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REPRODUCTIVE PATTERNS SHAPE INTROGRESSION DYNAMICS AND SPECIES SUCCESSION WITHIN THE EUROPEAN WHITE OAK SPECIES COMPLEX

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The reproductive system of hybrids is an important factor shaping introgression dynamics within species complexes. We combined paternity and parentage analyses with previous species characterization by genetic assignment, to directly identify reproductive events that occurred within a stand comprising four European white oak species. Comparing species status of parent pairs provided a precise quantification of hybridization rate, backcrosses, and intraspecific matings in two life stages. The detailed mating system analysis revealed new findings on the dynamics of interspecific gene flow. First, hybrids acted successfully as both male and female during reproduction. They produced acorns and seedlings that were as viable as those sired by purebreds. Second, species maintenance could be due to a relatively low level of interspecific mating contrasting with a large proportion of intraspecific crosses and backcrosses. Despite a high proportion of hybrids and extensive interspecific gene flow, partial species integrity is maintained by genetically controlled pollen discrimination, ensuring preferential matings within purebreds and high parental species fidelity in hybrid reproduction, which impedes complete collapse into a continuous hybrid swarm. Finally, we showed that pollen from the different species had unequal contributions to reproduction suggesting that introgression processes could ultimately lead to extirpation or expansion of some species.

KEY WORDS: **Hybridization, genetic assignment, microsatellites, parentage, paternity, *Quercus*.**

Hybridization is a widespread phenomenon among animal and plant taxa (Dowling and Secor 1997; Rieseberg and Carney 1998; Mallet 2005). In bringing together genomes from different species, interspecific reproduction can have very different consequences. On the one hand, species separated by strong reproductive isolation rarely hybridize and when formed, hybrids can be unfit, restricting hybridization to the first generation (Jiggins and Mallet 2000; Orr and Presgraves 2000). On the other hand, species separated by very weak reproductive barriers can collapse into a hybrid swarm when they come into contact, leading to the mixing of both species in a unique hybrid lineage, a process called reverse speciation (Seehausen 2006; Taylor et al. 2006). Between these two extreme scenarios, reproductive isolation between taxa can display intermediate levels, with prezygotic barriers hindering hybridization events and postzygotic barriers affecting the survival and fertility of hybrid individuals. The relative contribution of these two kinds of reproductive isolation together with the ecological context of hybridization will determine the outcome of interspecific reproduction. Among the factors shaping hybridization dynamics, the reproductive system of hybrids will have a significant impact. When hybrids are reproductively isolated from their parental species (as a consequence of ecological or geographical barriers or because of genetic incompatibility), they can form a new species, a phenomenon termed hybrid speciation (Rieseberg and Carney 1998; James et al. 2005; Chapman et al. 2007; Abbott et al. 2009). However, in other cases, hybrids reproduce with their parental species, producing second or later generation backcrossed individuals. Depending on the abundance of hybrids and parental species but also on the reproductive system of the interacting taxa, backcrosses between hybrids and parental species can lead to the recovery of one of the parental species types by recurrent backcrosses with the same parental species (Rieseberg and Carney 1998; Buggs 2007). Such later generation backcrosses can display pure parental phenotypes but still contain introgressed genes from the other parental species. Alternatively, when reproductive barriers are weak or absent, a hybrid swarm can form (Rhymer and Simberloff 1996; Seehausen et al. 2007). Hence, one of the most challenging issues regarding hybridization in such cases is the maintenance of species identity in spite of interspecific gene flow.

Because of their long life span, perennial habit, and predominantly outcrossed mating system, tree species are particularly prone to hybridization (Ellstrand et al. 1996; Petit and Hampe 2006). Examples of hybridization in tree species are numerous. Among the most frequently cited are examples from *Populus* (Martinsen et al. 2001; Lexer et al. 2005, 2007), *Pinus* (Williams et al. 2002), *Fraxinus* (Fernandez-Manjarres et al. 2006; Gérard et al. 2006), *Quercus* (Arnold 2006; Lepais et al. 2009), *Salix* (Hardig et al. 2000), *Eucalyptus* (Potts and Reid 1988), *Morus* (Burgess et al. 2005) and *Aesculus* (Thomas et al. 2008). Hybridization within the European white oak complex has been the focus of many recent studies that have demonstrated the importance of this phenomenon both in the past, including during postglacial recolonization (Petit et al. 1997, 2004), and more recently, as inferred from genetic assignment or parentage studies (Streiff et al. 1999; Valbuena-Carabana et al. 2005, 2007; Curtu et al. 2007, 2009; Salvini et al. 2009; Lepais et al. 2009). Despite extensive hybridization, species remain morphologically and ecologically well differentiated. To date, the mechanisms of maintenance of species integrity are still poorly understood. Introgressive hybridization was proposed as a mechanism of species dispersal in eucalypts (Potts and Reid 1988) and in oaks (Petit et al. 2004). In this model, established population of a postpioneer species could be invaded by a later successional species by recurrent and asymmetric hybridization and introgression through pollen swamping (Petit et al. 2004). However, in spite of intensive studies analyzing introgression patterns among adult oaks and estimating hybridization rate in natural populations, the significance of introgression as a dispersal mechanism remains unclear. Further studies that follow the fate of hybrid lineages and encompass several life stages are thus needed to uncover the consequences of introgression within the white oak complex.

In this article, we contribute new insights into species maintenance in the face of hybridization by studying the mating system of four white oak species and their hybrids in a natural population. Despite its major importance for the outcome of hybridization within the oak species complex, hybrid mating system has never been studied before, except in the case of a single hybrid produced by artificial pollination (Olrik and Kjaer 2007). Our study included the four species of the European white oak species complex that occur in France: *Quercus robur* L., *Q. petraea* (Matt.) Liebl., *Q. pubescens* Willd., and *Q. pyrenaica* Willd. We took advantage of recent methodological advances that allow a precise identification of hybrids to study in detail the reproductive fate of hybrid compared with purebred individuals. We used a combination of genetic assignment, paternity, and parentage analyses to track back reproductive events that have occurred within the stand. However, such methods cannot characterize precisely the pollen coming from outside the stand, a serious drawback when dealing with highly outcrossing species with pollen dispersed over long distances (Streiff et al. 1999; Valbuena-Carabana et al. 2005). We therefore applied an indirect mating system analysis approach (Smouse et al. 2001) to compare allele frequencies of pollen pools received by each studied oak (Curtu et al. 2009). In addition, we compared results obtained at different life stages (seed and seedling) to investigate potential selective mortality in the critical phase of the early life stages. We addressed the following questions: What kind of reproductive events are observed and what are their frequencies within the species complex? Have hybrids produced viable acorns? Can the reproductive patterns explain species persistence despite interspecific gene flow? What are the consequences of the observed pattern of introgression at the species level? We conclude by discussing the evolutionary significance of introgressive hybridization in terms of species dynamics within this species complex.

Materials and Methods

STUDIED SPECIES AND POPULATION

Our study includes all four species of the European white oak species complex that occur in France. *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. are keystone species of temperate European forests, whereas *Q. pyrenaica* Willd. and *Q. pubescens* Willd. are more thermophilous species mostly found in Atlantic and Mediterranean forests in southern Europe. In addition to climatic factors, the distribution ranges of the species are shaped by edaphic factors (Rameau et al. 1989). *Quercus robur* occurs in rich, deep soils and can tolerate flooding whereas *Q. petraea* is found on poorer and dryer soils. *Quercus pubescens* grows on limestone whereas *Q. pyrenaica* is commonly found on sandy soils. Three of the four species. *Quercus robur*, *Q. pubescens*, and *Q. pyrenaica* are postpioneers capable of colonizing open land, whereas *Q. petraea* is a late-successional species that grows in stable and well-established forest environments.

The studied stand (called Briouant) is located in the southwest of France (43°18′23.26″N; 1°2′51.93″E) within an old woodland managed in coppice. Historical maps indicated that the woodland was already present in the 18th century, but probably stand for an ever longer period of time.

All adult oaks have been genotyped and genetically assigned to one of the species or their hybrid as detailed in a previous study (Lepais et al. 2009). This analysis indicates that the stand is composed of 240 *Q. robur*, 235 *Q. pyrenaica*, 83 *Q. pubescens* and three *Q. petraea* individuals. In addition to these 561 individuals classed as pure species there were 246 hybrid oaks (30.5% of the adult oaks). These were classified into one of the six possible hybrid classes corresponding to all possible pairs of parental species (28 *Q. robur* × *Q. petraea*, 35 *Q. robur* × *Q. pubescens*, 48 *Q. robur* × *Q. pyrenaica*, 7 *Q. petraea* × *Q. pubescens*, 40 *Q. petraea* × *Q. pyrenaica* and 88 *Q. pubescens* × *Q. pyrenaica*; Lepais et al. 2009). Hybrids between all species pairs were identified in this stand Hybrids involving rare species are at higher frequencies than expected on the basis of random hybridization between pure species (see Fig. 1). The high proportion of hybrids in this stand could be due to the unequal frequencies of the four species, thereby increasing hybridization opportunities (Lepais et al. 2009) and to the forest management of the stand (coppice) that correspond to a disturb environment potentially increasing hybridization rates (Anderson 1948).

PROGENIES SAMPLING AND GENOTYPING

Acorns on mother trees

We collected a total of 623 acorns from 16 mother trees (mean: 38.9, SD: 15.5) evenly located within the stand (Fig. 1). Acorns were collected in different parts of the crown of each tree. Among the sampled mother trees, six were purebreds (three *Q. robur*, one *Q. pubescens* and two *Q. pyrenaica*; Table 1) whereas 10 were hybrids (three *Q. robur* × *Q. petraea*, two *Q. robur* × *Q. pubescens*, one *Q. robur* × *Q. pyrenaica*, three *Q. petraea* × *Q. pyrenaica*, and one *Q. pubescens* × *Q. pyrenaica*; Table 1).

Each collected acorn was weighed (Table 1) and individually labeled. About one-fifth of the basal part of the acorn, containing mostly cotyledon tissues, was cut for subsequent DNA isolation. For 10 progenies, the remaining part of the acorn containing the embryo was placed in a 100-mL pot filled with potting compost and watered as required. Germination success was measured during the following six months (Table 1). Mean acorn weight and germination rate were computed for each mother tree and differences between purebred and hybrid mothers tested by analysis of variance (ANOVA).

DNA isolation was performed following a modified CTAB/24:1 chloroform:isoamylalcool extraction methods (Lepais et al. 2006b) with an addition of 0.5 mg/mL Proteinase K and 0.2% β-mercaptoethanol in the extraction buffer. After extraction, DNA was diluted in distilled water (1:400) and typed at 10 highly polymorphic microsatellite markers (QrZAG11, QrZAG39, QrZAG96, QrZAG112, QpZAG110, QrZAG5b, QrZAG7, QrZAG20, QrZAG65, and QrZAG87 (Steinkellner et al. 1997; Kampfer et al. 1998)) using two multiplex PCR amplifications as described elsewhere (Lepais et al. 2006b). Separation of PCR fragments was carried out using a MegaBACE 1000 96-capillaries automated sequencer (Amersham Biosciences, Uppsala, Sweden) and alleles were scored with Fragment Profiler version 1.2 (Amersham Biosciences). In 23% of the individuals (146/623), more than two alleles were encountered at one or more loci, which we attribute to the presence of a small amount of maternal tissue in the material from which DNA was isolated. In such cases, these genotypes were transformed into diploid genotypes by randomly removing one of the two maternal alleles.

Established seedlings

We exhaustively sampled 208 established seedlings, from 1- to 10-year old, in five 1 m^2 plots evenly distributed within the stand (Fig. 1). Each seedling was tagged and one of its leaves was sampled and stored at 4°C until subsequent long-term storage at −80°C in the laboratory. DNA isolation and genotyping of 10 microsatellite loci followed the protocols described elsewhere (Lepais et al. 2006b).

DIRECT ESTIMATION OF REPRODUCTIVE EVENTS

Paternity analysis on acorns

A maximum-likelihood paternity analysis was performed using the software FaMoz (Gerber et al. 2003) to identify adult oaks within the stand that had sired the acorns collected on the 16 mother trees. We first computed identity and exclusion probabilities based on the allele frequencies of the 10 microsatellites among adult oaks. The most likely fathers were then identified on the basis of the log of the odds ratio scores (LOD scores) with an optimal threshold determined by simulation (as described in Gerber et al. 2000, 2003). When two oaks had an equal LOD score, that is, an equal probability to be the father of a particular seed, no father was assigned to the corresponding acorn. The error due to mistyping (estimated, by mother–offspring mismatches, to occur in 2% of monolocus genotypes) was accounted for by introducing an error rate of 0.000001 in the simulation procedure and in the assignment of the most likely father. This error rate parameter allowed for one mismatch between the offspring and the potential parent genotypes, avoiding undue exclusion (Christie 2010).

Within each progeny, the number of fathers belonging to each species and hybrid class was reported. As each mother and

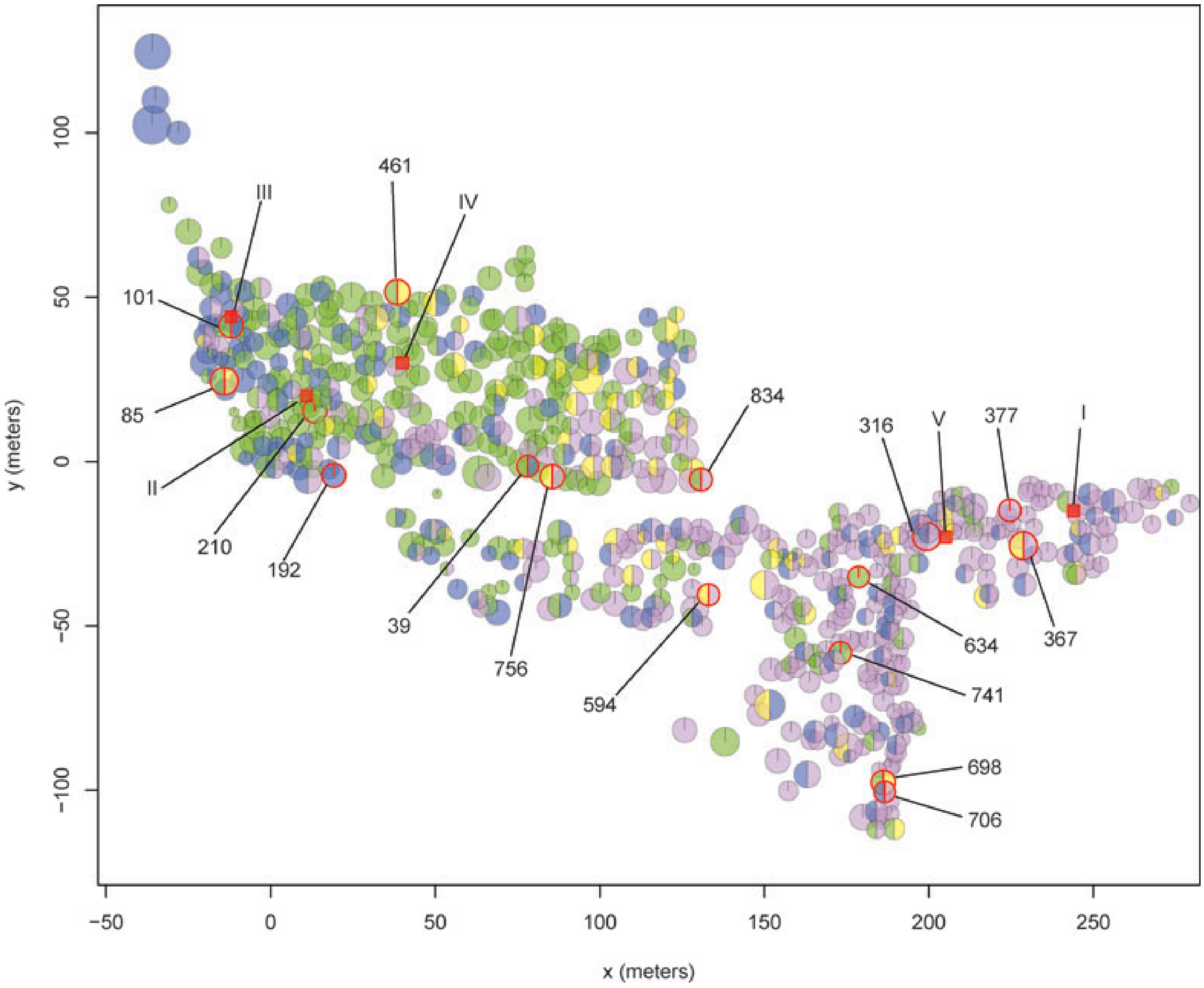

Figure 1. Map of adult oaks within the Briouant stand. Each oak is represented by a pie chart with colors corresponding to the species as determined by genetic assignment (green: *Q. robur*, purple: *Q. pyrenaica*, blue: *Q. pubescens* and yellow: *Q. petraea*). Hybrid oaks correspond to pie charts with two colors indicating their two parental species. The size of the pie charts is proportional to the stem diameter at breast height. Oaks sampled for acorns and plots sampled for established seedlings are identified by Arabic and Roman numbers, respectively.

father tree was classified into a particular species or hybrid class, the reproductive event could be precisely described. For progenies with a purebred mother, the possible reproductive events observed were: intraspecific (parents from the same species), interspecific F1 (parents from different species), backcross (the father is a hybrid between two species, the mother species being one of these), or trihybridization (the father is a hybrid between two parental species, the mother is from a third, different species). For progenies with a hybrid mother, the possible reproductive events observed were: F2 hybridization (both the mother and the father belong to the same hybrid class), backcross (the father is a purebred from one of the parental species of the hybrid mother), or trihybridization (with either a father from a purebred species different from the two parental species of the mother or a hybrid father involving one species different from that of the mother, both cases leading to the admixture of three different species in the genome of the progeny). Reproductive events are reported by mother tree and summed over purebred or hybrid mother trees.

Parent pair analysis on established seedlings

FaMoz was also used to implement a maximum-likelihood parentage analysis to identify potential single parent or parent pairs of the sampled seedlings. We computed the exclusion probabilities for a single parent or a parent pair. The most likely parent or parent pair was determined using an LOD score threshold value determined by simulation (Gerber et al. 2000, 2003). When for a particular seedling two putative parent pairs had an equal LOD score for a particular seedling, we did not assign the relationship. The same error rate as in the paternity analysis was included to

Table 1. Summary of the 20 analyzed half-sib progenies.

Mother tree ID	Species	Sampling	*N*	Mean acorn weight (g)	Germination rate (%)
210	rob	acorns	16	1.86	nd
634	rob	acorns	32	1.70	nd
741	rob	acorns	46	1.72	37.5
192	pub	acorns	69	0.83	72.4
377	pyr	acorns	17	1.28	nd
316	pyr	acorns	18	1.61	48.1
316	pyr	seedlings	21		
388	pyr	seedlings	45		
211	rob	seedlings	11		
443	rob	seedlings	24		
85	hyb_robpet	acorns	41	2.40	44.2
461	hyb_robpet	acorns	29	0.64	nd
698	hyb_robpet	acorns	52	2.22	32.1
39	hyb_robpub	acorns	56	2.08	66.1
101	hyb_robpub	acorns	28	0.52	nd
834	hyb_robpyr	acorns	50	2.94	61.0
367	hyb_petpyr	acorns	28	1.75	72.7
594	hyb_petpyr	acorns	43	1.31	nd
756	hyb_petpyr	acorns	50	2.53	69.1
706	hyb_pubpyr	acorns	48	1.91	79.2
Overall		20 progenies	724		

Seedlings refer to the four half-sibling family identified by the parent pairs analysis (see Result section). *N*=number of offspring. Species are abbreviated as followed, rob=*Q. robur*, pyr=*Q. pyrenaica*, pub=*Q. pubescens*, pet=*Q. petraea*, hyb_xy=hybrid between species x and y. Nd=not determined.

account for potential genotyping errors. The different types of crosses observed for seedlings are reported as described above.

Comparison of observed and expected reproductive events

We compared the number of reproductive events observed with the number expected under two different mating models. The first model, called Full Panmixia (FP) model, consists of a random mating between each studied mother tree and all trees within the stand. The second model, called Constrained Panmixia (CP), is more realistic given the reproductive events revealed by paternity analyses (see results section). Purebred mothers are allowed to mate randomly with conspecific individuals (producing intraspecific crosses) and with their hybrids (producing backcrosses), whereas hybrid mothers mate randomly with hybrids of the same type (producing F2) and with their parental species (producing backcrosses). The advantage of computing expected number of reproductive events under two mating models is that we take our sampling design into account. We have indeed a limited sampling of mother trees that is potentially not representative of the stand composition, thus our computation takes this potential bias into account.

For each maternal tree studied, we computed the expected number of reproductive events under the FP model and under the CP model, taking into account the species or hybrid class of the focal mother tree, the number of acorns studied and the number of seedlings assigned to it, as well as the frequencies of adult species and hybrids in the stand. For seedlings with two assigned parents, the spatially closest parent was considered to be the mother because in oaks, seed disperse at much smaller distance than pollen (Dow and Ashley 1996). We then summed all observed and expected number of reproductive events over all purebred and all hybrid mother trees and compared the observed and expected numbers under the two mating models independently with a Fisher exact test.

INDIRECT CHARACTERIZATION OF INTROGRESSION PATTERNS

Genetic clustering of progenies

We used Structure software version 2.1 (Pritchard et al. 2000; Falush et al. 2003) to characterize the genetic composition of the progenies. A total of 2107 adult individuals previously analyzed in Lepais et al. (2009) were included, comprising the 807 oaks of the present study. The mean species sample size is high

(406 individuals, with a minimum of 146 for *Q. pubescens*), facilitating species delimitation. In particular, combining all populations allows an accurate assignment of locally rare species, which would have not been detected if the stand had been analyzed separately (as in the case of *Q. petraea* in Briouant, for instance; see Duminil et al. 2006; Fogelqvist et al. 2010). We performed the genetic clustering with the Popflag parameter set to 0 for the offspring and the option UpdateAlleleFrequency for PopFlag = 1, which removes the potential confounding effect of the offspring family structure in the clustering analysis (Anderson and Dunham 2008). We run Structure with the Admixture and Correlated Allele Frequencies models, the number of clusters (K) was set to 4, the actual number of species in the dataset, each cluster corresponding to one species (Lepais et al. 2009), and a burnin length of 100,000 followed by 1,000,000 repetition of the MCMC. As some of the progenies must be second or later generation hybrids and given the low power of genetic assignments with only a limited number of loci in such cases (backcrosses indistinguishable from purebred individuals; Vähä and Primmer 2006; Lepais et al. 2009), we did not analyze the clustering results at the individual level. Instead, we report each of the four admixture coefficients of the offspring within half-sibling families as an estimation of their global genetic composition.

Genetic differentiation among pollen pools

To further characterize the species origin of pollen received by each mother tree, we studied the genetic differentiation among pollen pools that sired each sampled mother. We performed this analysis on acorns from 16 half-sibling progenies and on four seedling groups that were half-siblings according to the parentage analysis. Because we were interested in the pollen characteristic at the species level, we only kept one offspring per full-sibling group to minimize the genetic structure due to multiple pollination by the same father in each sibship (a total of 672 progenies, including 597 acorns and 75 seedlings, were used for this analysis). We used the TwoGener method (Smouse et al. 2001) implemented in R (Hirao et al. 2006; R Development Core Team 2009) to compute the pairwise genetic differentiation among pollen pools received by mother trees (Φ_{FT}). We modified the TwoGener R script to test for significance of pairwise Φ_{FT} by performing 4000 randomizations of the pollen genotypes among each pair and by computing Φ_{FT} in each randomization to construct a null distribution of the estimate. We extracted the P-value as the proportion of Φ_{FT} obtained with randomization that was equal or above the observed Φ_{FT}. Finally, we computed the Euclidian distance matrix between rows of the Φ_{FT} matrix and used it to construct a Neighbor-Joining tree with the R package APE (Paradis et al. 2004).

Results

ACORN WEIGHT AND GERMINATION

Mean weight of acorns collected on hybrid individuals did not significantly differ from those sampled on purebred mothers (mean acorn weight on hybrids: 1.83 g, standard deviation: 0.79 g purebreds: 1.50 g, standard deviation: 0.38 g; $F = 0.89$, df = 1, $P = 0.36$). Similarly, mean germination rates did not significantly differ between acorns sampled on hybrids (60.6%) or on purebreds (52.7%; $F = 0.46$, df = 1, $P = 0.52$). Thus, acorns produced by hybrids do not exhibit any detectable disadvantage compared to those from purebreds.

PATERNITY ANALYSIS ON ACORNS

Our set of 10 microsatellites yielded a cumulated exclusion probability of 1.0 for paternity analysis. Applying the optimal LOD score threshold resulted in a type I error (excluding a true father) of 0.5% and a type II error (assigning a wrong father) of 1.5%. Among the 623 acorns analyzed, we were able to assign a father for 167 (26.1%) of them. The percentage of unidentified fathers ranged from 43.7% to 100% among progenies (Table S1). Pollen came from a total of 126 oaks producing 167 acorns. Only 15 of the 126 trees sired more than one acorn (12 trees fathered two acorns, one three acorns, one four acorns and one 10 acorns; Table S1). Selfing was detected in four mother trees including two *robur* × *petraea* hybrids and two *Q. robur* purebreds, producing one acorn each.

According to the species of each mother and father trees, we could classify reproductive events into five categories detailed above (Material and Methods section). Among purebred mother trees, the majority of reproductive events were intraspecific crosses (35 events, 63%) followed by backcrosses (13 events, 23%; Table 2). Five first-generation hybridizations (F1) were observed (9%) and only three trihybridization events (5%) were detected. Under the FP model, we would expect a significantly smaller number of intraspecific crosses and a much higher number of F1 hybridization (Fig. 2A), indicating that conspecific matings are favored at the expense of interspecific ones. On the contrary, the CP model, which allows purebred species to be fertilized by conspecifics and their hybrids, provides a good fit with the distribution of observed reproductive events (Fig. 2A).

In the progeny of hybrid mother trees (Table 2), we identified 76 backcrosses (68%), 21 trihybridizations (19%), and 14 crosses between hybrids of the same class (F2, 13%). Under the FP model, this represents a significant excess of backcrosses and a significant deficit of trihybridizations (Fig. 2B). Hybrids were thus not fertilized randomly but were instead preferentially pollinated by their parental species. Furthermore, mating between hybrids is constrained by their parental species, as shown by

Table 2. **Summary of paternity analysis for acorns sampled on six purebreds and on 10 hybrid mother trees, and corresponding reproductive events.**

Mother tree species	Mother ID	Number of acorns with one father identified	Reproductive events Intra.	F1	F2	Bc.	Trihyb.
rob	210	8	6	0	-	2	0
rob	634	10	4	3	-	1	2
rob	741	19	14	1	-	3	1
pub	192	11	8	0	-	3	0
pyr	316	1	0	1	-	0	0
pyr	377	7	3	0	-	4	0
Purebreds	total	56	35	5	-	13	3
			62.5%	**8.9%**	-	**23.2%**	**5.4%**
hyb robpet	85	12	-	-	1	11	0
hyb robpet	461	8	-	-	0	7	1
hyb robpet	698	12	-	-	1	9	2
hyb robpub	39	17	-	-	1	13	3
hyb robpub	101	7	-	-	1	6	0
hyb robpyr	834	9	-	-	1	6	2
hyb petpyr	367	0	-	-	0	0	0
hyb petpyr	594	3	-	-	0	0	3
hyb petpyr	756	16	-	-	5	2	9
hyb pubpyr	706	27	-	-	5	22	0
Hybrids	total	111	-	-	14	76	21
			-	-	**12.6%**	**68.5%**	**18.9%**
Overall	**16 mother trees**	**167**	**35**	**5**	**14**	**89**	**24**
			21.0%	**3.0%**	**8.4%**	**53.9%**	**14.4%**

Reproductive events are abbreviated as follow, Inta=intraspecific reproduction, F1=first generation hybridization, F2=reproduction between hybrids sharing the same parental species, Bc=backcrosses, Trihyb=trihybridization (see Materials and Methods section for detailed explanations). Species are abbreviated as followed, rob=*Q. robur*, pyr=*Q. pyrenaica*, pub=*Q. pubescens*, pet: *Q. petraea*, hyb_xy=hybrid between species x and y.

the smaller than expected number of observed trihybridization events. In contrast, random mating between hybrids of the same type could not be ruled out, as the number of observed F2 did not depart from expectations. The CP model, which allows hybrids to be fertilized by their parental species and by hybrids of the same type, but not by other species, demonstrated the existence of a deficit of backcrosses due to an excess of trihybridization events that are not accounted for in the model (Fig. 2B). On the other hand, the expected number of F2 events did not significantly differ from the observations. Overall, the observed number of reproductive events showed a clear deviation from panmixia (FP model), as a consequence of assortative mating between conspecific individuals. This results in a higher proportion of intraspecific crosses than expected, at the expense of F1 crosses, and a preferential mating between hybrids and their parental species, leading to a higher proportion of backcrosses than expected at the expense of trihybridization. Our CP model, characterized by mating constrained by species membership, provides indeed closer (although imperfect) expectations of reproductive events than the FP model.

PARENT PAIR ANALYSIS ON ESTABLISHED SEEDLINGS

The cumulative exclusion probability of our 10 microsatellite loci is 0.999984 for a single parent and 1.0 for a parent pair. With the optimal LOD score thresholds, type I and type II errors were 0.5% and less than 0.1% (for a single parent and a parent pair, respectively).

Among the 208 sampled seedlings, 110 had two identified parents, 80 had one assigned parent, and only 18 had no parent identified. Four oaks were parents of 70 seedlings among the 110 with two parents (Table 3) and were considered to be mother trees because of their immediate proximity with the sampled plots. Considering the species status of both parents, we identified 47 intraspecific mating events (67%), 21 backcrosses (30%), 2 first-generation hybrids (3%), and no trihybridization event (Table 3). Considering all 110 seedlings with two identified parents (Table 3), 62 intraspecific crosses (56%), 38 backcrosses (34%), four mating events between hybrids sharing their two parental species (4%), three trihybridizations (3%), and three first-generation hybrids (3%) were observed. These observations

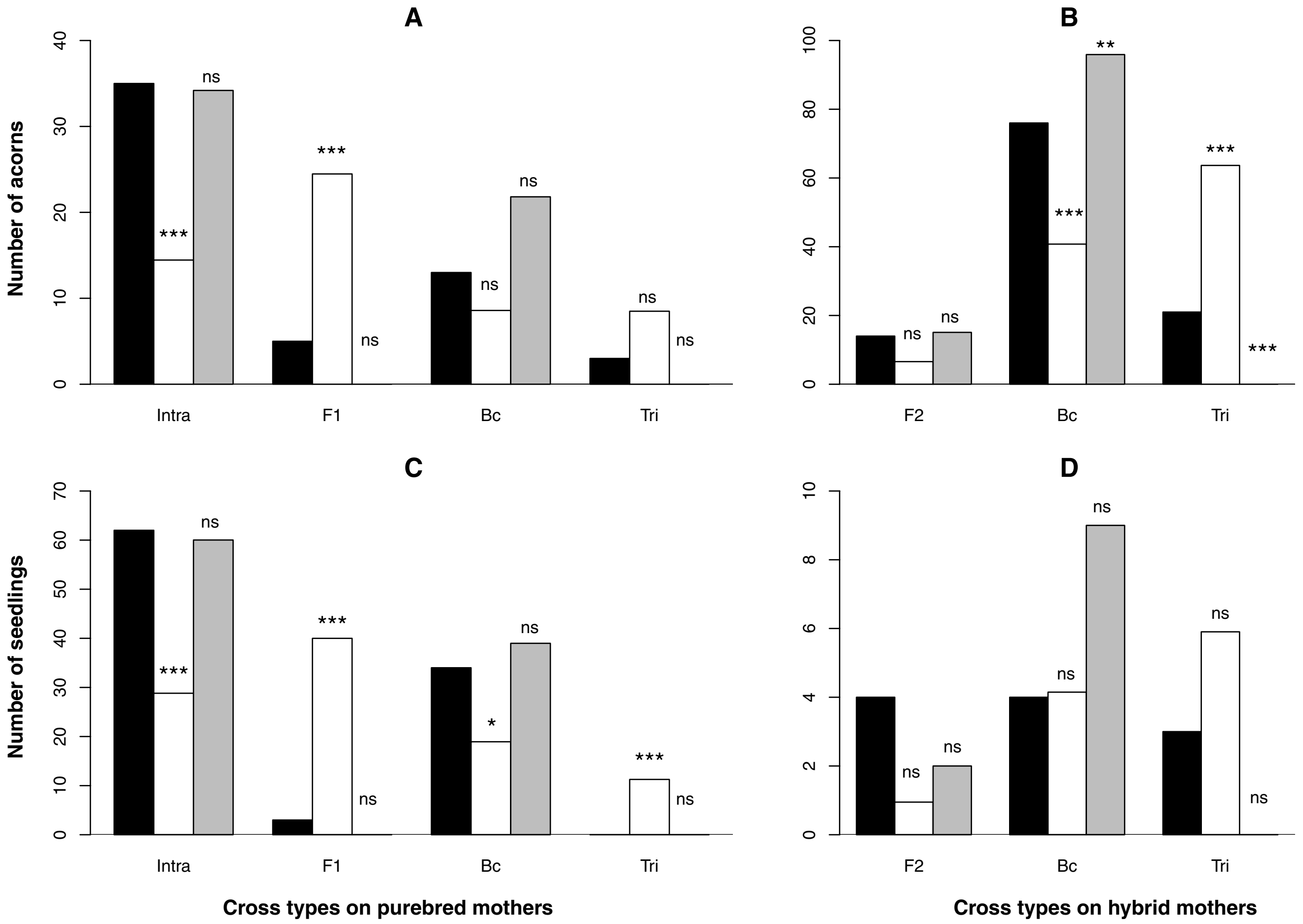

Figure 2. Observed (black) and expected numbers of reproductive events under the Full Panmixia (FP) and Constrained Panmixia (CP) models (white and gray, respectively) for acorns collected on purebred (A) and hybrid (B) mother trees and seedlings originating from purebred (C) and hybrid (D) mother trees. Expected numbers of reproductive events are compared to observations with a Fisher exact test (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$ and ns: not significant). Intra: intraspecific cross, F1: first-generation hybridization, Bc: backcross, Tri: trihybridization, F2: cross between hybrids of the same type.

Table 3. Summary of parent pair analysis for established seedlings sampled in five plots and corresponding reproductive events.

Mother tree species	Mother ID	Number of seedlings with two parents identified	Reproductive events				
			Intra.	F1	F2	Bc.	Trihyb.
Four half-siblings seedling families identified							
Pyr	388	38	25	0	-	13	0
Rob	443	15	13	0	-	2	0
Rob	211	8	8	0	-	0	0
Pyr	316	9	1	2	-	6	0
Total	**4 mother trees**	**70**	**47**	**2**	**-**	**21**	**0**
			67.1%	**2.9%**	-	**30.0%**	**0%**
Overall		**110**	**62**	**3**	**4**	**38**	**3**
			56.4%	**2.7%**	**3.6%**	**34.5%**	**2.7%**

Reproductive events are abbreviated as follows: Intra=intraspecific reproduction, F1=first generation hybridization, F2=reproduction between similar types of hybrids, Bc=backcrosses, Trihyb=trihybridization (see Materials and Methods section for detailed explanations). Species are abbreviated as follows: rob=*Q. robur*, pyr=*Q. pyrenaica*, pub=*Q. pubescens*, pet=*Q. petraea*, hyb_xy=hybrid between species x and y.

departed significantly from the FP model expectations with an excess of intraspecific crosses and backcrosses and a deficit of F1 hybridizations and trihybridizations for seedlings originating from purebred mothers (Fig. 2C). On the contrary, the CP model provided a correct picture of reproductive events for seedlings from purebred mother trees. As for acorns, intraspecific crosses and fertilization of purebred individuals by their respective hybrids were the prevalent events. The analysis of seedlings produced by hybrid mothers was not conclusive due to limited sample size (Fig. 2D).

Finally, among the 80 seedlings with one identified parent, 31 were assigned to one of the four mother oaks mentioned above and were included in the subsequent analysis at the family level.

GENETIC CLUSTERING OF OFFSPRING

The genetic clustering analysis corroborated the results of the paternity analysis (see for instance the left-most clustering plots in Fig. S1). In general, the genetic composition of an offspring corresponds to the average of the genetic composition of its parents (see pie charts and bar plots in Fig. 3). Note however that the genetic characteristics of offspring from hybrids fluctuate widely, with contributions predominantly from one of the parental species or from the other (Fig. 3). Additionally, offspring with no identified father showed a somewhat different genetic composition from that with a father in the stand (compare right and left clustering plots in each panel in Fig. S1). A common pattern among several mother trees was that incoming pollen mostly originated from *Q. pubescens* (Fig. S1).

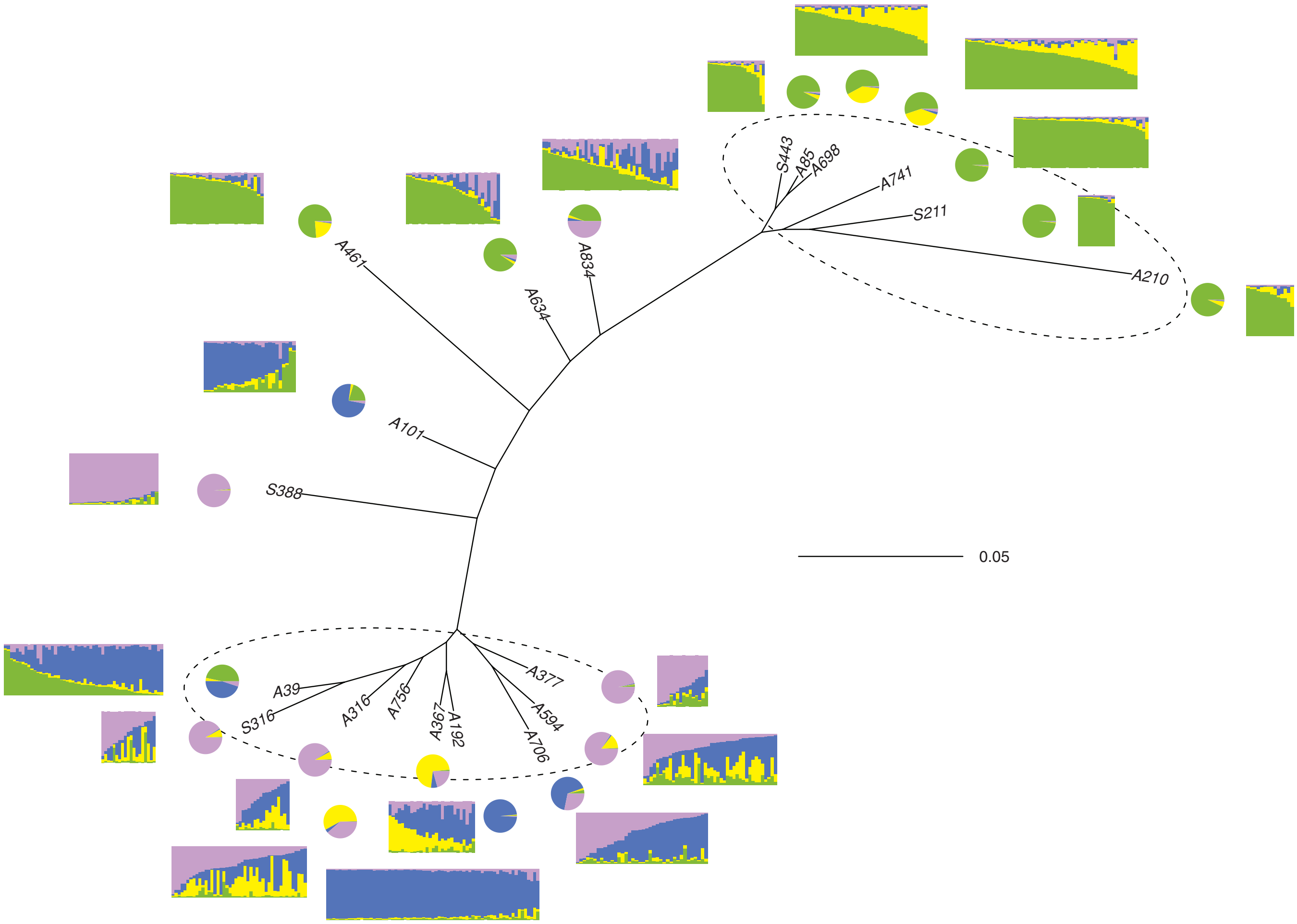

Figure 3. Introgressive hybridization pattern inferred from the pollen pool genetic differentiation between progenies represented by a neighbor-joining tree computed on Euclidian transformed pairwise Φ_{FT} matrix. Progenies surrounded with a dashed line were sired by pollen pools not significantly differentiated from each other. The tips of the branches are labeled by A for acorn or S for seedling progenies, followed by the identification number of the mother tree. Pie charts correspond to the genetic assignment of mother oaks, bar plots represent the genetic clustering of the corresponding progeny and the colors correspond to the proportion of the genomes originating from each of the four species (green: *Q. robur*, purple: *Q. pyrenaica*, blue: *Q. pubescens* and yellow: *Q. petraea*).

GENETIC DIFFERENTIATION AMONG POLLEN POOLS

The global genetic differentiation between all pollen pools (Φ_{FT}) was 0.042 (standard error estimated by jackknife over loci: 0.009). In more detail, genetic differentiation between pairs of pollen pools ranged from −0.013 to 0.116 (mean: 0.044, SD: 0.029). At a significance level of 0.05, 84% of the pairwise Φ_{FT} differed (160/190), and still 61% (116/190) at the 0.001 level. The pairwise Φ_{FT} (Table S3) was used to compute an Euclidian distance matrix and to construct a Neighbor-Joining tree (Fig. 3), which represents the genetic distances between pollen received by the different mother trees. Both extremities of the tree (Fig. 3) are composed of mother groups that were pollinated by identical pollen pools in terms of species composition. As shown by the genetic characteristics of the mother trees (Fig. 3, pie charts) and their progenies (Fig. 3, bar plots), uppermost branches of the tree group mother trees that mostly received pollen from *Q. robur*, whereas lowermost branches group mother trees that mostly received *Q. pubescens* pollen. Mother trees fertilized by *Q. robur* pollen were either purebred *Q. robur* or *robur* × *petraea* hybrids (upper part of the tree, Fig. 3). Mother trees fertilized by *Q. pubescens* pollen were either purebred *Q. pubescens*, purebred *Q. pyrenaica*, *petraea* × *pyrenaica* hybrids, or *pubescens* × *pyrenaica* hybrids (lower part of the tree, Fig. 3). Five mother trees, located at intermediate places on the tree plot, were characterized by pollen pools significantly different from the others. Among these oaks, one was almost exclusively fertilized by *Q. pyrenaica* pollen (seedlings from mother 388), whereas the other received pollen from different species constituting an unique and genetically differentiated pollen pool. Overall the species composition of the pollen pools differed markedly from the species composition of the stand, indicating an unequal contribution to pollination between species. In particular, pollen from *Q. pyrenaica* was sparsely represented in the pollen pool, despite the local abundance of this species, whereas pollen of *Q. pubescens* was abundant in the pollen pool, despite its relative rarity in the stand.

Discussion

Our detailed analysis of the mating system in a mixed stand of European white oaks revealed new findings on the dynamics of interspecific gene flow. First, hybrids appeared fully fertile and acted both as male and as female at reproduction. Second, species maintenance, despite interspecific gene flow, could in part be due to a relatively low level of interspecific mating contrasting with a large proportion of intraspecific crosses and backcross events. Finally, we showed that pollen from the different species had unequal contributions to reproduction, suggesting that introgression processes could ultimately lead to extirpation or expansion of some species, at least if the patterns of gene flow observed one year were representative of patterns of gene flow during another year.

REPRODUCTIVE EVENTS SHAPING HYBRIDIZATION DYNAMICS

Combining paternity and parentage analyses with genetic assignment of the identified parents allowed us to classify reproductive events in six different categories. Adult oaks can only be broadly assigned to purebreds or to hybrids due to the inherent difficulties to differentiate first- and second-generation hybrids with our set of markers (Lepais et al. 2009). Hence, the reproductive event classes are necessarily simplified but are still meaningful to understand the consequences of hybridization within the species complex. Despite high opportunity for hybridization given the presence of four interfertile oak species, first-generation hybridization remains limited (9% and 3% of reproductive events observed on purebred mother trees at the acorn and seedling stage, respectively). These results are consistent with estimates obtained by different paternity analyses (7.4% in Streiff et al. 1999; 8.4% in Curtu et al. 2009; an average of 15% in Salvini et al. 2009). Most of the purebreds reproduce preferentially with conspecific individuals (62% and 67% of intraspecific crosses at the acorn and seedling stage, respectively). Hybrids were mostly pollinated by one of the parental species (68% of the pollination events observed on hybrid mother trees at the acorn stage), indicating that prezygotic reproductive barriers are not totally lost in hybrids, as they remain largely reproductively isolated from nonparental species. These proportions depart significantly from expectations under random reproduction between all trees within the stand (Fig. 2). In particular, the excess of intraspecific crosses and backcrosses (and the deficit of F1 hybridizations and trihybridizations) indicates that genetically based pollen discrimination maintains a certain level of assortative mating within species and between species and their hybrids, avoiding a total collapse of species into a hybrid swarm. The high fidelity of hybrids toward their parental species, illustrated by the low departure observed with the CP mating model (Fig. 2), indicates that genes from different species are brought together by rare F1 hybrids reproductively compatible with their parental species, producing numerous backcrosses. A high rate of intraspecific gene flow is an important factor for keeping species cohesion (Mayr 1963) and for mitigating introgression (Currat et al. 2008; Petit and Excoffier 2009). Because of the high fidelity of hybrids toward their parental species, backcrosses are recurrent in both directions, with hybrids acting either as female or male, resulting in the recovery of purebreds within a few generations. It thus appears that the hybrid state is a transitory phase that connects pairs of species by gene flow, producing diverse offspring types and offering an opportunity for selecting successful genetic combinations. The combinations that are closest to the parental types can speed up recovery of purebred genotypes while allowing for some gene exchanges between species, with potential evolutionary benefits (Arnold 1992, 2006; Rieseberg and Carney 1998).

CONTRASTING HYBRIDIZATION PATTERNS ACROSS LIFE STAGES

Studies of mating system in natural populations usually target one particular life stage, that is, seeds (Streiff et al. 1999; Curtu et al. 2009; Salvini et al. 2009) or seedlings (Dow and Ashley 1996; Valbuena-Carabana et al. 2005). Analyzing only seeds could in principle overestimate the consequence of hybridization because seedling establishment is a critical stage in which strong competition and selection could remove unfit hybrid genotypes (Muir and Schlotterer 2005). Focusing on two life stages, seed and established seedlings, allowed us to compare the fate of hybridization across a longer life period. We found only slight differences in hybridization patterns between the two life stages. In particular, a comparative study of acorns and established seedlings originating from *Quercus pyrenaica* tree #316, using both genetic clustering and pollen pool analysis, showed that the genetic composition of progenies at both life stages are comparable. Despite the purebred nature of the mother tree (*Q. pyrenaica*), progenies contained quite a high proportion of *Q. pubescens* genes and the pollen pool that fertilized this tree is not significantly differentiated from the purebred *Q. pubescens* pollen pool (Fig. 3).

More generally, reproductive events involving purebred mother trees at the seed and established seedling life stages showed similar trends (Tables 2 and 3, Fig. 2). Intraspecific crosses dominate (62% and 67% in acorns and seedling respectively) whereas backcrosses are the second most frequent type of reproductive events (23% and 30%). These reproductive events are in excess according to our FP model (Fig. 2), both at the acorn and at the seedling stages.

Direct comparison of reproductive event numbers between the acorn and seedling stages showed only few significant differences. Trihybridization events that mix the genomes of three species in a single individual were found on purebred mother trees in 3 of 56 crosses at the seed stage but were absent at the seedling stage (0 out of 99 crosses, Fisher exact P value = 0.046). In addition, backcrosses observed on hybrid mother trees were significantly more abundant at the seed stage than at the seedling stage (76 out of 111 crosses and 3 out of 11 crosses, respectively, Fisher's exact test: P-value = 0.046). These differences could indicate that during seedling establishment, unfit genetic combinations are eliminated by natural selection. Otherwise, no significant differences were found between the seed and seedling stages, indicating that postzygotic barriers, at least in the early life stage, play probably a minor role compared to prezygotic barriers in the maintenance of species integrity. Additional analyses focusing on genetic determination of physiological traits linked to species ecological adaptation could help determine phenotypic trait expressions in hybrids and their evolutionary consequences.

Finally, we found that hybrids produced acorns that were at least as viable as those produced by purebreds, a result consistent with that obtained with artificial pollination on a single *Q. petraea* × *Q. robur* hybrid (Olrik and Kjaer 2007). In addition, we demonstrated that hybrids were successful pollinators, confirming that they produce viable pollen. Similar results were found after analyzing pollen viability and germination ability of hybrids between *Q. petraea* and *Q. pubescens* (Salvini et al. 2009) and paternity analysis in a stand containing four oak species and their hybrids (Curtu et al. 2009). In accordance with previous findings showing that hybridization is not limited to the first generation (Valbuena-Carabana et al. 2005; Curtu et al. 2007, 2009; Lepais et al. 2009), hybrids can be fully fertile in this oak species complex. So far, hybrid oaks have not been found to be sterile, either for the male or for the female sex. This contrasts with earlier results showing low pollen viability of morphologically intermediate oaks (Rushton 1993). However, intermediate morphology does not necessarily reflects hybridity at the individual level (Viscosi et al. 2009), in contrast to the situation observed for other species in hybrid zones (Sweigart et al. 2006; Rieseberg and Willis 2007; Long et al. 2008; Mallet 2008). Hybrid fertility could well be a general trend among oaks, explaining the widespread hybridization in the complex (Lepais et al. 2009).

INTROGRESSIVE HYBRIDIZATION AS A SPECIES INVASION PROCESS

We observed an unequal contribution of the species to the overall pollen pool. *Quercus pyrenaica* and *Q. petraea* were not well represented, whereas *Q. robur* and *Q. pubescens* were particularly successful pollinators (Fig. 3).

Quercus pyrenaica was found to be a poor pollinator within the stand compared to its dominance among adult oaks. This could be explained by the delayed phenology of this species, with a flowering period occurring about three weeks after the other species. However, due to a high variability in flowering time within individuals and to the protandrous nature of oak species, some *Q. pyrenaica* pollen efficiently fertilized some *Q. pyrenaica* hybrids with an intermediate phenology (Lepais 2008). Phenological shift, a potential prezygotic barrier, affects in part hybridization and introgression dynamics but does not result in a complete reproductive barrier in the studied stand.

Quercus robur individuals were mostly pollinated by intraspecific pollen, except one individual that hybridized with *Q. pyrenaica* and *Q. pubescens* (Fig. 3). Pollination of hybrids by *Q. robur* resulted in numerous backcrosses, especially for *Q. robur* × *Q. petraea* hybrids.

Quercus petraea was notably absent from the pollen pool due to the low number of remaining adult individuals within the stand and in the neighborhood. Interestingly, however, *Q. petraea* hybrids were not rare among adult oaks (Fig. 1) and were mostly fertilized by the more abundant species. In particular, *Q. robur* × *Q. petraea* hybrids were fertilized by *Q. robur*, producing numerous backcrossed hybrids. Together with the coppice forest management, unsuitable for *Q. petraea*, massive introgression will lead, in the long term, to dilution of *Q. petraea* genes with those of the dominant species and to species extirpation. However, *Q. petraea* genes could persist long after the species will have disappeared in its purebred form. This cryptic presence of *Q. petraea* genes means that potential receptors of *Q. petraea* pollen, that is, *Q. petraea* hybrids, will remain for several generations within this stand, providing a way for this species to quickly to recolonize the stand by pollen swamping (Lepais et al. 2009).

Quercus pubescens pollen fertilizes a wide range of hybrids in our stand. This should allow the species to expand by directional introgression at the expense of the other species, especially because most pollen coming from outside the stand is from *Q. pubescens*, reinforcing the apparent invasive nature of this species. This trend holds for the seed life stage but also for the established seedlings, suggesting a recurrent process not restricted to a particular pollination season. Although our analyses shed light on contemporary processes, the expansion of *Q. pubescens* by pollen could be a more ancient phenomenon according to the geographical pattern of chloroplast DNA within this species (Dumolin-Lapègue et al. 1999; Petit et al. 2002a,b). *Quercus pubescens* was assumed to have expanded from Italian refugia after the last glacial maximum, because the chloroplast of this species is closely associated with the lineage originating from the Apennine Peninsula (Petit et al. 2002b). Because it is a thermophilous species, its expansion could have occurred after the expansion of *Q. robur*, *Q. petraea*, and *Q. pyrenaica* from the Iberian refugium, all of them characterized by chloroplast types belonging to lineage B (Petit et al. 2002b). Western *Q. pubescens* populations are characterized by lineage B haplotype, an unusual haplotype for this species. These populations were suggested to represent the outcome of a secondary expansion of this species by pollen swamping at the expense of other previously established species (Petit et al. 2002c). The region of our studied stand could represent such a zone of secondary expansion of *Q. pubescens* as it was shown that all species share the same chloroplast haplotype (lineage B, Lepais et al. 2006a). The ecological features of *Q. pubescens* provide additional explanations for the successful expansion of this species. First, it is a pioneer thermophilous species, resilient to drought stress, which could be favored by a warming climate (Damesin and Rambal 1995; Tognetti et al. 1998). Second, it is mostly found at the edge of forests and can accommodate well fragmentation and small-scale coppice management (Rameau et al. 1989; Timbal and Aussenac 1996). *Quercus pubescens* could thus have been indirectly favored by massive human forest exploitation during the last 2000 years, expanding at the expense of late successional species such as *Q. petraea*. The contemporary introgression dynamics observed in our forest could be representative of a long-term process of regional species dynamics in response to disturbance and environmental changes.

Introgressive hybridization was proposed as a mechanism of dispersal in eucalypts (Potts and Reid 1988) and in oaks (Petit et al. 2004). According to this hypothesis, pioneer species first colonize available land by seeds and establish a dense population. A successional species can then colonize the population with its pollen by means of hybridization and unidirectional introgression. The later successional species can eventually be recovered as a purebred through recurrent backcrossing. This potential phenomenon has important biological implications as it suggests that plant species could disperse by pollen in an area already colonized by a sister species. Although initially proposed as a mechanism speeding up species migration rate at the end of the last glacial period, such mechanism could also play a significant role in species range shift nowadays, where migration is a way to cope with changing climates and environmental disturbances.

Using a combination of paternity, parentage and genetic clustering analyses, we characterized the pattern of hybridization and introgression across life stage within the European white oak species complex. Our results demonstrate that hybrids produce viable pollen and acorns, ruling out hybrid incompatibility as a postzygotic reproductive barrier in this system. Instead, natural selection, acting against unfit genetic combinations, could play a more significant role, as shown by the slightly different distribution of reproductive events observed at the seed and seedling stage. We found however that prezygotic barriers had a major impact on hybridization and introgression pattern in comparison to postzygotic reproductive barriers. First of all, prereproductive barriers play a significant role in the directionality of introgression. Second, genetically based pollen discrimination was found to represent a major barrier as it greatly increases assortative mating within species and parental species fidelity of hybrids. Both reproductive trends contribute to the maintenance of species despite interspecific gene flow. Further attempts to identify the genetic mechanisms involved in pollen discrimination in these species should shed new light on the consequences of hybridization, from the genomic level to the community scale, in this species complex.

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Supporting Information

The following supporting information is available for this article:

Table S1. Detailed results of the paternity analysis.
Table S2. Pairwise genetic differentiation (Φ_{FT}) between pollen pools (below) and associated *P* value (above) obtained by 4000 randomizations of pollen genotypes among each pair.
Figure S1. Combination of parentage analysis and genetic clustering of the acorns genotypes.

Supporting Information may be found in the online version of this article.