of the postulated influence on cork quality, this has not been considered of major economic importance because early detection and elimination of the initial hybrids makes them uncommon, and also because cork from Portuguese mixed stands is generally good.

This lack of concern is somewhat misleading. The *Q. ilex* × *suber* hybrid seedlings are macroscopically indistinguishable from those of *Q. suber* until they develop the bark, i.e., not before the trees are 10 to 15 years old (TOUMI & LUMARET 1998). Thus all hybrid or introgressed propagation materials will remain undetected in juvenile stages, posing many problems regarding the quality control in nursery-propagated trees. On the other hand, avoiding mixed stands for the collection of acorns for propagation might seriously curtail the available genetic diversity available for propagation, especially for the fact that mixed stands with holm oak represent one end of the ecological gradient occupied by cork oak, at least in Portugal. A molecular approach would be necessary to control such materials.

Earlier isoenzyme analyses have been undertaken for the assessment of interpopulation diversity, both within *Quercus ilex* (YACINE & LUMARET 1989, MICHAUD *et al.* 1995) and *Q. suber* (VON WUEHLISCH & MUHS 1995, NÓBREGA 1997, TOUMI & LUMARET 1998, JIMÉNEZ *et al.* 1999), thus establishing a repertoire of allelic variation that could be useful for the discrimination between the two species. Introgression of holm oak genes in cork oak trees using this approach has been suggested from isoenzyme analysis (ELLENA-ROSSELLÒ *et al.* 1992, TOUMI & LUMARET 1998) or plastid DNA haplotypes (BELAHBIB *et al*. 2001, COLLADA *et al.*, in press), but in all cases the sampling design did not allow to rule out alternative explanations. In particular for the need to certify propagation materials of Portuguese provenance used by nurseries, a dedicated strategy was needed. The present work was designed to detect first generation hybrids between holm oak and cork oak in mixed stands containing both species, using discriminating isoenzymes that are amenable for future investigations on a larger scale.

MATERIALS AND METHODS

Plant materials

Locations

Table 1 Table 1 summarizes the geographical data and designations adopted for all plant materials sampled, and figure 1 Figure 1 includes a map of their relative positions. The listed locations fall into three categories: reference stands, which are "pure" populations of either cork oak or holm oak separated from trees of the other species by at least 3 km; mixed stands, where roughly equal numbers of each of these two species were present in a continuous area; and hybrid trees. The reference cork oak stand, henceforth named Rc, and the reference holm oak stands, Rh1 and Rh2, were located well apart from the mixed stands and can be taken as representatives, for South Portugal, of the more typical biotopes for the species they represent. The three mixed stands were located relatively close to each other, with local distribution of the two species mainly as intertwining zones (M1 and M2) or completely interspersed (M3).

All hybrids are spontaneous individuals that were pointed out in the field by landowners, forest technicians or local people alike, mainly from popular knowledge of distinguishing morphological characters for these trees (roughly described as cork oaks with atypical bark). Most were confirmed by isoenzyme analysis and are the 9 considered here. As figure 1 shows, the three mixed stands are located within the distribution of these hybrids.

Leaf and seed genetic materials

During the fruiting season of 1998 (October to November), acorns were collected from the reference and mixed locations, as well as from three hybrids (SM1, SM2 and SES), then sown on peat in containers at Sto. Isidro nurseries, Pegões (cross in figure 1). In the reference stands the acorns were bulk-collected, but in the mixed stands, like for the hybrids, the collections were traced on mother tree, thus allowing the progenies to be identified by half-sib family in the containers. Acorns were sown to Forest-Pot F/P 400 (400 cm^3) containers, as described by VARELA *et al.* (2003), except that peat was used as substrate. Leaf samples, from these plants as well as from the adult trees marked in the mixed stands and from the hybrids, were taken mostly between September 1999 and December 2000. A few acorns from the fruiting season of 2000 were taken from one holm oak and from several hybrids and used immediately for analysis. To distinguish them from the hybrids, seedlings from SM1, SM2 and SES, as well as the latter acorns taken from the hybrids, will be called "backcross progenies" all through the present work, based on the *a priori* assumption that the hybrids were mainly cross-pollinating with individuals of either of their parent species.

Reagents

Hydrolysed starch for electrophoresis was obtained from Sigma (St. Louis, MO) in two different types, one with higher gel strength (catalogue reference S-4501), the other with lower viscosity (S-5651) along the guidelines by MAY (1991). Polyvinylpyrrolidone-40 was purchased from Sigma (PVP-40T). Chemicals were of analytical grade, and deionised water purified with a mixed bed resin (Permutit, Paramus, NJ) with 0-2 µS conductivity was used thoughout. Stock solutions of protease inhibitors were prepared in water at 10 mM for AEBSF, 1 mM for E-64, 100 mM for iodoacetate, and 500 mM for EDTA. They were stored at –20 ºC (E-64 and iodoacetate), 4 ºC (AEBSF) or room temperature (EDTA).

Enzyme extraction and conservation

Leaves were processed the same day they were collected from the plants. Approximately 400 mg were placed in a mortar, liquid nitrogen added and reduced to a fine powder with a pestle. While still very cold, the powder was transferred to a 2 mL tube and 1 mL of extraction solution was added, agitated to ensure thorough mixing, and placed on ice. The extraction solution is a slightly viscous yellow medium containing 25 mM histidine, 25 mM ACES, 250 mM sodium ascorbate, 12.5 % (w/v) PVP40, 25 % (v/v) ethylene glycol, 8 % (v/v) dimethyl sulfoxide, 10 mM EDTA, 0.2 % (w/v) BSA and 5 mM DTT, having a final pH 6.5–6.7. After 10 seconds homogenization at full speed (on ice to prevent heating, and using a rotating blade homogenizer, model X120, CAT, Germany), the tubes were centrifuged at 5600 RCF at 4 ºC and 100 µL of the supernatant transferred to a 0.5 mL tube containing 5 µL of freshly prepared 20× protease inhibitor solution (100 µM AEBSF, 10 µM E-64, 1 mM iodoacetate and 10 mM EDTA final concentration), the tube tapped to ensure good mixing, and stored at –20 ºC until used. All enzymatic activities were in general stable and well resolved within at least one month of preparation.

Seed materials were cut from the side distal to the radicle, thus not destroying the germination potential. After removal of the integument, 400 mg of cotyledon material were weighed, cut to thin slices and processed as described for the leaves. Only acorns that were mature but still attached to the tree branches were used.

Electrophoreses

Starch gels were prepared as follows (adapted from MAY [1991], see also KEPHART [1990]): equal amounts of the two starch types (16.5 + 16.5 g) were mixed well and fully suspended in 100 mL the appropriate gel buffer in a Kitasato flask. The remaining 200 mL of this buffer were brought to a boil before being added to the suspension,

producing a translucent viscous liquid that was heated on a flame until large air bubbles started to roll on the side of the flask, then the flask was taken out of the flame, and vaccum applied to degas the liquid before pouring inside the gel mould. The buffer systems used depended on the enzyme activities to be assayed, and included the following: the Citrate system (abbreviated as C; citrate-morpholine, pH 6.1 [MAY 1991, MICALES *et al*. 1986]), Ridgway system (R; Tris-citrate-LiOH-borate, pH 8.5 [MAY 1991, MICALES *et al*. 1986]), Histidine system (H; Histidine-EDTA-tris-citrate, pH 7.0 [MICALES *et al*. 1986]), and Maleate system (9; maleic acid-Tris-EDTA-Mg pH 8.0 [MAY 1991]) were used.

Electrophoresis was performed essentially as described by MAY (1991). The separations lasted 3 hours for the C and 9 systems at 300 V, 2 ½ hours for the R system at 300 V, and 2 ¼ hours for the H system at 200 V, at 4 ºC, with "freeze packs" placed on top to dissipate the heat from the gel and replaced regularly.

Staining and visualization

Table 2

After electrophoresis the gels were sliced (MAY 1991, MICALES *et al*. 1986), producing three replicas that were placed in different enzyme staining solutions. Table 2 provides details on the enzyme activities that were assayed. As suggested by SOLTIS *et al*. (1983), the glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Tsenk.) Van Tiegh. was used in the staining reactions requiring this activity, thus enabling the more economic use of the NAD coenzyme instead of NADP (WHITE & WHITE 1997).

After destaining (MAY 1991), gels were placed on a white light transilluminator to improve the identification of bands colocalizing with the specific enzyme activities.

RESULTS

Figure 2

Isoenzyme patterns

For the purpose of detecting holm oak genes in cork oak, and given that the leaves from hybrids and backcross progenies can be almost indistinguishable from cork oak especially in young plants (NATIVIDADE 1936), the sensitivity connected with each enzyme system reported in this study relates to the relevant ability to discriminate hybrid from cork oak zymograms. Thus the quantifications given in this section do not pertain to the distinction between holm oak and the hybrids. No differences between adults and seedlings were observed for any of the leaf enzyme activities described herein.

Markers with full sensitivity

Leaf diaphorase (DIA) in gel C developed as a pattern of 1 to 4 anodic bands per sample in both species, with faster migration in the holm oak (figure 2a). In each individual the band distal to the origin of migration was always present, and in most cases it was the most intense. The fact that in the hybrids the activity seemed to form a single broad band, sometimes recognizable as as cluster of 3-4 very close bands or rather diffuse (but not too broad) otherwise, and that the backcross progenies would display either this hybrid pattern or the "pure" holm or cork oak patterns, is interpreted as follows: the enzyme is coded in a single locus and is probably monomeric (WHITE & WHITE 1997, MAY 1991) and invariant within species; the presence of several bands would probably result from posttranslational modifications that shift the isoelectric point of the enzyme (POLY 1997). This activity, while strong in leaves, is very weak in cotyledons and therefore it does not seem to be usable with seeds.

Leaf NAD-malate dehydrogenase (MDH) in gel C developed as a complex set of bands, all but one on the anodic part of the gel (figure 2b). The cathodic band migrated faster in cork oak than in holm oak, and in the hybrids both bands could be seen. However, in holm oak this band remains undetected in many samples because of its low intensity. On the anodic side, there were 4 bands in holm oak and 7 in cork oak extracts, four among these migrating faster than those of holm oak so that the two species were easily distinguished. In the hybrids, 5 bands were seen with a distinct distribution of intensities, but in the backcross progenies further patterns were seen, apparently through recombination among several loci (this enzyme is probably multimeric [MAY 1991]). Consistent with this, the cathodic band did not always co-segregate with the anodic bands in the hybrid progenies, thus making the genetic interpretation of MDH more complex than with DIA. In cotyledons the bands observed were similar except for two

aspects: the band on the cathodic part of the gel is not discriminating (and migrates faster than the band from leaves), and in backcross progenies the distributions of bands on the anodic part are different from those in seedlings leaves.

Both DIA and MDH, especially the latter in holm oak, sometimes showed a variation of the distribution of isoenzyme intensities. The hypothesis that this variation is hereditary was tested by analysing the progenies of mother trees with this kind of variation, but the results were inconclusive (not shown). For DIA at least this variation might be explained by differences in the degree of posttranslational modification of the enzymes. The distinction between some MDH variants and the patterns of some backcross progenies can leave some doubts, requiring well-trained observation.

Leaf glutathione reductase (GSR) in gel H developed as a complex pattern of 5 bands in cork oak, 7 bands in holm oak, and a very long diffuse activity in the hybrids (figure 2c). The activities in holm oak migrated faster than in cork oak, and the backcross progenies either segregated the "pure" patterns or displayed the diffuse hybrid pattern. Thus the genetic interpretation for this enzyme would be similar to that proposed for DIA. Sometimes developing this enzyme activity was not successful, hence the reduced number of samples that are reported in the analyses section. The expression in cotyledons was strong and with fewer bands, and would be a likely candidate for discrimination between the two species using seed materials.

Leaf phosphoglucomutase (PGM) in gel H developed as a single band or a pair of bands in holm oak, while in cork oak it appeared always as a single band, rather strong by comparison (figure 2d). This enzyme is generally monomeric (WHITE & WHITE 1997) and it would appear that it is monomorphic in cork oak (no variation in 929 individuals analysed) while in holm oak three common codominant alleles were identified. With the exception of one of the less common holm oak isoenzymes, their migration was faster than the cork oak form. The exception is rare (12 occurrences out of 782) and heterozygous, hence one of the discriminating holm oak alleles was present as well. The hybrids had in general both the cork oak enzyme and one of the common holm oak enzymes, but surprisingly SM2 had a single band that either was only of the cork oak type or heterozygous with the uncommon comigrating holm oak type, and its progeny always had a band at this position. The expression in cotyledons was strong but not similar to that in leaves, with a more complex banding pattern (possibly from the combination of 2 polymorphic loci).

A few other enzyme systems were not routinely assayed but the notion that they could be highly sensitive markers must be kept in mind. Fluorogenic acid phosphatase (F-ACP) in gel 9 was assayed once with leaf extracts from reference samples and appeared as a single band in both species, the cork oak form migrating faster. NAD-

glutamate dehydrogenase (GDH) in gel R developed only in cotyledons, as a single band in the parent species (the holm oak form migrating much faster) and one or two bands in backcross progenies.

Markers with partial sensitivity

Leaf chromogenic α-esterases (EST) in gel R appeared as a rather close set of dark grey bands on the anodic side of the gel. The pattern was actually very complex and difficult to resolve, but the appearance of a common faster-migrating band in holm oak that separated well from the position of the cork oak cluster was sufficient for routine purposes (figure 2e). However, 48 out of 829 holm oaks did not show this band, thus the sensitivity of this enzyme system was estimated to be 94.2 %. In cotyledons, this system gives analogous results.

Leaf phosphoglucose isomerase (PGI) in gel R developed as a system of 2 or 4 bands per individual in both species and the hybrids (figure 2f). The distal band was invariant in both species and had practically the same migration rate between the two species. The other bands formed patterns that were consistent with the interpretation of a dimeric enzyme coded by a single locus which, in the heterozygotes, forms one faster-migrating homodimer, the intermediate heterodimer with double staining intensity, and the slower-migrating homodimer (MAY 1991). The latter system appears to correspond to the *Pgi-1* locus described by YACINE & LUMARET (1989) for holm oak and TOUMI and LUMARET (1998) for cork oak. According to the proposed genetic interpretation, holm oaks showed a higher allelic diversity in this locus than cork oaks, which had two common alleles and an uncommon third allele. For discrimination purposes, most holm oak enzyme forms migrated faster than the cork oak forms, with the exception of a homodimer which present in 31 out of 481 holm oaks where this was recorded, comigrating with the fastest (and most common) cork oak form, thus giving a sensitivity of 93.6 % for this system. The hybrids reflected the allelic diversity in holm oak, while having a cork oak allele. The expression in cotyledons was similar to leaves.

Leaf phosphogluconate dehydrogenase (PGD) in gel H developed as 2 systems of isoenzyme bands probably corresponding to two loci; one is faster-migrating and invariant, and the other almost so except for an extra band that is present in 109 out of 831 holm oaks analysed, thus giving a sensitivity of 13.1 % (figure 2g). The enzyme is probably dimeric in the holm oak and the presence of this band, if due to an uncommon allele, appears to be part of a three-band system as proposed for the PGI heterozygotes, but the predicted slower-migrating homozygote is not always visible. Slight intraspecific variations were noticed but they had no impact on the purpose of discriminating between the two species. Cotyledons have a very strong PGD activity and the distributions observed are similar to those in leaves.

Other markers

Leaf peroxidases (PER) in gel C were visualized as a very complex pattern of bands, from very fast anodic to very fast cathodic. It was common to see 6 bands for each sample (figure 2h). The interest for discrimination was however reduced, in spite of early indications to the contrary. The fastest-migrating activity on the anode part of the gel in particular seemed absent in cork oak and constant in holm oak, but with time many exceptions were observed and the risk rates were estimated to go as high as 11.6 % holm oaks without this band and 28 % cork oaks with it. For this reason it seems advisable not to consider any isoenzyme marker as having discriminating value until enough samples have been analysed. This band was absent from all cotyledon materials analysed. On the cathode side, a few activities were occasionally observed in the vicinity of the intermediate constant band, 1.6 % in cork oak and 19.5 % in holm oak. Most acorns did not show any peroxidase activity on the cathode side.

Chromogenic esterases (EST) in gel C were assayed in a limited number of leaf samples only recently. Generally, a single band per individual (apparently different from those that were developed in R gels) appeared on the cathodic side of the gel, the cork oak form migrating faster, thus encouraging the inclusion of this analysis in routine screening, for example in replacement of PER. However, in the hybrids the cork oak form was consistently absent, and further work is needed to ascertain its value for routine analysis.

Analyses

Backcross progenies

A total of 100 seedlings descending from SM1, SM2 and SES were analysed. By definition, a hybrid tree is heterozygous for each discriminating locus, and each embryo sac produced contains a recombinant gene set. If the pollen tube is from one of the parent species, then the seedling that results may be similar to that species on one or more diagnostic loci. In table 3, a few examples are given to illustrate some combinations of zymotypes obtained and the identification of the pollen species. A few seedlings contained both cork and holm oak isoenzyme types, suggesting that recombinant pollen, probably by self-pollination of the hybrid trees, was involved. On the other hand, there was no segregation of any parent enzyme type that would enable a decision on the pollen source and were indeterminate (apomyxis remains a possibility in these cases). Two seedlings from SM1 were taken as contaminants from neighbouring trees since they could not be distinguished, morphologically or by isoenzyme analysis, from either parent species. The extract from another seedling gave no results.

Table 3

The identification of the pollen types that gave origin to the backcross progenies provided an insight into

Table 4

the crosspollination preferences of the hybrids, and as would be expected from the cross-pollinating preference of oak trees and the hybrid character of their mother trees, most were confirmed as being the result of backcrosses with pollen from either holm oak or cork oak. The relevant account is given in table 4, showing that the hybrids can be pollinated either by holm oak or cork oak.

The distinctive leaf morphology of holm oak was already visible in 8 backcross seedlings from SM1 and 3 from SM2, and all these cases, from their zymotypes, were considered to derive from holm oak pollen (seedling 33.5 in table 3 was an example of these).

Detection of hybridism

Table 5

Table 5 summarizes the results obtained by the routine analyses done so far. A total of 12 misplaced seedlings were detected from morphological observation and confirmed as being "pure" by isoenzyme analyses (noted as seed contaminations, "sc"). Of these, 8 were holm oaks mixed in the progeny of a cork oak that stands at the bottom of a slope in M3, in the vicinity of a few holm oaks. Regarding the occurrence of *Quercus ilex* × *suber* hybrids among the seedlings, none was observed. The probability P of successful hybridization can thus be stated as being lower than the value at which, for a given sample of size N, the odds that no hybrids are observed are below a critical value α. From the Poisson distribution it can be derived that $NP < -\ln \alpha$. Discounting the adults (64 holm oaks and 85 cork oaks) analysed and repositioning the seed contaminations detected, the sample size becomes 489 holm oak and 652 cork oak seedlings, 1141 overall, from which the estimates $P < 0.0062$, $P < 0.0046$ and $P < 0.0027$, respectively, are obtained for $\alpha = 0.05$.

Regarding the observations of diffuse distribution of DIA on the gel among seedlings, all (except for one case from Rc) had a very broad distribution that was not observed in hybrids and might be interpreted as rare cases of heterozygosity in the DIA locus rather than introgression. It was also present in 2 adult holm oaks in M3 (numbers 11 and 36) and 5 out of the 37 progeny from the latter. A second extraction was done on tree 36, confirming the distribution, ruling out the possibilibility of enzyme degradation.

The ambiguous sample in Rc could not be repeated because the seedling died in the meantime, so the presence of two "pure" holm oak isoenzymes in this cork oak seedling, from a location where most likely they would appear as hybrid at best, could not be verified nor explained.

Overall, the results indicated a very low probability of formation of first-generation hybrids in the season of 1998, for the locations under study.

Polymorphic loci

In addition to serving as discriminating activities, PGM and PGI activities were highly polymorphic in holm oak, and the latter in cork oak as well. Other than noting for these activities the type (cork, holm or hybrid) in each sample, the genotype for PGM in holm oak and for the polymorphic PGI (*locus Pgi-1* [TOUMI & LUMARET 1998] or *Pgi-B* [Jiménez *et al.* 1999]) in cork oak was also recorded (the PGI polymorphism in holm oak was too complex and was not analysed in detail). In holm oak, 5 different alleles at the *Pgm locus* were identified, each corresponding to a different band. Numbering the alleles according to the migration of the corresponding isoenzyme, in ascending order from the most anodal, the 3 most common were alleles 2, 3 and 4 (the rare isoenzyme 5 was the one comigrating with the cork oak PGM). The same numbering for the cork oak *Pgi-1* alleles was made, alleles 1, 2 and 3 probably corresponding to alleles .90, .70 and .50 described by TOUMI & LUMARET (1998).

The contingency test for homogeneity among locations M1–M3 (adult trees) did not invalidate using the frequencies obtained from pooled data as representative for a single population of each species (P = .41 for *Pgm* in holm oak and P = .14 for *Pgi-1* in cork oak). The seedlings data from the M1–M3 holm oak and cork oak populations were then used to investigate the tendency for inbreeding (MITTON 1993) in the 1998 flowering season and thus estimate the overall percentage *t* of intervening outbred pollen (BROWN & ALLARD 1970). Since the available seedlings were not from all analysed adults, and the sampling per progeny was very variable, the data from the previous generation relevant for comparison had to be based on the actual frequencies for the female gametophytes sampled, which were calculated from the progeny sizes for each mother tree genotype. Table 6 shows that, for both populations, these frequencies were very close to those of the seedlings; however, the results were very different between the two populations: in holm oak, the *Pgm* data suggested a nearly panmictic situation (practically zero variation of the fixation index *F*, *t* aproximately 100 %), but in cork oak the *Pgi-1* data suggested inbreeding (an increase of 0.31 for *F*, with only 53 % of outbred pollen). Assuming that both species have a similar degree of inbreeding at the pollination level, this increase is probably compensated by selection against homozygotes, either during seed development or even before fertilization (BROWN & ALLARD 1970, WASER 1993) in the case of holm oak *Pgm* (this would account for the apparent lack of deviation of its *F* from adults to seedlings), or after germination in the case of cork oak *Pgi-1* (MITTON 1993).

DISCUSSION

Molecular characterization of the hybrids and their backcrossing characteristics

The hybrid nature of the *Q. ilex* × *suber* trees, undisputed for quite long on the basis of the anatomical characters of their bark and leaves (NATIVIDADE 1936) and on their natural occurrence (COUTINHO 1888), has remained without genetic evidence[1]. The enzymes that in the present work were found to be discriminant between the two parent species have provided such evidence, since all displayed zymograms predictable under the assumption of this hybrid nature, with further support from the backcross progenies of three individuals, where the segregation of homospecific genotypes was observed.

The genetic interpretations proposed, though consistent with the observed patterns in the hybrids and backcross progenies, still require a formal confirmation based on controlled crosses. Nevertheless, those interpretations allowed to establish that the backcrossing can be with any of the parent species, with estimations of the relative contribution of the two parent pollen types. The percentage of identified cork oak parentage varied markedly among the three hybrids studied, with preferences that can be hypothesised to depend on how much exposure to cork oak pollen, in relation to the particular phenology of female flowering, each hybrid gets. However, since there was a significant proportion of seedlings that appeared to derive from recombinant pollen, and given the low number of markers available, it is possible that a few cases in which either of the parental types of pollen was identified might be in reality a recombinant type. Thus it seems that a significant proportion of the acorns produced result from self-pollination.

Detection of hybrids from the parent species and their breeding biology

The absence of F_1 hybrids between holm oak and cork oak, for the three mixed locations, provided an estimate of the probability of spontaneous hybrid formation below 1 %. However, during the flowering season that preceded the seedlings reported here, the periods of maximal pollen release by both species overlapped for 2 weeks in the M1 location (VARELA *et al*., in preparation). As pointed out by TOUMI & LUMARET (1998), this contrast between opportunity for cross-pollination and actual hybridization underlines the importance of barriers to hybridization

[1] After the original submission of the present paper, a similar approach using nuclear SSR markers imported from other oak species was published (SOTO *et al.*, 2003). Within a very limited sample, 6 markers were preliminarily proposed as effective, although to varying degrees.

between species of different subgenera of *Quercus* (*Cerris* for cork oak and *Sclerophyllodrys* for holm oak [SCHWARZ 1936-37, TUTIN 1964, FRANCO 1990, NIXON 1993, BELLAROSA 2003]). On the other hand, the relatively high seed set from controlled crosses between the two species (*Q. ilex* fertilized with *Q. suber* pollen) suggests that there can be circumstances under which hybridization can be successful (BOAVIDA *et al.* 2001).

At any rate, given that each adult oak can live for over 100 fertile years, yielding tens of thousand acorns on its own, it is not surprising that, in wide areas of contact between the two species like those that exist in Portugal, *Q. ilex* × *suber* hybrids can occur. Their prevalence may even be underestimated since many are eliminated by man, but their incidence seems to be, at least for the present sampling, very low. It is possible that some locations are more likely to produce hybrids than others, as suggested by the distribution of 7 of the 9 hybrids, detected in the present study, in 2 tight clusters (figure 1). The inventory of these hybrids within this vast domain is continuing.

In contrast to the apparent separation between the parent species making the formation of hybrids relatively unlikely, once a hybrid is formed it is presumed that it will backcross easily with either of the parent species. Our data on the progenies of 3 hybrids confirms the ability to backcross with both parent species, and also of self-fertilization. Thus a hybrid will generate lineages that phenotypically become closer to either of the type species, while retaining introgressed genes from the other species. Considering the presumably long generation time of the backcross lineage with cork oak (the parent species takes 25 years after germination to bloom), it is unlikely that such introgression will disappear shortly.

Due to the fact that very little is known on the reproduction biology of the hybrids, most of the introgression scenario remains speculative, but its implications are not trivial. According to NATIVIDADE (1936), the similarity of anatomical characteristics of the bark in the hybrids and certain individuals with cork of very bad quality is a strong indication of the introgressed nature of the latter. This hypothesis raises a very sensitive issue connected with the pressure by landowners to eliminate the economically less profitable holm oak from mixed locations. However, NATIVIDADE (1936) used as reference only the best cork-forming oaks, based on his assumption that only these are "pure" and that the polymorphism in cork oak is largely determined by introgression from other species (NATIVIDADE 1934, pages 126 and 128). This assumption is probably invalid and, given the natural variation in cork anatomy, the reference used would be highly biased — the hybrids could be merely at one end of the natural anatomical variation of cork in *Quercus suber*, which would invalidate the hypothesis above. Nevertheless, the kind of cork produced by some trees of this species, similar to that observed in the *bona fide* hybrids, suggests a hybrid-like intermediate state between the typical cork anatomy and the bark anatomy of other congeneric species.

Studies in Spain, using rather limited samples, have detected cork oaks with isoenzymes that could be of holm oak origin (ELLENA-ROSSELLÒ *et al.* 1997), a result suggesting that certain locations might have a high degree of introgression. Subsequently, the work of TOUMI & LUMARET (1998) on genetic variation in cork oak included the casual detection of unusual isoenzyme forms from provenances where holm oak was also present, and their correspondence with alleles from holm oak was also hypothesized. From our point of view, the sample sizes in those studies were not conducive to any decisive interpretations, or otherwise the rates of introgression would have to be alarming: for example, in two Moroccan locations three different alleles of the polymorphic *Pgi-1* locus were interpreted as being of holm oak origin, in samples of 32 and 29 plants only (TOUMI & LUMARET 1998). Neither the required analysis of holm oaks in those locations, nor the disproof that such "introgressed" alleles are local variants in true cork oak, were considered. The two Portuguese provenances represented in that study did not have any of such forms, but only 30 plants were sampled per provenance, and they were outside the main area of coexistence with *Quercus ilex*. It may be possible that the situation in Portugal is different from other regions but, given the fact that 50 % of the world cork is produced in this country, and the recent extensive afforestation efforts using nursery-propagated materials (some of which from seeds originating abroad), under incentives from the European Union as well as national, assessing the risk of introgression is not a task of minor importance for the sector.

More recently, the analysis of mixed stands in Morocco (BELAHBIB *et al.* 2001) and the Iberian Peninsula (COLLADA *et al.* in press) using combinations of cytoplasmic DNA markers has provided an astonishingly high prevalence of suspected introgression of holm oak cytoplasm into cork oak populations. Again, the limited representation of Portuguese provenances in the latter study was found to be "pure". Apparently, the interpretations given to the DNA data are not based on nucleotide sequence comparisons, making the assignment of haplotypes to "ilex" or "suber" tentative, even questionable. Thus under the assignments proposed, some of the "ilex" haplotypes in cork oak were not detected in holm oaks, and the complete substitution of "ilex" haplotypes for "suber" haplotypes was the dominant pattern of introgression. Fixation/elimination events, in spite of the high fecundity of oak trees, might have played a role for these outcomes.

Polymorphic isoenzymes and clues to the breeding biology in the parent species

The availability of genotypes for one polymorphic locus in each parent species, both in adults and seedlings from the three mixed locations (treated as a single population) enabled an analysis that can be relevant with regard to the reproduction biology of these species and, by extension, that of the hybrids. The increase of the fixation index *F* for

the cork oak *Pgi-1* locus suggests significant inbreeding which might take place through either self-pollination or crossing among genetically related trees. To our knowledge, there is at present no direct evidence of the incidence of self-pollination in this species, but adopting as reference the results from detailed assignments of parentage using six hypervariable DNA *loci*, within a *Quercus petraea* (Mattuschka) Liebl. + *Q. robur* L. mixed stand in France (STREIFF *et al.* 1999), then self-pollination could be rather low. One is left with the possibility that a significant proportion of pollination would originate from genetically correlated trees, most probably those nearby since these were naturally regenerated stands. The same parentage study (STREIFF *et al.* 1999) derived, for both oak species, a dual distribution of mating events over distance from the pollen origin, implying that the nearest trees would be those individually most represented in the matings (indeed, one of the *Q. petraea* trees in that study, completely surrounded by *Q. robur*, had relatively high amounts of hybridization). The studies by LEXER *et al*. (2000) on *Q. robur*, and the *Q. alba* L. data treatment by SMOUSE *et al.* (2001), arrived at similar conclusions regarding the sources of pollen involved in matings. Thus in naturally regenerating stands, it is predictable that genetically related trees tend to cluster together and, given the observed increase of *F* for cork oak *Pgi-1* from adults to seedlings, an important share of the pollen that fertilizes a given tree is genetically related. The same line of reasoning would predict that successful hybridization is favoured wherever a nearby tree from the other species releases its pollen at the appropriate time. Our evidence from phenological studies at the M1 mixed stand indicates that such did not happen in 1998 (VARELA *et al*., in preparation).

The use of isoenzymes for the study of hybridization

In spite of the increasing availability of DNA markers, with higher genetic resolution, isoenzymes still provide a cutting edge when reliable DNA markers for a given species are lacking, or when the costs and time involved in large-scale surveys are critical (MAY 1991). Given the growing demand for certified propagation materials for afforestation projects, the need to clarify the actual extent of hybridization and introgression in cork oak, and more importantly where it most likely occurs, can be well served by the routine large-scale monitoring of seedlings using the isoenzyme markers identified here. Starch gel electrophoresis is probably the most economical means to achieve this, and the extraction and conservation procedures developed in the present study for the most readily available material, the leaves, were designed for the amenability of assaying each plant extract repeatedly, within a reasonable time (1 month at least). Within the limits of current data, one electrophoresis (preferably system C with revelation of DIA, MDH and possibly chromogenic EST, but alternatively system H with GSR, PGM and PGD) will be enough

to detect hybrids, but the number of markers for detecting introgressions can hardly be enough, although the tentative number of 10 at present (excluding PER and with an additional marker from seeds, table 2) can be said to achieve a good degree of sensitivity. Further testing is needed on samples from other countries as well as from Portugal to verify its broader applicability. The use of cpDNA haplotypes to detect introgression is limited to the cases where hybridization involved a holm oak as the mother tree, even then missing all subsequent backcrosses involving cork oak female gametophytes, and they also provide no insight on the proportion of introgressed holm oak nuclear genes in cork oak. On the other hand, the complementarity of isoenzyme analysis with nuclear DNA-based methods such as the recently introduced use of simple sequence repeats ("microsatellite") markers (SOTO *et al.*, 2003) or AFLP analysis (CERVERA *et al*., 2000), is expected to be of great advantage for introgression studies.

CONCLUSIONS AND PROSPECTS

Cork oak and holm oak, although separated by slightly different ecological niches and by almost nonoverlapping blooming periods, coexist on a large area in Portugal and form hybrids, but the occurrence of these appears to be, in the light of our data, strongly avoided through the evolutionary establishment of fertilization barriers. For the moment, the elimination of holm oaks in mixed stands is not warranted, not until the locations where such barriers might be less effective are defined with some precision, the overall prevalence of hybridization is estimated, and the reproduction biology of the first generation hybrids is known. Discriminant isoenzymes are an effective means for the large-scale detection of hybridization and introgression, which is critically important to support the certification of propagation materials. A systematic survey of such materials is made heretofore possible in order to settle the issues connected with the management of the ecologically invaluable mixed stands in this country.

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TABLES

Table 1. Coding, localization and general morphology of the materials used in the study. Rc, Rh1 and Rh2, reference stands (seedlings only); M1, M2 and M3, mixed stands (adults and seedlings); remainder sources are spontaneous hybrid trees. The total number of plants analysed from each source are given.

Code	Property name	Location	Reference toponym	Comments
Rc	Monte Branco	38º 00' N 8º 41' W	National Woods of Sines	"pure" cork oak stand, 217 seedlings
Rh1	Azeiteiros	39º 05' N 7º 07' W	Campo Maior	"pure" holm oak stand, 233 seedlings
Rh2	Testa	37º 36' N 8º 04' W	Almodôvar	"pure" holm oak stand, 147 seedlings
M1	Mitra	38º 32'N 8º 01'W	University of Évora	mixed stand; cork oak: 39 adults (ad.), 19 half-sib families (fam.) for a total of 125 seedlings (s.); holm oak: 25 ad., 19 fam. for a total of 200 s.
M2	Feijoas dos Ramos	38º 24'N 7º 50'W	Torre de Coelheiros	mixed stand; cork oak: 32 ad., 16 fam. for a total of 187 s.; holm oak: 20 ad., 11 fam. for a total of 90 s.
M3	Alfaiates	38º 17'N 7º 51'W	Santana	mixed stand; cork oak: 20 ad., 14 fam. for a total of 348 s.; holm oak: 20 ad., 10 fam. for a total of 191 s.
SM1	Outeiro	38º26.8'N 7º54.9'W	S. Marcos da Abóbada	Well-grown tree, corky bark; 42 seedlings
SM2	Outeiro	38º26.0'N 7º55.8'W	S. Marcos da Abóbada	Well-grown tree, slightly corky bark; 45 seedlings
SM3	S. Marcos	38º26.1'N 7º53.9'W	S. Marcos da Abóbada	Well-grown tree, thin bark
SM4	S. Marcos	38º25.9'N 7º54.3'W	S. Marcos da Abóbada	Young tree, but setting seed, thin bark
SES	Sesmarias	38º17.5'N 8º00.3'W	Alvito	Large tree, corky bark;13 seedlings
MRM	Marmeleira	38º47.5'N 7º42.1'W	Evoramonte	Well-grown tree, thin bark
VR	Vale de Reis	38º54.6'N 8º00.0'W	Malarranha	5 well-developed offshoots growing at the base of a cut tree, thin bark
ALC	Alcarou de Baixo	38º50.4'N 8º02.15'W	Pavia	Well-grown tree, no seeds, corky bark
CAB	Cabeção	38º57.9'N 8º05.95'W	Mata Nacional de Cabeção	Well-grown tree, corky bark

Table 2. Enzymatic activities that are mentioned in the text for discrimination of cork oak from holm oak. Those marked with asterisk (*) in the comments were not used routinely.

EC number	Symbol	Gel	Recipe reference	Comments
1.1.1.37	MDH	C	MAY 1991	NAD-Malate dehydrogenase, ≥ 2 markers
1.6.4.3 [a]	DIA	C	MAY 1991	NADH-diaphorase
1.11.1.7	PER	C	SOLTIS *et al*. 1983	Peroxidase; non-discriminating
3.1.1.x [b]	EST	R	BULT & KIANG 1991	Chromogenic esterases (also gel C*)
5.3.1.9	PGI	R	BULT & KIANG 1991 [c]	Phosphoglucose isomerase
2.6.1.2 [a]	GSR	H	MAY 1991 [c]	NADPH-glutathione reductase
5.4.2.2	PGM	H	MAY 1991 [c] (adapted)	Phosphoglucomutase
1.1.1.44	PGD	H	SOLTIS *et al*. 1983 [c]	Phosphogluconate dehydrogenase
3.1.3.2	F-ACP	9	WEDER & KAISER 1995	Fluorogenic acid phosphatase *
1.4.1.2	GDH	R	MAY 1991 (adapted)	Glutamate dehydrogenase * from seeds only

[a] Numberings as in the reference given, however the name NADH-diaphorase is more commonly applied to EC 1.6.99.2 (and 1.6.4.3 is replaced by 1.8.1.4), sometimes dubbed as menadione reductase, and NADPH-glutathione reductase to EC 1.6.4.2

[b] x = 1, 2, 6

[c] agarose overlay

Table 3. Examples of seedling progenies from three hybrids, SM1, SM2 and SM3. The + and – signs on MDH refer to the anode- and cathode-migrating bands, respectively. Types that contribute to identification of the pollen type are underlined.

Hybrid	Seedling	DIA	MDH+	MDH–	GSR	PGM	others	Pollen‡
SM1 (25¶)	33.16	cork¶	hybrid¶	cork	cork	25¶		cork
	33.5	holm¶	hybrid	n.o.¶	holm	35		holm
	38.8	hybrid	hybrid	n.o.	n.o.	25	heteroz *Pgd*	holm
	38.3	hybrid	cork	cork	holm	5		rec
SM2 (5)	36.13	cork	hybrid	n.o.	cork	5		cork
	36.4	hybrid	hybrid	n.o.	hybrid	5	PGI b (cork)	cork
	34.26	holm	hybrid	hybrid	n.o.	n.o.		holm
	34.23	hybrid	hybrid	hybrid	n.o.	n.o.		?
SES (45)	38.26	cork	cork	n.o.	n.o.	5	*EST*: 12§	cork
	38.27	hybrid	cork	hybrid	n.o.	4		rec

¶ Each seedling zymogram is coded either as cork oak type, holm oak type or hybrid type, except for PGM, given also for each hybrid mother tree, using the allele numbering as explained in the polymorphic loci section (the cork oak allele is coded as 5). n.o., not observed.

‡ Inferred pollen type; r ec (recombinant) pollen is one possible explanation for the occurrence of holm and cork types in the same seedling; ?, indeterminate pollen

§ Notice that seedling 38.26 was not considered a "pure" cork oak seed contamination because of the EST isoenzyme 1.

Table 4. Probable pollen origins for the backcross progenies from three hybrids. Indeterminate cases were those that did not provide any indication on the pollen source, see example ("?") in table 3 and text. Two apparently "pure" seedlings (1 cork oak and 1 holm oak) were considered to be contaminations in the SM1 family.

Mother tree (sample size = B)	SM1 (39)	SM2 (45)	SES (13)
Cork oak pollen (A)	15	34	10
Holm oak pollen	20	5	0
Recombinant pollen	3	5	2
Indeterminate	1	1	1
Incidence of cork oak pollen (A/B)	38 %	76 %	77 %
Contaminations	2	0	0

Table 5. Results from the routine analyses with the samples from the present study. For each population, the number of analysed families / individuals (adults + seedlings) is given in the first column. The 7 enzyme columns show the number of unexpected observations / total visualized activities, and the comments column clarifies whether they are from seed contaminations ("sc", see text) or otherwise. Pooled results per species are included in the bottom rows.

	Population(families/ adults+seedlings)	DIA	MDH	EST	PGI	GSR	PGM	Comments
holm oak	Rh1 (–/0+233)	0/233	0/233	0/199	0/232	0/184	0/225	
	Rh2 (–/0+147)	0/147	0/143	0/145	0/147	0/147	0/134	
	M1h (19/25+200)	0/225	0/225	0/194	0/222	0/148	1/160	ambiguous [a]
	M2h (11/20+90)	1/109	1/98	0/84	0/99	0/48	1/72	1 sc (3 cork oak enzymes; EST and PGI concordant but not diagnostic)
	M3h (10/19+191)	7/208	0/201	0/197	0/204	0/190	0/180	Trees 11 and 36 and part of the progeny of tree 36 with diffuse DIA
cork oak	Rc (–/0+217)	4/204	2/217	2/189	2/180	2/126	2/199	2 sc (5 holm oak enzymes in both + GSR in one) 1 with diffuse DIA, 1 ambiguous (DIA GSR holm oak, PGI MDH PGM cork oak)
	M1c (19/40+125)	1/165	0/163	0/137	0/158	0/83	0/164	diffuse DIA
	M2c (16/25+187)	1/208	1/209	1/182	1/192	0/176	1/208	1 sc (6 holm oak enzymes)
	M3c (14/20+348)	9/365	8/366	8/340	8/364	4/343	8/368	8 sc (5 holm oak enzymes in all + GSR in 4) 1 with diffuse DIA
Holm oak, pooled:		8/923	1/901	0/819	0/905	0/718	37316	1 sc + diffuse DIA in M3 + 1 ambiguity[a]
Cork oak, pooled:		15/942	11/955	11/848	12/894	6/728	11/939	11 sc + 3 with diffuse DIA (Rc, M1 and M3) + 1 ambiguity

[a] the seedling is the only individual from M1 that had the rare d allele apparently homozygous (it was analysed twice), being a normal holm oak for the rest.

Table 6. Estimation of *t* (percentage of outbred pollen) from the increase in the fixation index F from generation n–1 (adult trees) to generation n (seedlings), in the M1–M3 populations. The frequencies for generation n–1 are estimates for the female gametophytes in 1998, as explained in the text. $F = 1 - H_O/H_E$, $\Delta F = F_n - F_{n-1}$, $t = (1-F)/(1+F)$ (BROWN & ALLARD 1970).

a) *Pgm locus*, holm oak population

	allele frequencies								
Generation	1	2	3	4	5	H_O	H_E	F	t
n–1	0,001	0,186	0,164	0,649	0,000	0,457	0,518	0,117	
n	0,022	0,143	0,145	0,686	0,004	0,433	0,488	0,113	80%

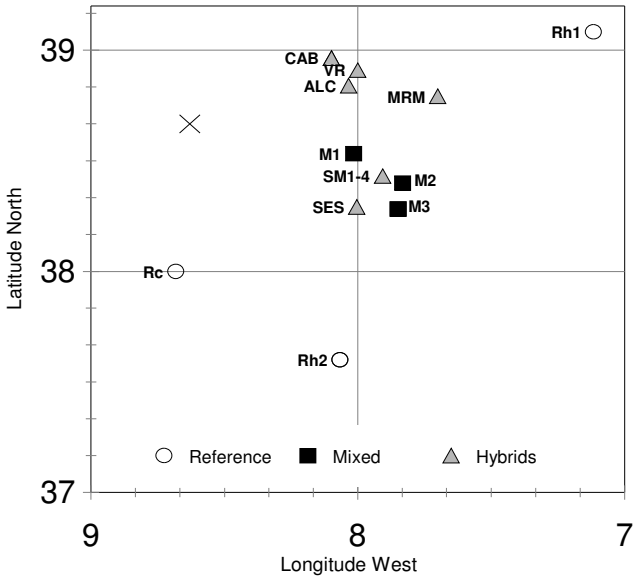
b) *Pgi-1 locus*, cork oak population

	allele frequencies						
Generation	1	2	3	H_O	H_E	F	t
n–1	0,662	0,338	0,000	0,545	0,447	–0,220	
n	0,693	0,306	0,001	0,387	0,426	0,091	83%

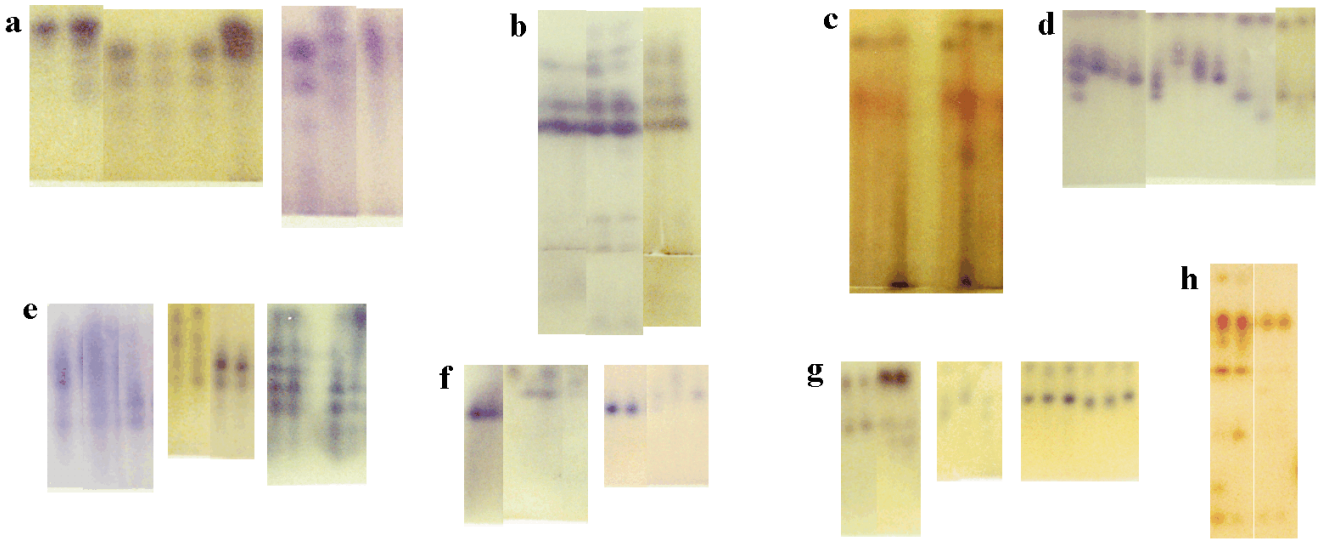
FIGURE LEGENDS

Figure 1 — Left: relative distribution of reference locations (circles), mixed stands (squares) and the hybrids (triangles), as designated in table 1 (SM1–4 represents 4 hybrids); right: placement in the geographical map.

Figure 2 — Examples of enzymatic activities discriminating cork oak from holm oak. All photographs oriented with anode on top. In each panel the samples are from the same gel, but not necessarily contiguous. a) DIA, left panel: 2 holm oaks (h), 3 cork oaks (c), 1 hybrid (x); right panel: 1c-1h-1x. b) MDH: 2h-2c-2x. c) EST: 3c-3h. d) PGI: 8h-4c (genotypes for *Pgi-1*: 11-22-11-12). e) GSR: left panel, 1h-1x-1c; middle, 2h-2c; right panel, 2h-2c. f) PGM: left panel, 2c-4h (genotypes 22-44-24-34); right panel, 2c-3h (genotypes 45-24-44). g) PGD: left panel, 2c-2h, the latter with the discriminant slow bands; middle, 3h, two with the extra bands; right panel, 6c showing variations of the migration of the slow band, presumably the 4^{th} and 5^{th} are heterozygotes. h) PER, 2h-2c.


Rh1
CAB
VR
ALC
MRM
M1
SM1-4
M2
SES
M3
Rc
Rh2
Reference
Mixed
Hybrids
39
38
37
9
8
7
Latitude North
Longitude West


SPAIN
Viana do Castelo
Braga
Leixoes
Porto
Aveiro
Coimbra
Covilhã
Portalegre
SPAIN
LISBON
Barreiro
Setubal
Beja
Faro


a
b
c
d
e
f
g
h