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Strength and variability of postmating reproductive isolating barriers between four European white oak species

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Abstract The identification and quantification of the relative importance of reproductive isolating barriers is of fundamental importance to understand species maintenance in the face of interspecific gene flow between hybridising species. Yet, such assessments require extensive experimental fertilisations that are particularly difficult when dealing with more than two hybridising and long-generation-time species such as oaks. Here, we quantify the relative contribution of four postmating reproductive isolating barriers consisting of two prezygotic barriers (gametic incompatibility, conspecific pollen precedence) and two postzygotic barriers (germination rate, early survival) from extensively controlled pollinations between four oak species (*Quercus robur*, *Quercus petraea*, *Quercus pubescens* and *Quercus pyrenaica*) that have been shown to frequently hybridise in natural populations. We found high variation in the strength of total reproductive isolation between species, ranging from total reproductive isolation to advantage toward hybrid formation. As previously found, *Q. robur* pollen was unable to fertilise *Q. petraea* due to a strong reproductive isolating mechanism. On the contrary, *Q. pubescens* pollen was more efficient at fertilising *Q. petraea* than conspecific pollen. Overall, prezygotic barriers contribute far more than postzygotic barriers to isolate species reproductively, suggesting a role for reinforcement in the development of prezygotic barriers. Conspecific pollen precedence reduced hybrid formation when pollen competition was allowed; however, presence of conspecific pollen did not totally prevent hybridization. Our results suggest that pollen competition depends on multiple ecological and environmental parameters, including species local abundance, and that it may be of uppermost importance to understand interspecific gene flow among natural multispecies populations.

Keywords Reproductive isolation · Controlled crosses · Pollen competition · Reinforcement · Hybridization · *Quercus*

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Introduction

One of the most fundamental issues in evolutionary biology is the development of reproductive isolation during speciation. The recent update of the *biological species concept* (Mayr 1942) that extends the phenomena from a whole genome process to a genic level process (Wu and Ting 2004) has led to a tremendous number of new developments in speciation biology, particularly through the identification of the reproductive barriers isolating species, and their genetic basis (Lowry et al. 2008; Widmer et al. 2009). Recent analytical developments allow the different types of reproductive barriers to be described and their individual contribution to the reduction of gene flow between species to be evaluated (Coyne and Orr 1997; Ramsey et al. 2003; Lowry

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et al. 2008). This analysis is very useful, giving access to the stages of speciation that these species experienced (Widmer et al. 2009) and to the consequences of hybridization on the evolution of a species complex (Chapman et al. 2005; Barbour et al. 2006; Taylor et al. 2009).

The European white oak species complex is a good example of a species complex with partial reproductive isolation and widespread interspecific gene flow in natural populations (Petit et al. 2004; Arnold 2006). There is an increasing number of studies reporting evidence of hybridisation between almost all sympatric oak species (Muir et al. 2000; Curtu et al. 2007; Valbuena-Carabaña et al. 2007; Lepais et al. 2009). Although the percentage of hybrids found seems to vary between species pairs and populations investigated, the relative frequency of the sympatric species was found to play a significant role in the hybridization frequency and the introgression pattern (Lepais et al. 2009). This relationship between species relative abundance and hybridization dynamics indicates that pollen limitation may increase interspecific crosses for rare species and subsequent pollen swamping of the resulting hybrids by abundant species. Reproductive system studies provide a more direct approach to infer hybridisation pattern within populations (Bacilieri et al. 1996; Streiff et al. 1999; Curtu et al. 2009; Jensen et al. 2009; Salvini et al. 2009; Lepais and Gerber 2011). Besides identifying first- and later-generation hybridization events and backcrossing, some of these studies also reported backcrosses dominant in one direction, resulting in introgression toward one of the studied species (Bacilieri et al. 1996; Salvini et al. 2009; Lepais and Gerber 2011). These results suggest that interspecific gene flow is an ongoing phenomenon within the species complex that may play a role in species adaptation to changing environmental conditions (Arnold 2004). Furthermore, directional introgression may also participate in the long run to species succession, as another form of multispecies adaptation at the species complex scale (Petit et al. 2004).

Despite the importance of the reproductive system in understanding the hybridization significance within the oak species complex, there are only a few experimental studies that directly investigated the reproductive isolation between species using an experimental approach (Steinhoff 1993; Kleinschmit and Kleinschmit 2000; Olrik and Kjaer 2007). Results from these crossing experiments between *Quercus robur* and *Quercus petraea* showed that hybridization can occur and that the success of hybridization is preferentially directional: the *Q. petraea* pollen being more successful on the *Q. robur* ovules than the *Q. robur* pollen are on the *Q. petraea* ovules. Although the causes of such directional compatibility remain unknown, it may have a complex genetic basis as it involves both prezygotic (pollen–pistil interaction) and postzygotic components (hybrid viability; Steinhoff 1993; Petit et al. 2004). Note, however, that *robur* pollen can fertilise the *Q. petraea* ovules, even at low rates, as illustrated by a recent experimental pollination on a *Q. petraea* × *Q. robur* hybrid, showing that hybrids can be viable and fertile Olrik and Kjaer 2007). In this study, the authors found again that pollination was preferentially directional as the fertilised tree accepted more pollen originating from the maternal species (i.e., the *Q. robur* in this case). Extrapolating such directional hybridization patterns in population suggests a potential way for one species to introgress within the range of the receiving species by extensive pollen swapping (Potts and Reid 1988; Petit et al. 2004; Lepais and Gerber 2011). However, additional experimental studies are needed to assess whether these findings can be generalised to other hybridising oak species and to increase our knowledge of the reproductive barriers involved in the decrease of gene flow between oak species.

In this paper, we report for the first time results from experimental pollinations between four oak species, *Q. robur*, *Q. petraea*, *Quercus pubescens* and *Quercus pyrenaica*. We took advantage of recently developed analytical tools to estimate the contribution of different reproductive barriers in isolating oak species and to quantify the total reproductive isolation under our experimental conditions. More precisely, we quantified two prezygotic barriers: the gametic incompatibility (the relative proportion of hybrid produced in the situation of heterospecific pollen fertilisation) and the pollen competition (the impact of the presence of both conspecific and heterospecific pollen on hybridization rate). This latter barrier may have a significant role in isolating the species as it has been shown that species frequency within natural populations impacts the hybridization dynamics within the species complex (Lepais et al. 2009). We then compared relative germination and survival rate between hybrids and purebred progenies as two early intrinsic postzygotic barriers. We finally compared these estimate between species pairs and discussed the likely consequences of these results for the hybridization and introgression dynamics within oak populations.

Materials and methods

Controlled pollination experiment

Controlled pollination on oaks has been performed since 1987 at the INRA Research Centre in Cestas-Pierroton, and the technique that was continuously improved over the years is described in details elsewhere (Roussel 1999; Roussel 2002; Abadie et al. 2012). The method was previously used to obtain individuals with known pedigree to construct genetic linkage maps (Barreneche et al. 1998), to detect quantitative trait loci (Saintagne et al. 2004; Porth et al. 2005; Brendel et al. 2008) and, more recently, to study postmating reproductive barriers between *Q. robur* and *Q. petraea* (Abadie et al. 2012). Most crosses involved *Q.*

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robur and *Q. petraea*, so additional plant material and modification of the method were necessary for our experiment with the four European white oak species.

Origin of plant material

Controlled crosses were conducted on two types of mother trees depending on the species. Mature 12-year-old grafted trees (Roussel 2007) located at the INRA Experimental Unit in Cestas-Pierroton were used as *Q. robur* and *Q. petraea* mother trees. In these cases, whole trees were bagged prior to flowering period to isolate them from external pollination. Pollination on one single tree (genotype) was made on several vegetatively replicated grafted copies of the same tree. Grafted trees allow a relatively easy access and handling of flowers for controlled pollination and increase the level of fruiting by allowing watering and fertilisation. However, as only *Q. robur* and *Q. petraea* were available as grafts, adult trees were selected in natural populations for controlled pollination on the two other species. For *Q. pubescens* and *Q. pyrenaica*, mature trees from natural stands located at Branne (lat. 44.838°, long. −0.202°, Gironde, France) and Cestas (lat. 44.755°, long. −0.711°, Gironde, France), respectively, were used as mother trees. In these cases, individual branches of the tree were bagged prior to flowering in order to isolate them from external pollen, and pollination was performed at the branch level with repetitions made on several branches of the same tree. A total of 14 genotypes were used as mother trees (four *Q. robur*, four *Q. petraea*, three *Q. pubescens* and three *Q. pyrenaica*; see Table S1).

A total of 20 different genotypes were used as pollen donors (Table S2). Anthers were collected on trees before dehiscence and then dried to extract the pollen grains using a system described in Roussel (2002). Pollen grains were then stored at −18 °C. For the controlled pollination, pollens from five different genotypes of the same species (thereafter called monospecific pollen mixture) were combined to constitute the pollen mixture used in the experiments (Ro, *Q. robur* pollen mixture; Pe, *Q. petraea*; Pu, *Q. pubescens*; and Py, *Q. pyrenaica*; Table S2). Pollen viability of each genotype was estimated using a method based on fluorescein (Heslop-Harrison and Heslop-Harrison 1970), and the viability levels were used to adjust the quantity of pollen from each genotype to constitute a mixture of pollen with the same percentage of viable pollen from each genotype (Table S2). This procedure was repeated each year before the crossing experiments. Lastly, the four species of pollen bulk collections were mixed to obtain an overall pollen mix (Mx=Ro+Pe+Pu+Py).

All parental trees used for the crossing experiment showed typical morphologic and genetic (as controlled by genetic Bayesian assignment) characteristics of their respective species (results not presented).

Crossing design

Controlled pollinations were performed in spring 2005 and 2006 and organised so that each mother genotype was fertilised with each of the pollen mixtures at least once. This resulted in 20 different cross combinations (Table 1) including intraspecific and all reciprocal interspecific crosses, using monospecific pollen mixtures (Ro, Pe, Pu and Py) and multispecific pollen mixture (pollen competition, Mx).

Progeny monitoring

All acorns were individually labelled, and about one fifth of the basal part of the acorn, containing mostly cotyledon tissues, was cut for subsequent DNA isolation. The remaining part of the acorn containing the intact embryo was glued on a plastic plate. Once 60 acorns were glued on a single plate, a picture was taken, and the plate was immersed in a wet vermiculite substrate and regularly watered thereafter. The plates were then monitored weekly. Germinated acorns were individually transplanted in labelled 4-L pots filled with potting compost and watered as required. After a 6-week period, acorns that failed to germinate became mildewed and were thus considered unviable. Seedlings were grown in these 4-L pots for a 2-year period during which height was regularly measured to estimate survival. Surviving seedlings were, thereafter, transplanted in an open field where they were measured 2 years later to estimate survival.

Parent identification

We adapted a CTAB-based DNA extraction protocol originally developed for pine mega-gametophyte (Bousquet et al. 1990) to the 96-well plate format by reducing the volume of chemical reagent used. We then amplified five microsatellite markers, QpZAG110, QrZAG11, QrZAG112, QrZAG39 and QrZAG96 (Steinkellner et al. 1997; Kampfer et al. 1998) using a multiplex PCR method described elsewhere (Lepais et al. 2006). Each parent exhibited unique allelic arrays for the different microsatellite loci allowing checking the maternal identity and determining the paternal origin for each acorn unambiguously. For crosses involving the Mx pollen pool, because the number of candidate father was relatively high, parentage analysis was performed using the FaMoz software (Gerber et al. 2003). When outlier alleles were detected (e.g. alleles not carried by the parents used in the crosses), corresponding acorns were removed from subsequent analyses (Table 1). Pollination from outlier pollen may have occurred due to earlier release of pollen by other trees prior to bagging and/or to pollen collection. Similarly, when some of the locus failed to amplify and the recorded acorn genotype could not allow determining the parentage unambiguously, acorns were removed from the subsequent

Table 1 Total number of acorns obtained for each cross and checked by microsatellite-based parentage analysis

		Monospecies pollination paternal species								Multispecies pollination paternal species									
Maternal species	Maternal genotype	rob		pet		pub		pyr		rob	pet	pub	pyr		[a]S	[b]PP	[c]NA	Total	
rob	3P	64	(1)	54	(3)	13	(1)	18	(1)	8	2	6	1	(1)	0	45	15	226	(7)
	11P	317	(1)	56	(2)	54	(1)	0	(2)	101	3	3	4	(1)	9	163	36	746	(7)
	A4	89	(1)	183	(3)	0	(1)	0	(2)	34	4	0	1	(1)	0	49	64	424	(8)
	A3									154	16	0	4	(4)	46	9	11	240	(4)
pet	QS11	0	(1)	13	(1)	49	(1)	0	(1)						0	2	2	66	(4)
	QS30	0	(3)	2	(2)	0	(2)	0	(2)	0	18	24	1	(1)	0	1	0	46	(10)
	QS32	0	(4)	22	(2)	26	(2)	0	(2)	0	0	0	0	(1)	1	2	11	62	(11)
	QS27									0	7	2	0		0	1	0	10	(3)
pub	Bra2	0	(12)	0	(12)	1	(12)	0	(12)	0	0	1	0	(12)	0	0	0	2	(60)
	Bra55	0	(13)	0	(12)	6	(13)	0	(12)	0	0	0	0	(12)	1	0	1	8	(62)
	Bra56	0	(12)	0	(12)	0	(12)	0	(12)	0	0	0	0	(11)	0	0	1	1	(59)
pyr	T9	4	(10)	10	(9)	5	(23)	1	(32)	1	0	1	3	(11)	0	4	6	35	(86)
	T29	12	(11)	11	(15)	8	(11)	59	(16)	3	3	6	11	(12)	0	28	28	169	(65)
	T30	33	(25)	18	(13)	21	(15)	2	(14)	0	1	1	6	(12)	2	15	14	113	(79)
Total															59	319	189	2,148	(465)

Numbers indicate the number of acorns obtained from each combination of maternal genotype and paternal pollen mixture. Numbers within parentheses show the number of crossing experiments attempted for each combination

S selfing, *PP* pollen pollution (pollen originating from outside the experiment), *NA* missing data (ambiguous parental assignment due to one or more missing genotypes)

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analyses (Table 1). Selfed acorns were removed from the analyses as only few were identified, except in one cross which hampered subsequent statistical treatments (Table 1). Because genetic analysis was performed on plant material collected before germination, we were able to track the parentage of acorns that failed to germinate and to determine a germination rate for each cross.

Statistical analyses

Descriptive statistics

Specific paternal contributions were summed up for each mother tree species either using acorns obtained by monospecific crosses, as an estimate of the gametic incompatibility between species, or multispecific crosses, to assess the impact of competition between pollens from different species. In the absence of a prezygotic barrier, we would expect equal paternal contributions from the four species in each cross type. At the opposite extreme, in the case of total gametic incompatibility, we would expect no paternal contribution from heterospecific pollen. An intermediate case would be a prezygotic barrier only due to conspecific pollen precedence which would be detected by equal paternal contributions from the four species in monospecific crosses (no pollen competition) and only conspecific paternal contributions in multispecific crosses (pollen competition leading to conspecific pollen advantage).

We reported the mean and standard deviation of the paternal contribution by mother tree species (each genotype within species considered as a replicate). For *Q. robur* and *Q. petraea* mother trees, we computed the reproductive success by crossing experiment (i.e. pollination of one graft). For *Q. pyrenaica* mother tree, because the number of acorns produced by crossing experiment on one branch of the tree is low, we reported the mean number of acorns produced for nine pollinations, which corresponds to the lowest number of experiments for any cross performed on this mother tree species (Table 1). For each mother tree species, we tested for differences in the number of acorns produced by cross type with a generalised linear model using a Poisson error distribution for count data.

Due to the high longevity of oaks, only early postzygotic barriers could be assessed. We used all available crosses (i.e. monospecific and multispecific) to compute germination rate and survival rate after 4 years of growth as a proxy for progeny viability. Germination and survival rate were compared between cross types as resolved by parentage analysis, i.e. independently of the crossing experiment they originated from (monospecific or multispecific) using a generalised linear model with a binomial error distribution for binary data. Note that overall fertilities could not be assessed because oak sexual maturity occurs at least at age 10 to15 years. However, progenies were planted and are still growing in the field, so fertility estimation for these individuals may be obtained in the future, allowing a direct comparison of hybrids and species fitness.

Estimation of the different components of the postmating reproductive isolation

We attempted to assess the absolute contribution of different reproductive barriers involved in the total reproductive isolation. These estimates were then used to compute their relative contribution and the degree of reproductive isolation between species according to the method described in Ramsey et al. (2003).

For each pair of parental species, the reproductive isolation due to gametic incompatibility (RI_i) was computed based on specific paternal contribution in monospecific crosses:

$$RI_i = 1 - \left(\frac{\text{interspecific paternal contribution}}{\text{intraspecific paternal contribution}} \right)$$

This parameter equals 1 when no acorn was produced by a particular cross, 0 if an interspecific cross produces the same amount of acorns as the corresponding intraspecific cross and a negative value when the interspecific cross yield more acorns than the intraspecific cross. This component of the reproductive isolation integrates numerous interaction steps between the gametophyte and the sporophyte from the adhesion of the pollen grain onto the stigma to the fertilisation (Johnson and Preuss 2002; Swanson and Vacquier 2002; Swanson et al. 2004). The gametic incompatibility estimated here involves both uncoordinated genetic interactions between the gametophyte and the sporophyte (inactive response), and rejection of the pollen by the gametophyte (active response). Note that postreproductive abortion may also contribute to lower the number of acorns produced, and this component of the reproductive isolation is also included within RI_i as the abortion rate could not be specifically monitored during the experiment due to logistical reasons.

The presence of pollen from several species on the stigma could have contrasting effects. On the one hand, conspecific pollen could reduce the efficiency of pollen discrimination mechanism (mentor effect; Knox et al. 1972; Pandey 1977; Gaget et al. 1989; Desrochers and Rieseberg 1998) by increasing the chance of pollen germination, even for heterospecific pollen, and increasing hybrid production. On the other hand, growth of conspecific pollen tubes may be faster than the growth of heterospecific pollen tubes (conspecific pollen precedence; Howard 1999; Chapman et al. 2005; Rahmé et al. 2009; Montgomery et al. 2010), leading to a decrease in hybrid production. We estimated the effect of conspecific pollen precedence using results from multispecific crosses

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where pollen competition occurred. The parental contribution of each species in this kind of cross comprises both the gametic incompatibility as described above in addition to the pollen competition. We, thus, computed the reproductive isolation due to conspecific pollen precedence alone by contrasting the observed parental contribution by the expected parental contribution without pollen competition (i.e. monospecific crosses; Martin and Willis 2007):

$$RI_C = 1 - \left[\frac{\left(\frac{\text{interspecific paternal contribution in multispecific cross}}{\text{interspecific paternal contribution in monospecific cross}} \right)}{\left(\frac{\text{intraspecific paternal contribution in multispecific cross}}{\text{intraspecific paternal contribution in monospecific cross}} \right)} \right]$$

We estimated two early postzygotic barriers using germination and survival rates after 4 years of growth in a common garden. The reproductive isolation due to differential germination rate between hybrid and species is computed as follows:

$$RI_g = 1 - \left(\frac{\text{hybrids germination rate}}{\text{purebred germination rate}} \right)$$

and the reproductive isolation due to differential survival of hybrid and purebred:

$$RI_S = 1 - \left(\frac{\text{hybrids survival rate}}{\text{purebred survival rate}} \right)$$

We then computed the relative contribution of these different postmating reproductive barriers using the method originally developed by Coyne and Orr (1997) for two barriers and generalised to *n* barriers by Ramsey et al. (2003). Our estimations were computed using the spreadsheet Reproductive Isolation Calculator (Ramsey et al. 2003; http://www.plantbiology.msu.edu/schemske.shtml). Shortly, for *n* reproductive barriers, each absolute contribution to the total isolation is

$$AC_n = RI_n \left(1 - \sum_{i=1}^{n-1} AC_i \right).$$

The total reproductive isolation is computed as follows:

$$T = \sum_{i=1}^{n} AC_i$$

and the relative contribution of each reproductive barrier is

$$RC_n = \frac{AC_n}{T}.$$

Results

A total of 465 individual crossing experiments were undertaken (Table 1), yielding 2,846 acorns. Because crosses A3×Mx and 11P×Mx produced a large number of acorns, 240 acorns from each cross were randomly subsampled; a total of 2,148 acorns were, thus, kept for subsequent analyses (Table 1). Acorn production was low for crosses on *Q. pubescens* mother trees with only 11 acorns produced, hampering further analysis of the maternal mating system of this species. Genotyping was successful for 2,018 acorns (93.9 % of the total analysed) from which 319 acorns (15.8 %) were fathered by trees from outside the experimental crosses (Table 1). The paternity could be unequivocally determined for the remaining 1,640 seeds (Table 1) from which 59 (3.6 %) originated from self fertilisation (mostly from genotype A3 used as the maternal tree, Table 1) that were removed from further analyses.

The outcome of monospecific pollination varied greatly between the mother tree species (Fig. 1). Heterospecific pollen fertilised significantly less acorns by cross compared to conspecific pollen (Fig. 1a). Notably, *Q. pyrenaica* pollen produced a low number of acorns on *Q. robur* mother trees. While conspecific pollen also tends to produce a higher number of acorns on *Q. pyrenaica* (Fig. 1c), the difference is not significant: heterospecific pollen still produces a high number of hybrid acorns on this mother tree species. At the opposite, *Q. petraea* produced a surprisingly high number of acorn when fertilised by *Q. pubescens* pollen compared to conspecific pollen (Fig. 1b), while *Q. robur* and *Q. pyrenaica* failed to produce hybrid acorns.

When pollen competition is acting on the stigma (multispecific pollination), the proportion of successful pollination is lower in heterospecific versus monospecific crosses (Fig. 1d–f). As a consequence, mentor effect can be ruled out in favour of conspecific pollen precedence. Note, however, that hybrids are still produced even when heterospecific pollen is in competition with conspecific pollen. In detail, the effect of pollen competition seems more pronounced in *Q. robur* (Fig. 1d) compared to *Q. pyrenaica* mothers (Fig. 1f). The proportion of *Q. pubescens* pollen fertilising *Q. petraea* mother trees is still higher than conspecific fertilisation (Fig. 1e), although not significant. Note that *Q. robur* pollen failed to produce hybrids on *Q. petraea* (Fig. 1e), while *Q. pyrenaica* pollen produced only one acorn (Table 1).

Results of germination rates (Fig. 2a–c) show some similarities to those of fertilisation success (Fig. 1). For instance, *Q. robur* × *Q. pyrenaica* hybrids showed a significantly lower germination rate (Fig. 2a), *Q. petraea* × *Q. pubescens* hybrids had a better germination rate than conspecific crosses (Fig. 2b) and conspecific *Q. pyrenaica* crosses had a much better germination rate than any other hybrids (Fig. 2c). In general, however, there were fewer differences between heterospecific and conspecific crosses for germination rates than there were for fertilisation successes, except for *Q. pyrenaica*. Germination rates were high for acorns produced by *Q. robur* and *Q. petraea* but notably lower for acorns produced by *Q. pyrenaica*. This could be explained by a more favourable environment for grafted trees compared to naturally standing

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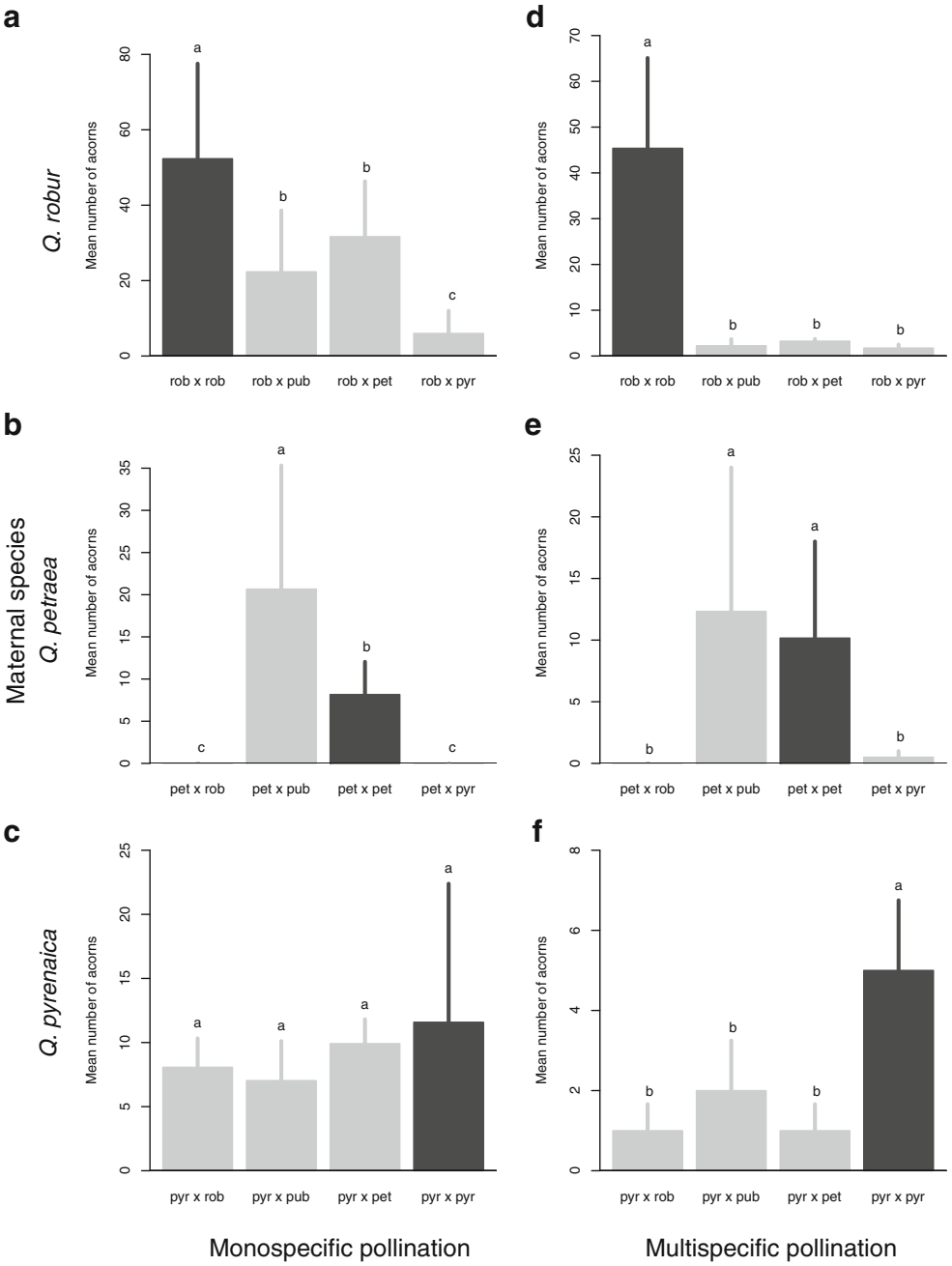

Fig. 1 Mean number of acorns obtained per crossing experiment (single cross for a–**d** or nine crosses for **c** and **f**) in monospecific (**a**, **b** and **c**) and multispecific (**d**, **e** and **f**) pollination for each cross type. Maternal species are *Q. robur* (**a** and **d**), *Q. petraea* (**b** and **e**) and *Q. pyrenaica* (**c** and **f**); paternal species are indicated under each *bar* (crosses coded as female × male species). *Black bars* highlight intraspecific crosses, while *grey bars* identify interspecific crosses. *Lines over the bar* represent the standard error, and *different letters* indicate significant differences at 0.05 probability level

tree (*Q. pyrenaica*) exposed to environmental stress like drought. This could also explain the very low number of acorns produced by *Q. pubescens* (Table 1).

The same general trends were observed for the early survival (up to 4 years) of the progenies. *Q. robur* × *Q. pyrenaica* hybrids showed a lower survival compared to other crosses involving *Q. robur* as a mother species (Fig. 2d). *Q. petraea* × *Q. pubescens* hybrids were characterised by a slightly higher, yet not significant, survival rate compared to conspecific *Q. petraea* crosses (Fig. 2e). There were no significant differences in survival between hybrids and conspecific individuals originating from *Q. pyrenaica* crosses (Fig. 2f).

Accordingly, we found a high variation in the different contributions of the reproductive barriers between the species (Table 2). In general, gametic incompatibility and pollen competition contributed more than germination and survival to the total reproductive isolation (Table 2). The amount of total reproductive isolation between species, that only takes into account the postmating reproductive barriers we tested, ranged from 1 (total reproductive isolation) between *Q. robur* as a paternal and *Q. petraea* as a maternal parent to −1.03 (indicating hybrid advantage) between *Q. pubescens* as a paternal and *Q. petraea* as a maternal species (Table 2). For the other pairs of species tested, total

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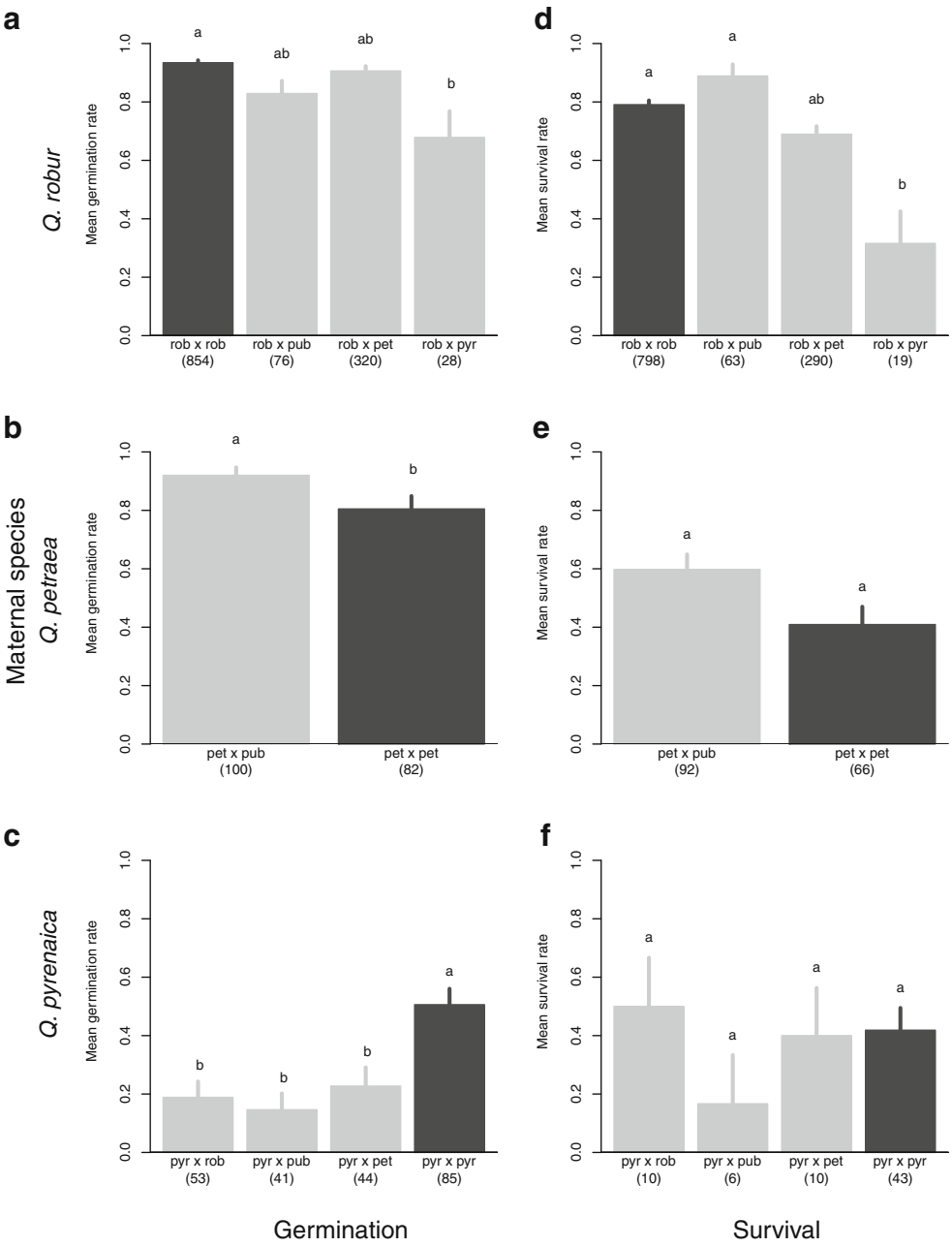

Fig. 2 Mean germination (**a**, **b** and **c**) and survival rate (**d**, **e** and **f**) including monospecific and multispecific crossing experiments for each cross type. Maternal species are *Q. robur* (**a** and **d**), *Q. petraea* (**b** and **e**) and *Q. pyrenaica* (**c** and **f**); paternal species are indicated under each *bar* (crosses coded as female × male species). Number of individuals in each cross used in the analysis is indicated under *brackets*. *Black bars* highlight intraspecific crosses, while *grey bars* identify interspecific crosses. *Lines over the bar* represent the standard error, and *different letters* indicate significant differences at 0.05 probability level

Table 2 Absolute contributions to total reproductive isolation of studied postmating reproductive barriers

Reproductive barriers	rob × pet	rob × pub	rob × pyr	pet × rob	pet × pub	pet × pyr	pyr × rob	pyr × pet	pyr × pub	Mean (SD)
Gametic incompatibility	0.39	0.57	0.88	1.0	−1.53	1.0	0.30	0.14	0.39	0.35 (0.77)
Pollen competition	0.53	0.38	0.08	/	1.32	/	0.50	0.66	0.21	0.52 (0.40)
Germination	0.00	0.01	0.01	/	−0.17	/	0.13	0.11	0.28	0.05 (0.14)
Survival	0.01	0.01	0.02	/	−0.64	/	−0.01	0.00	0.07	−0.08 (0.24)
Total reproductive isolation	0.94	0.97	0.99	1.0	−1.03	1.0	0.91	0.91	0.95	

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reproductive isolation ranged from 0.91 to 0.99. Gametic incompatibility seems to be the main barrier that contributes to reproductive isolation between *Q. robur* mothers and heterospecific pollen (increase in gametic incompatibility and decrease in pollen competition along the increasing total reproductive isolation gradient: *Q. robur* × *Q. petraea* < *Q. robur* × *Q. pubescens* < *Q. robur* × *Q. pyrenaica*; Table 2). However, pollen competition and particularly differential germination rate between hybrid and purebred seems to play a relatively important role in interspecific crosses involving *Q. pyrenaica* as the maternal species to reproductively isolate the species (Table 2).

Discussion

Strength of reproductive isolation between species

Our extensively controlled pollination experiment within and between four oak species confirm that European oak species are not totally reproductively isolated. However, our data give access to a striking observation by revealing the variability in the strength of total reproductive isolation between species (Table 2).

Q. petraea as a maternal species showed a total reproductive isolation when pollinated by *Q. robur* and, to a lesser extent, by *Q. pyrenaica* pollen. Previous crossing experiments already demonstrated a strong reproductive isolation when *Q. robur* pollen was used to fertilised *Q. petraea* (Steinhoff 1993; Kleinschmit and Kleinschmit 2000). However, in these cases, the reproductive isolation seemed to be due to an additive effect of several isolating barriers as few hybrid seeds were obtained, but these hybrid seeds subsequently showed weak germination and survival (Steinhoff 1993). Our results show a more direct impact of early reproductive barriers as no acorns were obtained. This may indicate a gametic incompatibility due to active rejection of heterospecific pollen by *Q. petraea* stigma or an uncoordinated gametic interaction during one of the numerous steps prior to fertilisation (Hiscock and Allen 2008). Given that we did not record the number of fertilised flowers or seed abortions for logistical reasons, an alternative explanation of these unsuccessful crosses may be an early postzygotic barrier acting between fertilisation and seed maturity causing seed abortion due to abnormal embryo development (Marshall and Folsom 1991). In contrast, there seems to be no reproductive barrier at all for *Q. pubescens* pollinating *Q. petraea*. For this cross, the estimated total reproductive isolation is negative, indicating a higher efficiency for heterospecific crossing. The cause of such result remains unknown and deserves additional investigations to rule out a potential genotypic or experimental design effect because of its far-reaching consequences in the hybridisation dynamics between the two species in natural populations. Note, however, that mating system analysis in an Italian population showed a reverse situation with a very low pollination of *Q. petraea* by *Q. pubescens* pollen, but relatively high pollination of *Q. pubescens* by *Q. petraea* pollen (Salvini et al. 2009). Directionality of interspecific compatibility and outcome of pollen competition may indeed be context-dependent in line with previous findings in other species, showing that environmental condition such as soil calcium content (Ruane and Donohue 2007; 2008) or geographical context (Aldridge and Campbell 2006) may have an impact on interspecific pollination success. Such environmental effects may play some role in the reproductive barrier between *Q. pubescens* and *Q. petraea* as the two species stand at the extreme opposite of the forest transition gradient, with *Q. petraea* being a late successional species found in old stable forests, while *Q. pubescens* is a postpioneer species found on disturbed coppice managed forests with a preference for calcium rich soil (Rameau et al. 1989; Timbal and Aussenac 1996). The high interspecific fertilisation success of *Q. pubescens* pollen found in the southern part of France (Lepais and Gerber 2011), which results in a directional introgression, may be due to a different ecological context compared to the Italian one or more broadly in other parts of the range where the species co-occur. In addition, it has recently been shown that the reproductive isolation between *Q. robur* and *Q. petraea* was sensitive to microenvironmental variation, suggesting some plasticity for the expression of the reproductive isolation between these species (Abadie et al. 2012). It is, thus, likely that environmental variation at the scale of the distribution area of the species can lead to contrasting expression of reproductive isolation between *Q. petraea* and *Q. pubescens* and more generally as well for any pair of species.

Regarding the other species pairs, the amount of total reproductive isolation was strong but not integral (ranging from 0.91 to 0.99) for crosses involving *Q. robur* and *Q. pyrenaica* as maternal species. Close inspection of the contribution of the different reproductive barriers show that prezygotic barriers are major contributors decreasing interspecific pollination in *Q. robur*, while postzygotic barriers plays some role, although minor, to reduce hybridization in crosses involving *Q. pyrenaica* as a maternal species (Table 2). The fact that postzygotic barrier are stronger in isolating *Q. pyrenaica* may indicate that this species diverged more anciently from the other species with postzygotic barriers evolving as a by-product of genetic drift leading to a higher genetic incompatibility. We are not aware, however, of any phylogenetic study with a scale sufficiently fine to resolve the divergence pattern between these closely related species. In addition, this higher isolation may result from greater ecological and distribution divergence compared to the other species. Indeed, *Q. pyrenaica* distribution range is limited to the southwestern part of Europe, a distribution more restricted than the one of the other species, resulting in a more limited sympatry

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zone between *Q. pyrenaica* and the three other species. *Q. pyrenaica* is sympatric with *Q. robur* and *Q. petraea* in the southern part of their range, while its sympatric zone with *Q. pubescens* is limited to the western part of France (Jalas and Suominen 1988). Additionally, *Q. pyrenaica* geographical distribution and its sympatric relationship mirrors its ecological requirement with *Q. pyrenaica* mostly restrict to warmer and drier sandstone soil with almost no overlap with the limestone soil requirement of *Q. pubescens*. Finally, *Q. pyrenaica* is flushing a couple of weeks later than the other species, leading to some disjunctions of the timing of flowering with other white oak species. To sum up, it is not clear whether the stronger effect of postzygotic barrier in *Q. pyrenaica* results from a more ancient speciation process (resulting in a higher genetic incompatibility) and/or from a greater ecological specialisation and a relative geographical isolation (causing a higher ecological divergence), leading to a reduced reproductive interaction with the other species.

Overall, reproductive isolation was mostly due to prezygotic barriers with a negligible effect of postzygotic barriers in total reproductive isolation. Indeed, we found a mean strength of prezygotic isolation of 0.76±0.39 (mean±SD) for a mean strength of postzygotic isolation of −0.02±0.37 over species pairs. This result contrasts sharply with a meta-analysis compiling results from 19 plant species (Lowry et al. 2008) that estimated a mean strength of prezygotic isolation of 0.84±0.06 for a mean strength of postzygotic isolation of 0.41±0.18. The fact that prezygotic barriers strongly contribute to total reproductive isolation in oak species could be due to several biological characteristics of the species, leading to high potential for heterospecific mating. Firstly, oak species are wind-pollinated, and this passive way of pollen movement is undiscriminating, compared to insect- or animal-mediated pollination, where pollinators are involved in flower choice and reproductive isolation (Lowry et al. 2008; Hopkins and Rausher 2012). Secondly, these species show an overlapping flowering time, with the exception of *Q. pyrenaica*, the flowering season of which still overlaps with the ones of other species due of the high variability of the flowering time between individuals within species (Lepais 2008). Thirdly, species distribution mostly depends upon the soil characteristics which can vary at the very local scale, meaning that several oak species are generally located close by. This fact, along with the long-distance pollen dispersal characteristics of the species, means that trees may receive an important proportion of heterospecific pollen that needs to be efficiently discriminated to keep species apart. The strongest contribution of prezygotic isolation probably results from the reinforcement phenomena, i.e. the increase in prezygotic barriers due to selection against hybrid formation (Coyne and Orr 1997; Yukilevich 2012). However, the strength of selection pressures to evolve prezygotic isolation should be a function of the loss of fitness from mating with heterozygotic species. Our results showed relatively low cost of hybridization for early fitness traits (germination rate and early survival), resulting in nearly negligible postzygotic isolation in an artificial setting. In addition, viable and fertile hybrids can be experimentally produced (Olrik and Kjaer 2007; Kremer et al. unpublished data) and are often found in natural populations (Curtu et al. 2007; Curtu et al. 2009; Lepais and Gerber 2011). Although these observations could point to a low probability of reinforcement in this species complex, selection against hybrid genotypes cannot be ruled out because it is tightly linked with microenvironmental characteristics which could lead to increase reinforcement in heterogeneous environment that is commonly found in natural populations. Comparison of fitness-related traits between hybrids and parental species need to be conducted across different ecological conditions to conclude on the maintenance of hybrids in nature and the role of reinforcement in the development of prezygotic barriers in these species complex.

Pollen competition and its interaction with ecological context in shaping hybridisation dynamics

Simultaneous application of pollen from four different species to the different mother tree species demonstrated that pollen competition was one of the most important components of the reproductive isolation between the studied oak species. This general result is probably explained by the difference in pollen tube germination and/or pollen tube progress between intra- and interspecific crosses as experimentally shown for *Q. robur* × *Q. petraea* crosses (Abadie et al. 2012). Interestingly, the latter study showed that pollen tube germination and progression in the stigmas were not correlated, indicating different mechanisms at stake. In the present study, we found a high variability in the absolute contribution of pollen competition as a reproductive barrier from as low as 0.08 in *Q. robur* × *Q. pyrenaica* crosses to as high as 1.32 for *Q. petraea* × *Q. pubescens* crosses. Such variation in pollen competition outcomes probably reflects different mechanisms involved during pollen tube germination and/or progression that may depend on the species pair considered.

Although using a mixture of pollen from different species allow us to assess the importance of conspecific pollen precedence as a significant reproductive isolation barrier, our experimental design hardly reflects realistic configurations that may be found in natural populations. On the one hand, application of pure heterospecific pollen to a stigma mimics the unrealistic situation of an isolated oak within a stand of another oak species, providing the most advantageous situation for hybrid production. In such a case, there is no pollen competition and no opportunity for conspecific pollen precedence (selfing excluded), and hybrids were produced. On the other hand, the multispecies pollen mixture we used (equal proportion of viable pollen from each

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species) allowed for pollen competition. In such a situation, we showed that conspecific pollen precedence was a significant (albeit variable) isolating barrier between species pairs. Such an experiment simulates an ideal situation of a stand composed of the four species at the same frequency and randomly located within the forest. In real populations, premating reproductive barriers would increase the strength of pollen competition. Indeed, conspecific oaks share numerous ecological preferences that would result in a higher proportion of conspecific pollen received. An obvious example of such environmental factors is the typical species distribution in spatial clusters within the stand shaped by soil characteristics such as composition and moisture. The less easy to assess is the flowering phenology differences either due to direct genetic effects (i.e. species characteristics, local family structure) and/or indirect microenvironmental effect (for instance, common exposure to sun of neighbouring trees) that may play some role in the specific composition of the pollen pool received by a stigma at a particular time in a particular place. Numerous complex factors can, thus, impact the strength of pollen competition and its consequences on hybrid formation. Pollen competition in natural populations would, thus, necessarily have a greater effect than the one estimated in our experimental crosses. As shown in several natural stands, locally rare species receiving a high proportion of heterospecific pollen would experience high hybridization rate due to pollen limitation and low opportunity for conspecific pollen precedence (Lepais et al. 2009). In contrast, hybridization opportunity will be reduced in stands composed of a balanced mixture of species because conspecific pollen precedence would be the rule in such situation (Lepais et al. 2009). Reproductive barriers like pollen competition can also play some role in the direction of backcross and subsequent introgression beyond the first generation of hybridization. Hybrids are typically reproductively compatible with their parental species, and thus, species composition of the pollen pool received by hybrids will have direct impact on the genetic composition of the next-generation hybrids (Lepais and Gerber 2011). Furthermore, hybrids are typically reproductively isolated from nonparental species whose pollen will compete with pollen from the parental species (Lepais and Gerber 2011). This relative reproductive isolation expressed in hybrids will trigger reproductive events toward backcrosses with the parental species reducing complete species mixture in a hybrid swarm. However, as conspecific pollen precedence (or parental species pollen precedence in hybrids) is a significant reproductive barrier in oaks, opportunity for trihybridization (admixture of genes from three different species in hybrids) will be increased when a hybrid between two locally rare species receive a high amount of pollen from another more abundant species, resulting in low parental species pollen precedence opportunities. In summary, pollen competition is an important component of reproductive isolation between oak species and the resulting hybridization dynamics because it is strongly affected by multiple premating factors linked to environmental local conditions and species ecological preference.

Although environmental factors appear to be of primary importance in the expression and the variability of reproductive isolation between oak species (premating but also postzygotic reproductive isolation), they have not been assessed in this study. If long-term common garden experiments and complex crossing design may seem to be unrealistic and unpractical for long-generation-time species such as oaks, modelling approaches of reproductive system in natural populations taking advantage of a multidisciplinary approach linking genetic and genomic data with relevant biological and environmental measurements at various scale may be the way forward for an integrative perspective to better understand hybridization dynamics and its consequence in the oak species complex.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments presented in this manuscript comply with the current law of the country in which they were performed.

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