Opportunity for hybridization between two oak species in mixed stands as monitored by the timing and intensity of pollen production

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ABSTRACT

The opportunity of cross-pollination in mixed stands of two oak species (cork oak and holm oak) was studied by characterizing individual phenologies of flowering. In the spring of 1998 at one stand consisting of 64 marked trees, there was a period of 19 days when maximal pollen release in one species and stigma receptivity in the other occurred simultaneously, enabling interspecific gene flow in either direction. This happened in spite of an average time separation of 22 days between the two species, reflecting a considerable intraspecific variation in the timings of flowering. Flowering intensities (as estimated from male flower abundance) were high, but fruiting intensities were comparatively low. Shortly after pollination, considerable abortion of female flowers and early fruits was recorded. In 2000, the interspecific overlap of phenologies was drastically reduced due to a delay in cork oak flowering. On the other hand, the individual timings were repeatable for most trees, at least in holm oak. Two other mixed stands were subject of parallel studies, with similar results in all traits except for a less dramatic reduction in fruiting intensities. In spite of the high opportunity for cross-pollination in 1998, and given the lack of hybrids among the progenies from the subsequent fruiting season [Oliveira, P., Custódio, A.C., Branco, C., Reforço, I., Rodrigues, F., Varela, M.C., Meierrose, C., 2003. Hybrids between cork oak and holm oak: isoenzyme analysis. *Forest Genet.* 10, 283–298], it can be concluded that the prerequisite of cross-pollination is clearly insufficient for hybridization to succeed. Post-pollination processes must play an important role in the maintenance of reproductive isolation between the two species.

1. Introduction

Holm oak (*Quercus ilex* L.) and cork oak (*Quercus suber* L.) are two interbreeding evergreen species with major areas of overlap in the western Mediterranean, in spite of some differences in ecological preference. These differences not withstanding, mixed stands are common and provide the opportunity for the formation of hybrids (*Q. morisii* f. *mixta* Franco & Vasc.).

Cork oak covers an area of 736,700 ha in Portugal (provisional results of the 2005/2006 Forest Inventory), mostly thanks to the economical value of quality cork that maintains a sector producing an equivalent of over one million Euro each year, some 32% of all forestry products from this country (DGRF, 2001). Since the industrial use of cork in nineteenth century, its production has expanded mainly in the impoverished Southern part of the country and has acquired an overwhelming impact on the local economy.

Holm oak covers 388,300 ha in Portugal (provisional results of the 2005/2006 Forest Inventory), and although its economical and social role is not as fundamental as that of cork oak, it is still exploited for energy (coal and firewood) and for feeding (acorns) to field-raised domestic animals, especially the increasingly coveted black pigs, as well as wild game. Very frugal on soil use and more tolerant to drought than cork oak, holm oak remains the dominant forest species over wide areas in the South of Portugal, and the two species together form a vast and invaluable ecological reserve that needs to be safeguarded.

The genus *Quercus* is recognized as comprehending many different hybrid combinations (Burger, 1975; Sedgley and Griffin, 1989; Franco, 1990; Ducousso et al., 1993; Williams et al., 2001; González-Rodríguez and Oyama, 2005), including those between holm oak and cork oak (Natividade, 1936, 1950; Vasconcelos and Franco, 1954; Boavida et al., 1999, 2001). These hybrids are known to be fertile, as already demonstrated genetically (Oliveira et al., 2003) and previously suggested by cpDNA analysis (Belahbib et al., 2001). The backcross with cork oak would thus generate introgressed progenies which might not be as easily discriminated from the type species, and this possibility in cork production

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stands is construed as a potential threat to the quality of the product (Natividade, 1936, 1950; Belahbib et al., 2001; Jiménez et al., 2004; Lumaret et al., 2005). Early histological studies of the periderm layer of the trunk in both species and the hybrids confirmed that the intermediate morphology of the cork produced by the hybrids results from the intercalation of ritidome-like outgrowths, similar to those of holm oak (Natividade, 1936). The cork produced by hybrids is not suitable for the cork industry and it is suspected that some cork of bad quality produced in Portugal comes from cork oak individuals that contain introgressed holm oak genes to varying degrees (Natividade, 1950).

In practice, however, the possibility of such cork resulting from hybridization between cork oak and holm oak has never been a priority of research. Mixed stands can be an advantageous exploitation system and no real impact on cork quality is apparent, to producers and technicians alike, from this contact between the two species. Although the incidence of these hybrids among the natural regeneration could be rather low (Oliveira et al., 2003), their occurrence should not be neglected. Some less well advised producers would even welcome more legal freedom to eradicate holm oaks from their mixed stands, therefore a better knowledge of the phenomenon could forestall their possible influence on future decisions.

Among the natural barriers to hybridization between holm oak and cork oak, the separation between the pollen emission periods by both species is generally regarded as an important factor. The present study aimed at observing in detail the phenology of flowering in a set of cork oak and holm oak mixed stands. The results suggest that this separation is rather incomplete.

2. Materials and methods

A permanent stand (Mitra) belonging to the University of Évora, located at latitude 38°32′N and longitude 8°01′W, containing 25 holm oak and 39 cork oak trees, was chosen for the most detailed observations. Its delimitation was defined by reference to a central tree (number 1), and tree numbering was according to distance in all azimuths. With this system some trees (numbers 6, 26, 28, 38, 39, 43, 52 and 56, cork oaks all of them) were still juveniles, and indeed no flowering was detected in trees numbers 6, 28 and 43, and premature drying occurred in trees numbers 26 and 52.

This stand is very characteristic of the "montado" system used in South Portugal: natural regeneration (at present very limited by goat grazing) with human management, producing a multiaged and irregularly spaced distribution. The soil is poor (non-humic gneiss lithosoil, SROA, 1974).

The climate is classified as temperate transitional between iberomediterranean and submediterranean (Albuquerque, 1982), suitable for both species, with an average rainfall of 567 mm and 4 months of summer drought (defined as less than 30 mm rainfall per month). Thirty-year average temperatures and rainfall for the first 5 months of the year, covering the flowering periods of both species, are given in Table 1 (Mendes et al., 1990).

The observations were made in 1998 and 2000, and the climactic contrast between these 2 years was striking: according to the meteorological stations at Mitra and other places nearby (http://www.cge.uevora.pt/en/cge/observations-and-data.html), in 1998 the temperature was relatively high (above 10 °C for most of January and close to 15 °C in February), and cumulative rainfall slightly low (114 mm by the end of February, 143.5 mm on 13 April); in 2000, the average temperature in January was 6.9 °C and in February 11.8 °C, but by 19 March the cumulative rainfall was still at 23 mm (but followed by 195 mm for April only).

Both species are monoecious, the flowers generally unisexuate, with a strong sexual dimorphism (Varela and Valdiviesso, 1996; Brás, 1999), the pistillate flowers, only 3 mm in size, being much less visible than the male inflorescences (catkins). Pollination is essentially anemophilous.

Previous studies in cork oak stands have permitted a classification of the phenological phases in this species as well as the establishment of a relationship between the development courses of the floral structures of both sexes (Varela and Valdiviesso, 1996; Reforço, 1997). In the present study the same system was used on holm oak flowering without difficulty (Brás, 1999). Briefly, phases are defined from the initial differentiation of flower primordia (A) to full flower maturation (F), from phase D onward with separate nomenclatures for male (m) and female (f) flowers, and with a subdivision of phase F to distinguish the key phases for anemophilous pollination: Ff_2 for receptivity of female flowers and Fm2 for maximal pollen release (Varela and Valdiviesso, 1996). Phases Gm and Hm define post-anthesis drying and dehiscence, respectively.

Both species are protandrous: as a rule, in the same branch, female flowering comes after the corresponding phases of the male flowering, with a delay of 2 or 3 days in the case of cork oak (Varela and Valdiviesso, 1996). In the case of holm oak, a similar delay is observed (Vásquez, 1998). Although the receptivity Ff_2 phase lasts in a cork oak branch on average 5 days, the scattering of phenologies over time, within a tree crown, is readily apparent; thus at a given date several phases may be observed on the same tree, and the total length of the receptivity at the crown level can last much longer (Ducousso et al., 1993).

The present study, to assess the opportunity of hybridization between two oak species, must therefore focus on the individual trees, thus individual phenologies are described at the crown level, not at the branch level. Our field experience of almost two decades in recording flowering phenologies on cork oak has shown that male phenologies are easy to observe from the ground with the naked eye, with great accuracy (the only limitation being the observation of male flowers before phase Dm, on some tree crowns that are too tall due to cork oak pruning for improved cork production). Concurrently, we have learned that female phenologies cannot be recorded with comparable accuracy by direct inspection, due to the minute dimensions of the developing strobiles. On the other hand, because of the predictable delay within each branch, male flowering has proven to be a reliable predictor for female phenologies within each tree, thus becoming our standard for field recording of both phenologies (Varela and Valdiviesso, 1996; Reforço, 1997; Brás, 1999).

Fruit development required a very close observation, and for this reason the sample was restricted to a set of labeled branches from trees that were easily accessible (43 branches on 8 holm oaks and 24 branches on 4 cork oaks).

Two other mixed stands that formed with the one at Mitra the source for a genetic detection of hybrids (Oliveira et al., 2003) were also studied (Feijoas do Ramos, 38°24′N, 7°50′W; Alfaiates, 38°17′N, 7°51′W), but not as thoroughly for logistic limitations.

Intensities of flowering (1998–2000) and fruiting (1998 and 1999) were recorded in Mitra. Further observations were made in Alfaiates and Feijoas do Ramos in 1998 (fruiting only) and 2000 (flowering only). Except for the absence of data of holm oak fruiting

Table 1
Average (30 year) values of temperature and rainfall for Évora-Mitra (Mendes et al., 1990)

	Month				
	January	February	March	April	May
Temperature (°C)	8.6	9.5	11.2	13.4	17.0
Rainfall (mm)	98.0	95.0	88.3	52.6	43.0

at Feijoas do Ramos in 1998, both species were observed each season. Rating of intensities was made on a subjective integer scale, comparable between flowering and fruiting, ranging from a minimum of one (scarce production of flowers or fruits, if any) to a maximum of three (close to full production), with two representing an intermediate degree.

Since cork oak bears both annual and biennial fruiting (Corti, 1955; Díaz-Fernández et al., 2004), the latter was excluded simply by starting the observations simultaneously with holm oak fruiting.

3. Results

3.1. Phenological studies

3.1.1. Mitra in 1998

Observation of flowering phases at short intervals of time (2–3 times every week) produced a detailed picture of the intraspecific variation of time of flowering in both species. This scattering was observed through all phases of flower development, such that a considerable overlap of flowering times was produced between the two species, notwithstanding the average gap of 21–28 days (depending on the phase, 22 days for Fm2) between them (Fig. 1).

A feature of interest was the duration of the Fm2 phase, in general slightly longer in cork oak than in holm oak, but with a striking increase in trees with their date of Fm2 onset close to the overlap with the other species (Fig. 2).

3.1.2. Mitra, Feijoas do Ramos and Alfaiates in 2000

This year the records of flowering were made mostly over March and beginning of April, but now for three mixed stands. The flowering dates in the holm oaks at Mitra were almost identical to those recorded in 1998 (Fig. 1): most had reached phase Fm2 at or before 6 April at Mitra (18 out of 22, discounting those that aborted flowering), Feijoas do Ramos (19/20) and Alfaiates (13/17). Regarding the date of Fm2 onset, of 18 individuals that could be compared between both years only three deviated more than 1 week.

The interruption of observations barred the acquisition of comparable data in cork oak. However, on 5/6 April 2000 only 18 out of 39 trees at Mitra, 11/32 at Feijoas do Ramos and 15/20 at Alfaiates had attained phase C, in contrast to the observation at Mitra on 6 April 1998, when all that flowered were at least in phase Dm, and 17 were in phase Em (Fig. 1). On 28 April only one tree had reached phase Fm2, showing exactly the same flowering phases as it did on 6 April 1998. Overall, the date in 1998 that resembled the situation for cork oak of 5 April 2000 was that of 20 March. Hence, the cork oaks were further delayed from holm oaks by approximately 2 weeks.

As seen in Fig. 1, in 1998 at Mitra Fm2 occurred in holm oak at a very similar time span as Dm did in cork oak. An analogous comparison showed that in 2000, pooling the three stands together, the distribution of holm oaks at Gm was almost identical with cork oaks at Bm (Supplementary Fig. 1). Thus, the delay of cork oak flowering in 2000 must have reduced, relative to 1998, the opportunity for hybridization.

3.2. The time span for hybridization

The data from 1998 at Mitra allow an assessment of how many trees might be involved in hybridization, on account of their phases at each time point. Considering the protandry in both species, this involves the period of time from the earliest records of cork oaks at Fm2 (6 April) to the last estimated dates of receptivity by holm
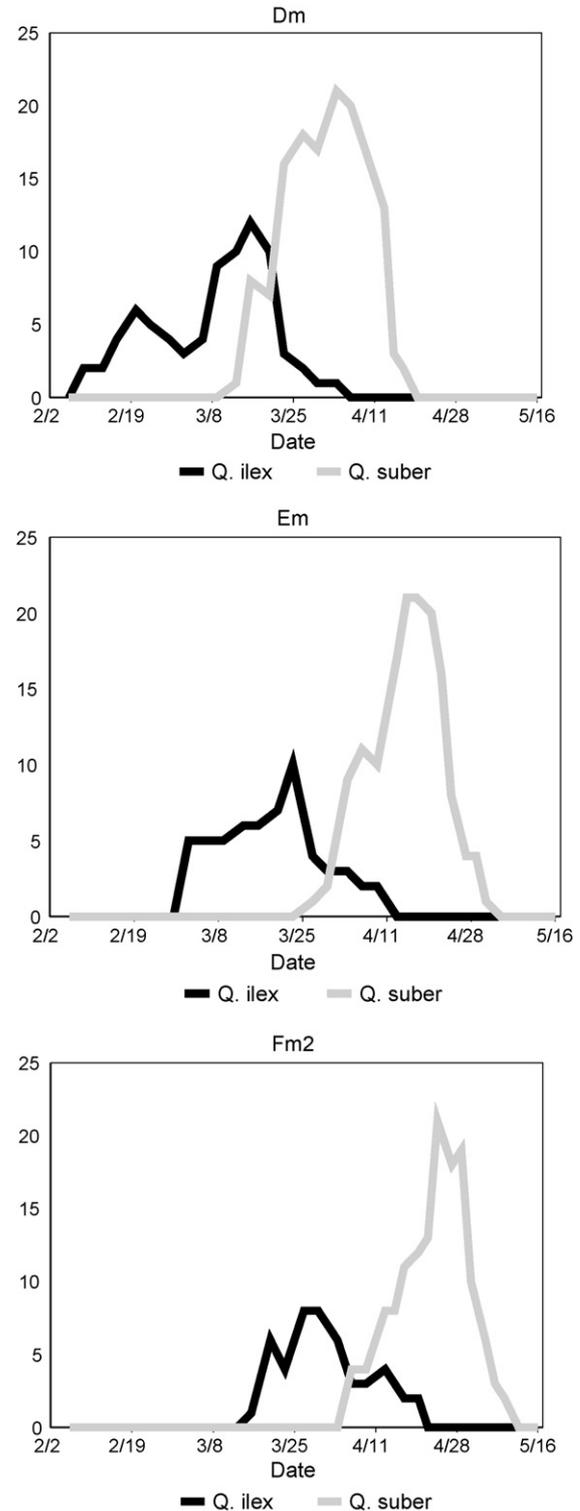
Fig. 1. Frequency distributions for time of occurrence of flowering phases. Dm, Em and Fm2 for holm oaks (*Q. ilex*) and cork oaks (*Q. suber*) observed at Mitra in 1998. Note that at a given date a tree could display flowers in different phases, and also that each phase might span, for a given tree, more than one date.

oaks (24 April). The last date of receptivity in each tree was assumed to lie, for both species, within 5 days after the last record of its Fm2, in most cases coinciding with the last observation of Gm.

Thus, within that time span, one could say that 10 out of the 25 holm oaks under study might be involved in cross-pollination with
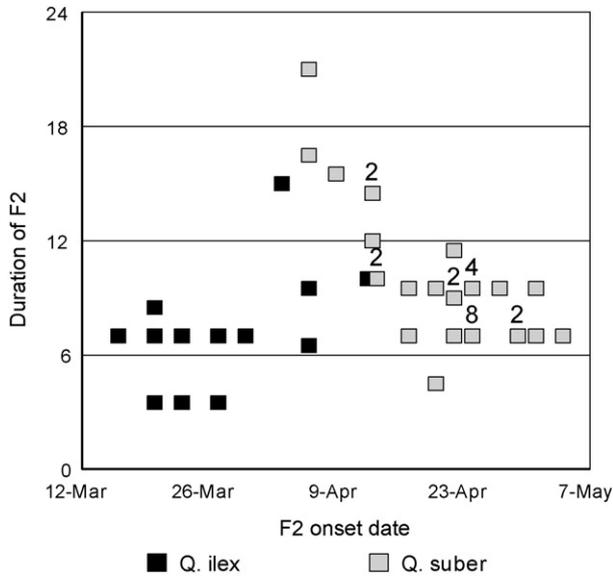
Fig. 2. Relation between observed date of Fm2 onset and its subsequent duration, as observed in Mitra in 1998. The number above each point is the number of trees, if more than one.

Fig. 3. Average number of female flowers (and developing fruits) per spike, in labeled branches of four cork oaks and eight holm oaks at Mitra, in 1998.

cork oak to some degree, five among them as possible pollinators of cork oaks since they were at Fm2 on 9 April or later. Reciprocally, 28/34 flowering cork oaks had reached Fm2 at or before 24 April, and 10 among them already at 17 April or earlier; the latter were probably receptive to pollen released by the 20, which included pollen from holm oak. Thus, at least regarding the opportunity for cross-pollination, it could have happened in both directions, i.e. through pollination of holm oak by cork oak and vice versa.

3.3. Outcome of female flowers after pollination

Labeled branches from a few trees at Mitra, each containing one or two spikes of female flowers, were observed past the pollination period in 1998 in order to analyze their outcome. In all cases a decrease in the number of developing flowers per spike was observed over time (Fig. 3), to reach on 14 July an average of 0.71 among holm oaks (range 0.30–1.23 for different trees) and 0.80 among cork oaks (range 0.70–1.17). Using the Gm phase in each tree as reference for phenology alignment, two periods of decrease were identified, occurring around 30 and 60 days after Gm in holm oak and 12 and 24 days in cork oak.

3.4. Intensity of flower and fruit production

Flowering at Mitra, although at similar average levels for both species in 1998 (2.44 for holm oak and 2.48 for cork oak, in a scale ranging from 1 to 3, see Section 2), was not sustained by cork oaks in the following years (1.74 in 1999 and 1.64 in 2000), in contrast to holm oaks (2.28 and 2.68, respectively). However, in 2000 the average cork oak flowering at Feijoas (2.19) and especially at Alfaiates (2.42) was closer to the holm oak levels (2.85 and 2.35, respectively). Fig. 4 illustrates the distributions of intensities for both species at the three stands in 2000.

Practically all trees identified as potential cross-pollinators at Mitra in 1998, as described above, had high flowering intensities (average intensity 2.4 for 5 holm oaks and 2.5 for 26 cork oaks).

As expected (Varela, 1994, 1996), fruit production at Mitra in 1998 was at a lower level than the previous flower production (the two scales are comparable), especially for cork oak (average 1.41), but also for holm oak (average 2.17). The 1998 fruit production at
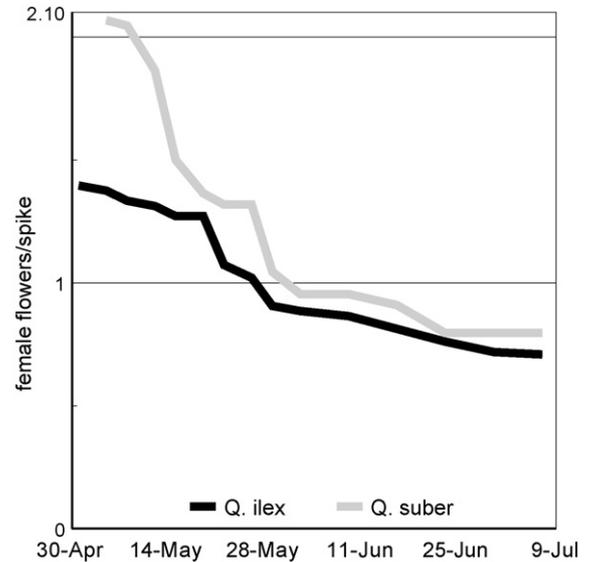
Alfaiates and Feijoas do Ramos was much higher than at Mitra: the averages were 2.55 and 2.11, respectively. By contrast, holm oaks at Alfaiates averaged at 2.10, similar to those at Mitra. Lower flower production in 1999 at Mitra was followed by an analogous reduction of fruit production (average 1.56 for holm oak, and an invariant 1 for cork oak).

Concerning fruit production by presumably receptive trees during the period where interspecific pollination might have occurred at Mitra in 1998, it was fairly weak: 1.4 average intensity for the holm oak set and 1.2 for the cork oak set.

4. Discussion

The present results document a set of phenomena regarding the occurrence of pollen release and subsequent fruiting in *Q. ilex* ssp. *rotundifolia* (the holm oak predominant in Spain, Portugal, Morocco and Algeria) and *Q. suber* (cork oak). As expected, holm oak developed flowers earlier (Vasconcelos and Franco, 1954; Franco, 1990), but the wide scatter of pollen release dates in both species blurred the separation from cork oak, such that in years like 1998 a significant overlap can occur.
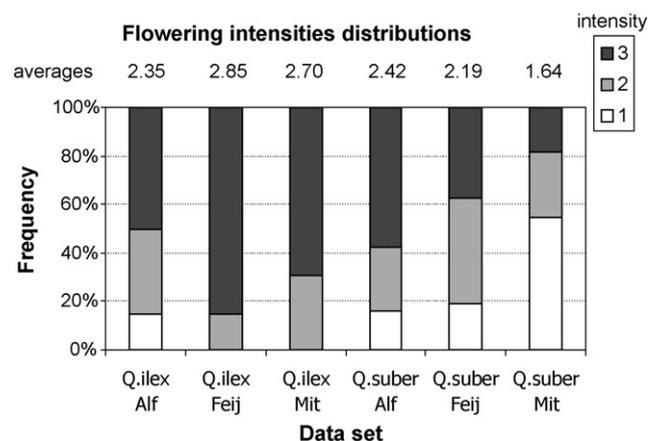
Fig. 4. Distribution of flowering intensities in 2000, as determined on a class scale (1–3) for holm oak and cork oak at Alfaiates (Alf), Feijoas do Ramos (Feij) and Mitra (Mit).

It seems clear, at least from pallinological studies (Brandão, 1996), that flowering dates can vary from year to year in both species independently, thus rendering the separation between their flowering periods variable. The comparison of flowering dates in 1998 and 2000 at Mitra suggests that there was a delay of about 2 weeks of cork oak flowering in 2000, possibly related to the cold temperatures in early winter and/or the lack of rain extending past mid March. With further studies relating this variation with climatic parameters, the likelihood of interspecific overlap of pollen emission might become more predictable.

Field observations of flowering phases can also be a good predictor: from this study it is suggested that if a comparable proportion of holm oaks at Fm2 and cork oaks at Dm are present simultaneously, as was the case in 1998 (Fig. 1), it should be a year of significant opportunity for cross-pollination.

However, the temporal overlap between pollen emission from one species and the stigma receptivity in the other species is merely a necessary factor, but not sufficient, for the actual incidence of hybrids. Post-pollination events are important for maintaining the barriers to hybridization between *Quercus*, even if pollen release by the two species is overlapping (Ducousso et al., 1993; Kanazashi et al., 1997; Boavida et al., 2001; Williams et al., 2001). The whole reproductive process that follows pollination involves the development of pollen tubes through the pistils, most of which do not reach the ovaries (Cecich, 1997; Yacine and Bouras, 1997; Boavida et al., 1999), competition for fertilization (Boavida et al., 1999), embryo development, seed viability and germination, each with a potential for selection against hybridization.

Experimental cross-pollination between these two species (Boavida et al., 2001) suggested a high tolerance to cork oak pollen by holm oak, but an increased proportion of abortive embryos relative to intraspecific pollinations. However, the inability to obtain seed from pollinated cork oak calls for an improvement of the methodology. Most regrettable, however, is the lack of pollinations using pollen mixes from both species, as done in other species of *Quercus* (Kanazashi et al., 1997), but with the use of recently developed discriminating molecular markers (Oliveira et al., 2003; Soto et al., 2003) such simulations of mixed pollination can now be traced to the seeds and seedlings.

The present study has also shown that the system proposed by Varela and Valdiviesso (1996) for classifying phenological phases in oaks enables meaningful characterizations, especially if the time points for observation are close enough. In our experience, also from more recent work with other colleagues, it is fairly easy to learn its use in the field for the observation of male flowering. It appears to be easily applicable to any species of *Quercus*, as would be predicted from the minor adaptations from earlier systems used for *Juglans* or *Castanea* species (Valdiviesso, 1991).

Both species being slightly protandrous, it is more likely that holm oak receptivity extends to the time of pollen release by cork oak than the opposite. Therefore, the preferential direction of hybridization involves pollination of holm oaks by cork oaks, and the common occurrence of "ilex" cpDNA haplotypes in cork oaks is indirect evidence of this (Belahbib et al., 2001; Jiménez et al., 2004; Lumaret et al., 2005; Magri et al., 2007). In contrast, hardly any evidence of "suber" cpDNA in holm oaks has been recorded (Belahbib et al., 2001; Collada et al., 2001).

Molecular analyses of the progenies from mixed stands should provide answers to questions on post-pollination phases. In a parallel study, over 1000 descendants from the Mitra, Feijoas do Ramos and Alfaiates fruiting season of 1998 were analyzed for diagnostic alloenzymatic markers, and not even one was a hybrid

(Oliveira et al., 2003). Considering that it was a year that appeared to favor hybridization (at least in Mitra), this suggests a strong post-pollination barrier to it, but more detailed studies would be needed. A more direct approach must involve a close look on post-pollination processes and embryo development, by coupling the observation of fruit abortion in the field with genetic analyses using diagnostic markers. The present study has already shown that two periods of significant abortion occur within the first few weeks after the Fm2 phase, and the possibility that the time points at which they occur can be predicted for each tree should be very helpful for that approach.

Physical barriers imposed by intervening tree crowns might also contribute to an effective reduction of anemophilous interspecific pollination (Sarvas, 1962); for example, the Mitra individuals from both species that could be involved in inter-specific pollination in 1998 were somewhat apart topographically (Supplementary Fig. 2). This would not rule out cross-pollination from remote sources, which tends to be predominant in oaks (Streiff et al., 1999; Valbuena-Carabaña et al., 2005). The relative concordance in timing among the three mixed stands in 2000 argues for the possibility that in 1998 the precocious cork oak pollinators were not only those at Mitra but also at surrounding stands.

However, it is known that a significant role in pollination of a tree is played by its closest neighbors, as can be expected from the anemophilous pollen transport and self-incompatibility (Streiff et al., 1999). Given the high repeatability of flowering timings in cork oak (Varela, unpublished results), and probably in holm oak as well (this study), we can hypothesize that the liability for interspecific pollination would involve the same individuals in each stand every year. In other words, one would expect hybridization to occur in spots where at least one late flowering holm oak were well exposed to pollen from early flowering cork oak (and vice versa). Another factor that might favor the incidence of hybrids, possibly relevant in some *Quercus* systems, is the virtual isolation from conspecific pollen. Published examples of this are the *Q. petræa* tree B of the study by Streiff et al. (1999) and the environmental emascula-tion model of Williams et al. (2001).

5. Conclusion

The overall separation between the pollen emission periods by holm oak and cork oak contributes to exclude a majority of individuals from cross-pollination events, even in years of significant overlap. A complementary role of post-pollination barriers for the avoidance of hybridization is very likely.

On the whole, potential negative impacts on cork quality, from hybridization between these two species in mixed stands, appears to be overrated. However, it is possible that a local juncture of various facilitating factors, with cross-pollination as a necessary component, may result in hybridization events, hence justifying research focused on the occurrence of introgressed trees that can be connected with current or past occurrence of such hybrids.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foreco.2008.06.049.

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