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Original article

The reproductive success of a *Quercus petraea* × *Q. robur* F1-hybrid in back-crossing situations

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Abstract – A 56 year old *Quercus petraea* × *Q. robur* F1-hybrid was back-crossed to both parental species. Pollen mixes were applied and paternity assigned to offspring based on microsatellite markers. The studied *Q. petraea* × *Q. robur* hybrid proved highly fertile and back-crossed well with both *Q. robur* and *Q. petraea* with slight but not significant preference for *Q. robur.* The results do not support the hypothesis about highly unidirectional gene flow between *Q. robur* and *Q. petraea* towards *Q. robur* as the observed back-crossing ability of the hybrid opens a route for nuclear gene flow from *Q. robur* to *Q. petraea*. However, *Q. petraea* × *Q. robur* hybrids may be rare in nature and the results do not tell us if the (probably more common) reciprocal hybrid also back-crosses easily to *Q. petraea*.

***Quercus robur* / *Q. petraea* / pollen fertility / reproductive success / introgression**

Résumé – Succès reproductif d'un hybride *Quercus petraea* × *Q. robur* en rétro-croisements. Un hybride de première génération de *Quercus petraea* × *Q. robur*, âgé de 56 ans, a été rétro-croisé avec chacune des deux espèces parentales. Un mélange pollinique a été appliqué et la paternité de la descendance a été déterminée grâce à des marqueurs micro-satellites. L'hybride étudié s'est révélé très fertile et se croise bien à la fois avec *Q. robur* et *Q. petraea* mais légèrement mieux quoique de manière non significative avec *Q.robur.* Nos résultats ne confirment donc pas l'hypothèse d'un flux génique unidirectionnel marqué entre *Q. robur* et *Q. petraea* en faveur de *Q. robur.* En effet, la faculté de rétro-croisement observée pour cet hybride ouvre aussi la voie à des flux géniques nucléaires de *Q. robur* vers *Q. petraea*. Cependant, il est possible que les hybrides *Q. petraea* × *Q. robur* soient rares dans la nature ; par ailleurs, les résultats obtenus ne nous disent pas si l'hybride réciproque (probablement plus fréquent) se croise facilement avec *Q. petraea.*

***Quercus robur* / *Q. petraea* / fertilité du pollen / succès reproductif / introgression**

1. INTRODUCTION

Sessile (*Quercus petraea* [Matt.] Liebl.) and pedunculate oak (*Quercus robur* L.) grow sympatric in many parts of their natural ranges, and possible hybridization and introgression between the two species has been subject to substantial interest from European dendrologists and forest geneticists for several decades. An interesting feature is the observed asymmetric hybridization pattern, where hybrids mainly are formed when *Q. petraea* is the pollen parent (father) and *Q. robur* the seed parent (mother) and not vice versa [6, 30]. This has lead to the hypothesis that nuclear gene flow between the two species is mainly unidirectional, going from *Q. petraea* to *Q. robur* (see [24] for review). However, the degree and direction of gene flow between the two species depends not only on the relative frequency of the F1-hybrids (*Q. robur* × *Q. petraea* versus *Q. petraea* × *Q. robur*). It is the reproductive fate of the hybrids – rather than their origin – that is important, and unidirectional introgression only takes place if the hybrids are fertile and show asymmetric affinity for back crossing with *Q. robur* in comparison to *Q. petraea.* However, nobody to our knowledge has measured this feature, and we therefore addressed this aspect by performing controlled back-crossings between a *Q. petraea* × *Q. robur* F1-hybrid and the two parental species. Thus, the objective of the present study was to investigate the fertility of the F1-hybrid in back-crossing situations and see if the findings could support the hypothesis of introgression through highly unidirectional gene flow from *Q. petraea* towards *Q. robur*.

1.1. Evidence of asymmetric hybridization and unidirectional gene flow

The level and significance of gene flow between *Quercus robur* and *Q. petraea* has been a subject for intensive research. Darwin ([7], loc. cit. p. 62 f.) used in *The origin of species* the European oaks as an example of taxa where species limits were difficult to draw and settle. Since then several investigations have focused on quantifying gene flow among the two species under natural as well as controlled conditions. Controlled crossing experiments have shown that hybrid crosses with *Q. robur* as mother have a significantly higher success

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rate than crosses having *Q. petraea* as mother [2, 21, 30]. This has lead to the conclusion that gene flow among *Q. robur* and *Q. petraea* is mainly unidirectional. This tendency of unidirectional gene flow has been confirmed from studies in natural stands as well [24]. E.g. in a French natural stand with equal proportions of *Q. robur* and *Q. petraea* (the pollen environment composed equal numbers of individuals from both species) the allozyme gene frequencies in seedlings as well as in seeds of both *Q. robur* and *Q. petraea* showed an asymmetrical shift towards more pronounced *Q. petraea* genetic characters [4]. These observed gene frequencies in progenies from *Q. robur* could be explained by fertilization of a proportion of female flowers by pollen of *Q. petraea* [4, 6]. A similar indication of unidirectional gene flow in the same stand was observed when using RAPDs [5].

The mechanisms responsible for the observed asymmetry in hybridization remain unknown. A potential explanation might be that different pre- and post-zygotic mechanisms are working in *Q. robur* and *Q. petraea*. Controlled crosses have thus lead to the observation that hybridization are more genotypic specific in *Q. petraea* compared to *Q. robur* [4, 29, 30] which can support the presence of an allele based incompatibility system that differs between the two species.

1.2. Levels of gene flow between *Q. petraea* and *Q. robur*

It has been repeatedly shown that *Q. robur* and *Q. petraea* can hybridize, but to what extend this hybridization actually occurs in natural populations is still subject to discussion (see e.g. [11, 22, 23, 26]). One possible explanation for this lack of knowledge could be that the extent of hybridization in natural populations might differ in different parts of the distribution area of the two species, either due to site differences or differences in (historic) anthropogenic effects (logging, deforestation, fires and agriculture). Disturbances produced by human activities have in other genera been shown to enhance the establishment of hybrids as such disturbances modify reproductive barriers [1, 16]. This phenomenon has also been observed within the genus *Quercus*, where the highest proportion of hybrids between *Q. crassifolia* and *Q. crassipes* were observed in areas with high levels of disturbance [32].

The reported differences in extent and level of hybridization might also be a result of different sampling strategies, sample sizes and data analysed in different ways, which makes it difficult to generalise from and compare studies. Results from a recent study in southern Sweden suggest that hybridization must be expected in populations where both species are present/coexists, although only at a low level.

Although not common, hybridization events can have substantial evolutionary consequences if the F1-hybrid proves to be fertile and able to back-cross with the pure species. Such a back-crossing pathway can explain the difficulties in finding species specific genetic markers in either the nuclear or cytoplasmic genomes of *Q. robur* and *Q. petraea* [4–6, 10, 15, 17, 20, 21]. Also, the fact that several morphological characters are needed to separate species support the theory of evolutionary significant introgression [5, 11] although the two species can be separated by using several morphological characters in combination (e.g. [2, 18, 22]).

It is still subject for discussion whether *Q. robur* and *Q. petraea* should be regarded as two separate or as one very polymorphic species (for different opinions see e.g. [3, 6, 13, 14, 19]). However, we take the existence of strong reproductive barriers between these species as a clear indication of true speciation. Also, the facts that the two species occupy different edaphic habitats in Denmark, and that a nationwide allozyme study of 26 Danish populations has shown variation between the two species to be ten fold the variation between populations within species [28], supports that we are dealing with two distinct species. But we are intrigued by the nature of the introgression because it might have had important consequences for the past and as well as for the future co-evolution of the two species. Also, recent silvicultural activities (such e.g. large scale planting of *Q. petraea* on sites previously only carrying *Q. robur*) encourage us to study the likelihood of hybridization/introgression.

2. MATERIALS AND METHODS

A series of controlled crosses between *Q. robur* and *Q. petraea* and vice-versa was carried out at two sites in Denmark during 1947–1949 by Helmut Barner, a Danish pioneer in forest genetics. One of the resulting hybrids was planted at the Hoersholm Arboretum (part of the Royal Veterinary and Agricultural University) in 1952. This *Q. petraea* × *Q. robur* F1-hybrid (Tree 1110-2440) formed the basis for the present pollination study in 2004, when the hybrid tree was 56 years old. Additionally, three mature *Q. robur* and three *Q. petraea* trees of known origin were selected in the Hoersholm Arboretum. Trees having many female flowers in spring 2004 were selected as mother trees, while pollen donor trees were selected among trees showing abundant male flowering (see Tab. I).

2.1. Checking the hybrid nature of tree 1110-2440

To ensure the value and scientific soundness of the present study, it was of greatest importance that the hybrid origin of tree 1110-2440 could be supported and verified by morphological evidence in order to neglect the risk that the tree was not a true hybrid but merely a result of pollen contamination during the crosses. However, this aspect has been addressed in a [so far unpublished] separate study where the investigated *Q. petraea* × *Q. robur* F1-hybrid was compared with twenty-four other still existing artificial hybrids from the same series of controlled crossings performed in 1947–1949, as well as with fifteen samples from Danish collections at the Museum Botanicum Hauniense (including five specimens of *Q. robur*, five specimens of *Q. petraea* and five specimens classified as putative hybrids between *Q. robur* and *Q. petraea*. Based on Kissling [12] and Rushton [26], nine morphological characters (lamina length, petiole length, lobe width, sinus width, length of lamina from the lamina base to the widest point, number of lobes, number of intercalary veins, basal shape of the lamina and abaxial lamina pubescence) were assessed on five fully expanded and undamaged leaves from the first flush of

Table I. Paternity assigned by DNA genotyping of progeny compared to pollen mix composition (in brackets).

Mother tree	*Q. robur* (1114-2416)	*Q. petraea* (1113-2432)	*Q. petraea* × *robur* (1110-2440)
Pollen parent			
Q. robur [1114-2416]	0% (8%)	–	–
Q. petraea [1113-2432]	–	3% (17%)	–
Q. petraea × *robur* [1110-2440]	40% (33%)	97% (33%)	8% (0%)
Q. robur [1608-3629]	20% (17%)	0% (17%)	15% (25%)
Q. robur [1114-2416]	–	0% (17%)	40% (25%)
Q. robur [1115-2408]	26% (8%)	–	–
Q. petraea [1113-2432]	0% (17%)	–	13% (25%)
Q. petraea [1510-3719]	7% (8%)	0% (17%)	–
Q. petraea [1610-2221]	7% (8%)	–	23% (25%)
TOTAL			
Q. robur (total non-selfing)	46%	0%	55%
Q. petraea (total non-selfing)	14%	0%	37%
Q. petraea × *robur* (total non-selfing)	40%	97%	–
Selfing	0%	3%	8%
Sample size	15	30	60

Comparison of pollination success versus composition of pollen mix (in brackets).
'–' Indicates that the given pollen parent was not included in the pollen mix (trees were not emasculated making selfing possible even if not included in the pollen mix). Numbers in squared brackets refer to position registration numbers of the trees in the Arboretum. All trees are of Danish origin.

the year. Canonical analysis of variance was performed and cross-validation applied for grouping into pure species and hybrids, respectively. In order to test morphological evidence of hybrid origin of the F1-hybrid included in the present pollination study (tree 1110-2440), the canonical values of this specific tree were plotted together with the reference trees in a graphic presentation.

2.2. Collection of pollen

Pollen from *Q. robur*, *Q. petraea* and the *Q. petraea* × *Q. robur* F1-hybrid (cf. Tab. I) was collected in 2003 and 2004 by the following protocol: branches were cut down and put in water after the first elongation of catkins. Branches from each individual were kept isolated in separate, unventilated rooms allowing no penetration of air coming from outside. Then mature catkins were cut off into a fine sieve, thereby separating pollen grains from anthers and other components of the catkins. One sieve per individual was used to avoid pollen contamination. Pollen collected in 2003 was vacuum dried and stored at –18 °C in airtight glasses until use the following spring. Pollen collected in 2004 was stored in airtight glasses until use at 5 °C. Two years of pollen collection were required in order to obtain sufficient amounts of pollen.

A small amount of pollen was germinated prior to pollination in a 10% sucrose solution in order to test pollen viability. Pollen showing pollen tube growth and expansion was considered viable.

2.3. Pollen mixes

Low amounts of pollen limited the design of pollen mixes. Still, three mixes could be made. Pollen mix 1 and 2 contained pollen from *Q. petraea*, *Q. robur* and the F1-hybrid, while pollen mix 3 only contained pollen from the pure parental species (Tab. I). In all three mixes taxa were represented in equal amounts. Consequently, the mother trees of *Q. petraea* and *Q. robur* were given the option of being pollinated by same species, hybrid, alternative species or self, while the F1-hybrid had the option of being pollinated by either of the pure parental species. Only one hybrid was included, so hybrid-hybrid crossing could not be tested in the design as the hybrid cross in this case was selfing. Potato flour was added in order to dilute pollen concentration and thereby ease pollination.

2.4. Isolation of flowers and pollination

2.4.1. Isolation

Special designed bags were used for the isolation of female flowers at the three mother trees. To avoid entering of foreign pollen the bags were pollen tight and at the basis stuffed with water resistant cotton before safely tightened to the branches. Using a sky lift to enter the upper part of the crown female flowers was bagged the 10th of May 2004 several days before being receptive. Bags were put in the upper sunny part of the crown and each bag contained two or more female flowers. No emasculation was applied as isolation was performed before the emerging of the male catkins. A total of 269 bags were used for isolation of female flowers on the three mother trees, with 92 bags put on *Q. robur*, 85 bags put on *Q. petraea* and 92 bags put on the *Q. petraea* × *Q. robur* F1-hybrid. Due to wind a few bagged branches broke off, but on average less than ten bags per mother tree was lost in this way.

2.4.2. Pollination

Pollination was performed the 23rd of May 2004 when female flowers were assumed to be receptive (stigma being widely open, brownish and sticky). A pollen sprayer was used to spray pollen mix into the bags. Subsequently, a small piece of tape was used to cover the needle hole in the bags to avoid entering of pollen from outside. As pollination was only performed once due to the limited amount of available pollen, each bag had two injections of pollen mix to secure excess of pollen in the bags. Different pollen sprayers were used for different pollen mixes to avoid contamination.

Q. robur was pollinated by pollen mix 1, *Q. petraea* by pollen mix 2 and the F1-hybrid (*Q. petraea* × *Q. robur*) by pollen mix 3 (for pollen mix types, see Tab. I).

Three weeks after pollination bags were removed and branches subsequently labelled by numbered metal rings. At this time female flowers were no longer receptive and no pollinating trees in the local area could be identified (catkins brown, dry and falling of).

2.5. Sampling for paternity analysis

In August 2004 net bags were put around the developing acorns to avoid loss in case of early acorn dropping.

Acorns were collected the 14th of October 2004 and sown in boxes in a heated greenhouse (one progeny per box) the day after collection in a mixture of sand (60%), sphagnum (35%) and clay (5%) and covered by a thin layer of sand. Boxes were covered with plastic foil and irrigated regularly to avoid desiccation of the acorns. During the first three weeks the temperature was kept low (around 5 °C) to initiate germination and then elevated to 10–15 °C. After appearance of the root, the cover of plastic foil was removed from the boxes and the temperature elevated further (to 16–18 °C). One or two not fully developed leaves were subsequently collected per seedling and immediately stored in alufolio at –80 °C until extraction of DNA.

DNA was extracted from 15 seedlings of *Q. robur* (total amount germinating), 30 seedlings of *Q. petraea* and 60 seedlings of *Q. petraea* × *Q. robur* F1-hybrid, respectively using DNAeasy Plant Mini Kit from Qiagen. Extracted DNA was stored at 4 °C. Seedlings were genotyped using five microsatellite loci: ssQpZAG9, ssQpZAG36, ssQpZAG104 [31], MSQ4 and MSQ13 [8]. Primers were labelled with Beckman colours D2-black (ssQpZAG9), D3-green (ssQpZAG36 and MSQ4) and D4-blue (ssQpZAG104 and MSQ13) and used in a 25 μL reaction volume (10 ng template DNA, 20 pmol of primer, 200 μM dNTP, 10× reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 9,0) and 1 unit of *Taq* DNA polymerase). The cycling profile of the polymerase chain reaction (PCR) consisted of an initial denaturation step of 4 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 45 s at 50 °C , 45 s at 72 °C and a final extension step of 20 min at 72°C. PCR fragments were separated on a CEQ 2000 XL.

3. RESULTS

3.1. Is the investigated tree 1110-2440 a true hybrid?

Leaf shapes of the investigated *Q. petraea* × *Q. robur* F1-hybrid are shown in Figure 1. Generally, hybrid leaves are long and deeply lobed, but substantial variation was observed. The leaves do not look like pure *Q. petraea*. Results of the canonical analysis based on morphological characters are presented in Figure 2. From the plot can be seen that the trees cluster into three fairly distinct groups representing *Q. robur*, *Q. petraea* and hybrid individuals, respectively. The investigated hybrid (tree 1110-2440) clearly clusters in the hybrid group among the artificial and putative hybrids. Furthermore, tree 1110-2440 was classified as 'hybrid' when cross-validated in the canonical discrimination analysis (data not shown). Consequently, the morphological analysis strongly supports true hybrid origin of the investigated tree 1110-2440 – a finding which is important for the conclusions.

3.2. Pollen viability

Pollen showing pollen tube growth and expansion were considered viable. Vacuum dried as well as fresh (non vacuum dried) pollen showed good viability with high percentages (80–90%) of germinating pollen. All pollen lots were found to be viable according to these criteria.

3.3. Amount of acorns and germination

A total of 208 acorns were harvested from the three mother trees. Variation in numbers of acorns was observed among the mother trees, with *Q. robur* giving least acorns (48 acorns from 92 bags), *Q. petraea* being intermediate (75 acorns in 85 bags) and the *Q. petraea* × *Q. robur* F1-hybrid giving most acorns (85 acorns in 92 bags). For more details see Table II.

Morphology and appearance of acorns from the different mother trees were variable with big differences in size and colour. Generally, acorns of *Q. robur* were round in shape, yellow/green to dark brown in colour and varied significantly in size with few very big acorns. Furthermore, many of the *Q. robur* acorns were not fully mature and indicated early abortion. Acorns of *Q. petraea* were round to oval in shape, dark green in colour with a yellow tone at the basis of the acorns and varied in size, although not as much as observed for *Q. robur*. Also some undeveloped and early aborted acorns were found within bags from the *Q. petraea* tree but fewer than observed for *Q. robur*. Acorns of the hybrid tree were bigger than acorns from both *Q. robur* and *Q. petraea*, green/yellow to light brown in colour and clearly egg shaped. Only very few undeveloped and early aborted acorns were observed.

The difference in amount of early aborted acorns is also expressed in the percentage of acorn germination which varies significantly among the different mother trees (Tab. II), and the pattern follows the observations on early abortion. Thus, acorns of *Q. robur* had the lowest germination percent with only 33% of the harvested acorns germinating. In comparison, acorns harvested from *Q. petraea* and the hybrid showed a germination percent of 55% and 91%, respectively. This variation in germination percent was highly significant ($\chi^2(2) = 17.3^{***}$). As an aggregated result, the fertility (measured as seedlings obtained per bag) of the F1-hybrid was substantial higher than that of both *Q. petraea* and *Q. robur* (Tab. II) with differences being highly significant ($\chi^2(2) = 40.7^{***}$).

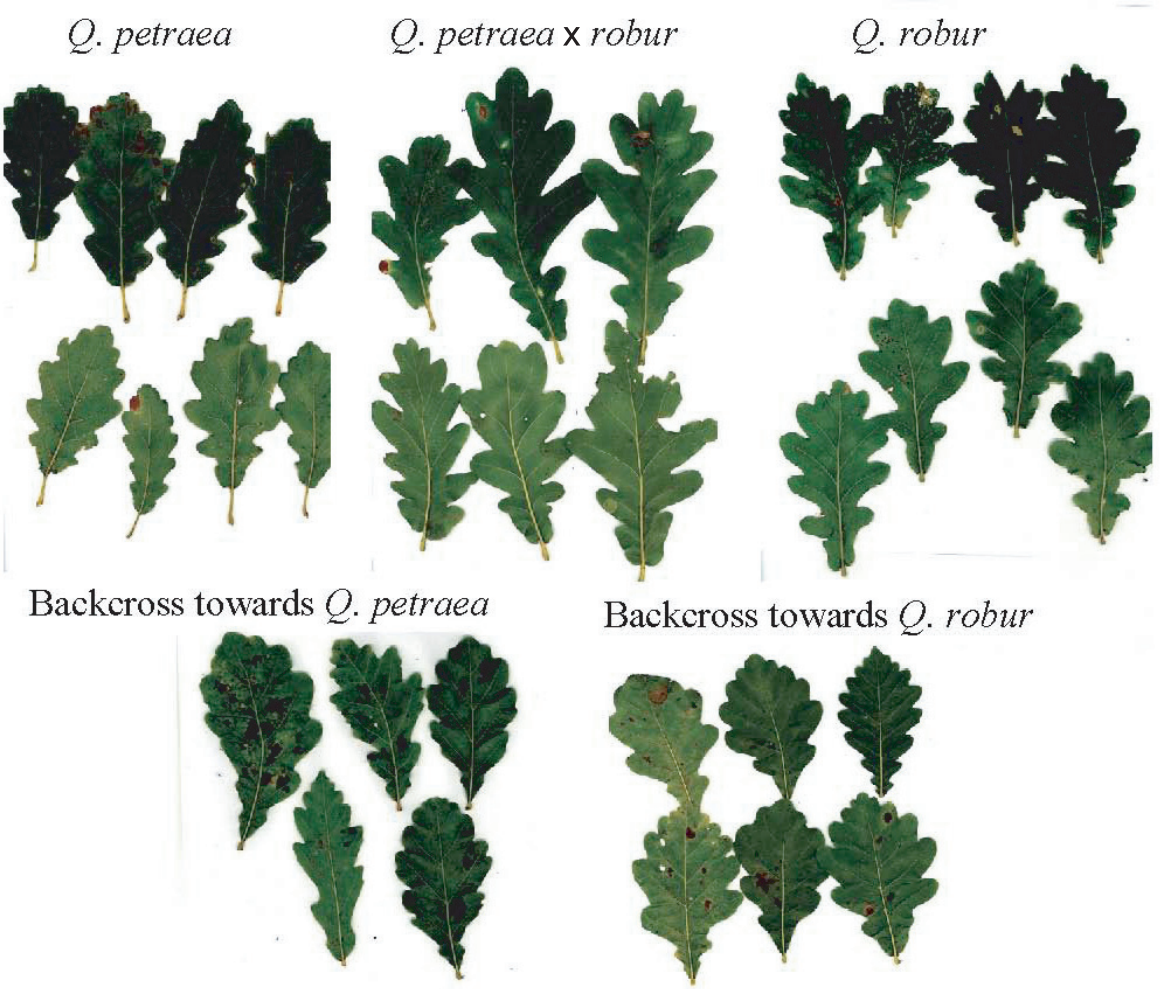

Figure 1. Leaf shapes of three parental trees. Top left: *Q. petraea* (1610-2221), top middle: *Q. petraea* × *Q. robur* (1110-2340) and top left: *Q. robur* (1114-2416). Below corresponding pair-wise one year old offspring.

Table II. Female reproductive success of the three mother trees.

Species (mother tree)	Number of bags	Average number of acorns per bag	Germination %	Average number of seedlings per bag
Q. robur (1114-2416)	92	0.52	33	0.17
Q. petraea (1113-2432)	85	0.88	55	0.47
Q. petraea × *robur* (1110-2440)	92	0.92	91	0.84
χ^2(*df* = 2)		11.54**	17.34***	40.67***

3.4. Paternity

Using five microsatellite loci, it was possible to unambiguously assign paternity to all genotyped seedlings (Tab. I).

The *Q. robur* tree showed a preference for crossing with either *Q. robur* (7/15 = 46%) or with the F1-hybrid (6/15 = 40%), while *Q. petraea* was found to be the pollen parent in fewer cases (2/15=14%). The deviation from 1/3:1/3:1/3 (corresponding to species composition of pollen mix 1) was highly significant, $\chi^2(2) = 12.6^{**}$ supporting the hypothesis that a reproductive barrier limits pollen flow from *Q. petraea* towards *Q. robur*. Results do not indicate that a similar barrier occurs against the hybrid.

The *Q. petraea* tree showed an almost exclusive preference for back-crossing with the hybrid (29/30 = 97%), indicating that this *Q. petraea* has strong affinity for the F1-hybrid and certainly possesses no barrier against crossing with it.

The F1-hybrid showed high ability to back-cross with both *Q. robur* (33/60 = 55%) and *Q. petraea* (22/60 = 37%). Preference (among the outcrossed 55 seedlings) for

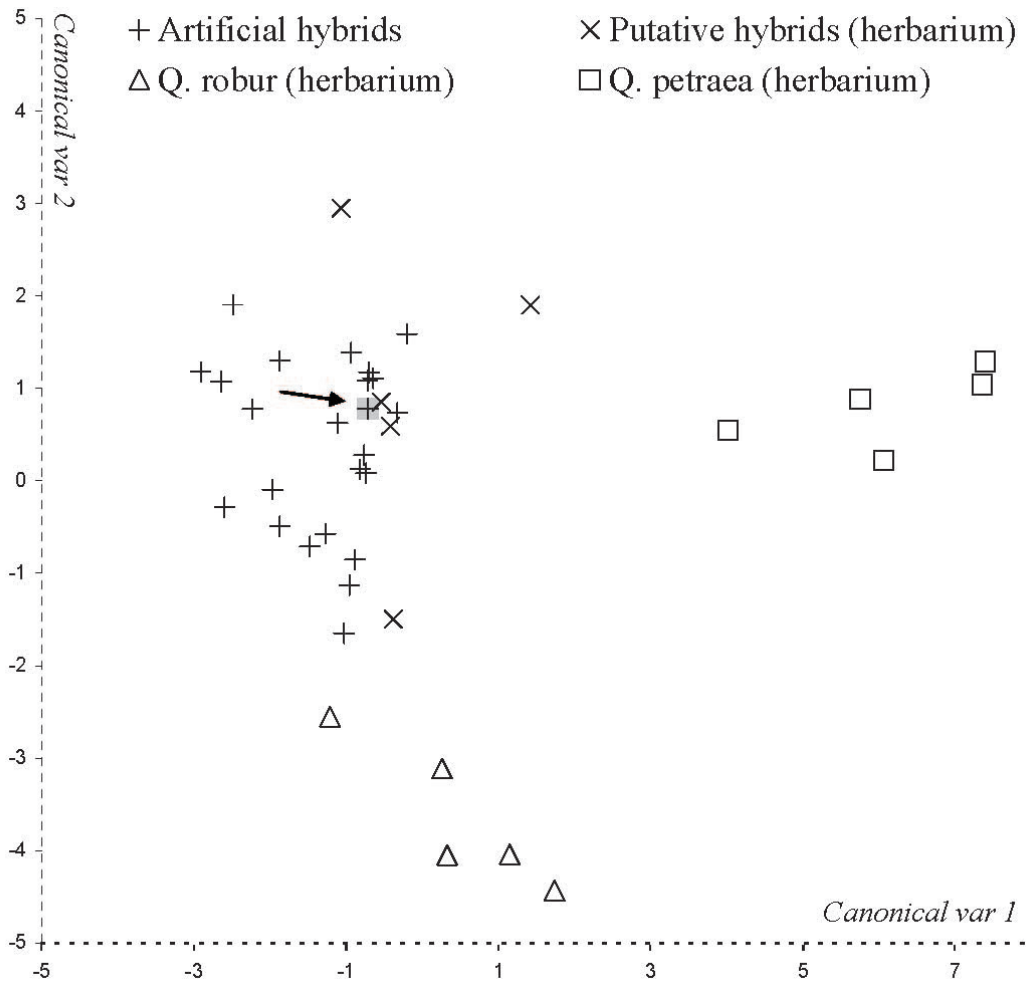

Figure 2. Canonical plot of artificial hybrids (+) including the investigated tree 1110-2440 (shaded at arrow), putative hybrids (×), *Quercus robur* (△), and *Quercus petraea* (□). Unpublished data.

back-crossing with *Q. robur* was not strictly significant $P(X \leq 22|X \sim b(55;0.5)) = 0.08$.

No selfings were found within the fifteen tested seedlings of the *Q. robur* mother tree. The 30 tested seedlings from the *Q. petraea* included one selfed offspring (3%) whereas five selfed seedlings (8%) were found in the 60 tested offspring from the F1-hybrid. However, these differences are non significant ($P < 0.59$ according to Fisher's exact test). No pollen from the hybrid tree itself was included in the pollen mix applied for pollination of the *Q. petraea* × *Q. robur* F1-hybrid (Tab. I). Thus, the five selfings found among the offspring from the hybrid tree most likely originate from male flowers within the pollination bags (male flowers were not emasculated).

4. DISCUSSION

4.1. Is the studied *Q. petraea* × *Q. robur* F1-hybrid fertile?

In our experiment we tested and found pollen viability in the hybrid to be as high as within the pure species. Furthermore, the paternity test showed that a high proportion of progenies from the pure species were the result of successful fertilization by the F1-hybrid. This proves that pollen viability was retained in the F1-hybrid even after up to a year of storage. The results do not coincide with the general observations made by Rushton who found that reduced pollen viability frequently could be observed in individuals classified as putative *Quercus* hybrids [26, 27].

In our study, acorns from the hybrid tree germinated substantially better than acorns from the *Q. petraea* and *Q. robur* tree, respectively, resulting in an overall fertility (in terms of viable seedlings produced per bag) of the hybrid much higher than that of the pure parental species. This shows that the investigated hybrid is viable and has a high fertility and consequently can not be considered to be a 'dead end'. Contrary, being both male as well as female fertile the hybrid is able to contribute to continued introgression between *Q. petraea* and *Q. robur*. Levels and significance of introgression will of course depend on the zygotic fitness of hybrids compared to pure species (from germination to maturity). Results from France indicate that hybrids are maintained in mixed stands for

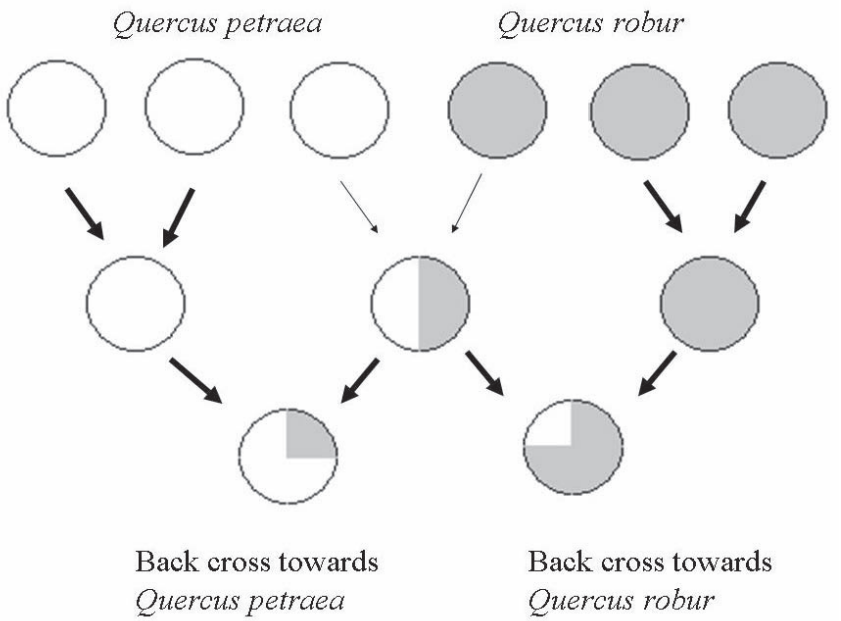

Figure 3. Introgression between *Quercus petraea* and *Q. robur.* Our results confirm that the formation of *Q. petraea* × *Q. robur* F1-hybrids is subject to substantial barriers (narrow arrows), but suggest that this rarely formed *Q. petraea* × *Q. robur* F1-hybrid easily can (at least in our case) back-cross with both parental species (bold arrows). This opens up for a two way route of introgression of nuclear genes.

at least three to six years [6]. Unpublished results from Denmark on fertile soils suggest that hybrids will grow as fast as the pure species up till maturity.

4.2. Direction of gene flow

Observations based on artificial experiments as well as in natural populations have lead to the conclusion that gene flow among *Q. robur* and *Q. petraea* is mainly unidirectional in favour of *Q. petraea* (see [24] for review). However, the route/direction of gene flow will depend on the reproductive fitness of the hybrids. The F1-hybrid in our study showed a distinct ability to back-cross to *both* parental species, thereby opening up a two way route of gene flow of nuclear genes between *Q. robur* to *Q. petraea* through the hybrid (Fig. 3). Thus, our results do not support the thesis that unidirectional hybridization can imply asymmetric back-crossing of the hybrid to the pure parental species.

Petit et al. [24] suggested the following model for explaining gene transfer between the two species: *Q. robur* is initially pollinated by *Q. petraea* resulting in an interspecific hybrid, *Q. robur* × *Q. petraea*. The nuclear genome of this hybrid will be a combination of genes from the two parental species with 50% coming from *Q. petraea* and 50% from *Q. robur* (assuming standard inheritance of nuclear DNA). But the organelle genome (chloroplasts, mitochondria) in the hybrid will be identical to that found in *Q. robur* as the organelle genome generally is maternally inherited [9] as in most other broadleaved tree species. Pollination of the hybrid (*Q. robur* × *Q. petraea*) with *Q. petraea* pollen will lead to individuals who have a nuclear genetic make-up consisting of 2/3 of genes coming from *Q. petraea* and 1/3 coming from *Q. robur* (again assuming standard inheritance) and an organelle genome exclusively made up of genes coming from *Q. robur*. This phenomenon – where individuals possess a nuclear genome predominantly of one species and the cytoplasmic genome of another – has been observed in several other plant species [25]. In Petit et al. [24] these observations are explained by assuming hybrids and their offspring to be male sterile, enabling them to transfer their organelle genomes. Such 'unequal' kind of gene flow can in theory end up in altering the cytoplasmic content in a given population, and could partly explain the observed patterns of variation in cpDNA and mtDNA of *Q. petraea* and *Q. robur* [10,17]. However, the F1-hybrid in our study was highly fertile and was able to produce viable offspring from pollination by either of the pure parental species. Furthermore, our hybrid could effectively pollinate both parental species – even when applied in competition with pollen from the same or the other species. As our hybrid almost equally well back-crosses with both *Q. robur* and *Q. petraea,* it leads to the conclusion that nuclear genes – at least in some cases – can move in the opposite direction from *Q. robur* into *Q. petraea* through *Q. petraea* × *Q. robur* hybrids (Fig. 3). But in spite of gene flow and ability of the hybrid to back-cross species limits seems to be maintained indicating that selection might be operating at one or several levels.

In our pollination study we found a surprising lack of *Q. petraea* × *Q. petraea* progenies from the *Q. petraea* tree although the pollen mix contained 1/3 *Q. petraea* pollen. However, the pollen mix only contained one additional *Q. petraea* father tree

as the rest of the *Q. petraea* pollen came from the mother tree itself (Tab. I). Thus, the results may be due to some kind of allelic based incompatibility system operating. Already, the high level of pollen selectivity within *Q. petraea* is well known [6].

4.3. Limitations

Our data are the result of artificial experiments from which it is not possible to fully generalize about level and significance of hybridization between *Q. petraea* and *Q. robur* in natural populations with more heterogeneous environment and pollination conditions. By pollinating with pollen mixes rather than doing single-tree crosses we have tried to introduce a degree of pollen competition, although only with few pollen parents involved.

The main limitation in our study relates to the fact that we have only investigated the back-crossing behaviour of a single *Q. petraea* × *Q. robur* F1-hybrid, and compared it to a single tree of *Q. robur* and *Q. petraea*, respectively. Studies on more hybrids are required in order to reduce the specific genotypic effects, and thereby obtain quantification of the back-crossing events on a scale that would correspond to population levels. Also, we need to investigate the reproductive fitness and behaviour of the reciprocal *Q. robur* × *Q. petraea* F1-hybrid. A significant different back-crossing pattern of the reciprocal hybrid (less affinity to back-cross to *Q. petraea*) would indicate involvement of cytoplasmic genes in the control of the reproductive barriers between the species. This is of course purely speculative at present as we still have not tested the back-crossing ability of the *Q. robur* × *Q. petraea* hybrid.

Our study is only of pilot nature. We therefore plan to establish a bigger experimental set up involving more hybrid individuals (including the reciprocals) in order to confirm if what we have observed here is a general trend or not. A number of additional hybrids from the controlled crossings made in Denmark between 1947 and 1949 still exist, and we hope studies of these – based on the approach applied in the present study – may contribute further valuable data to the on-going discussion concerning genetic diversity, introgression and gene flow within and between *Q. robur* and *Q. petraea*.

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