PATERNITY ANALYSIS IN A SEED ORCHARD OF *QUERCUS ROBUR* L. AND ESTIMATION OF THE AMOUNT OF BACKGROUND POLLINATION USING MICROSATELLITE MARKERS

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ABSTRACT

Gene flow was assessed using microsatellite analysis in a seed orchard of pedunculate oak (*Quercus robur*) in The Netherlands. Using six microsatellite loci and an exclusion procedure, pollen donors were inferred of 180 acorns collected from three adult trees in the seed orchard. About 70% of the pollen donors originated from outside the orchard. Self-pollination could not be detected. No evidence was found for a non-random pollination of the three maternal trees by the fathers within the orchard. However, the pollen clouds received by the maternal trees differed slightly. For the genetic composition of the pollen clouds from outside and inside the seed orchard no significant differentiation was found. Furthermore, the high gene diversity of the seed orchard consisting of 57 clones was similar to that of samples from two indigenous oak populations in The Netherlands.

Keywords: *Quercus robur*, pedunculate oaks, seed orchard, paternity analysis, background pollination, gene flow

INTRODUCTION

The aim of a seed orchard is the production of genetically improved seeds to be used for reforestation and afforestation purposes. Seeds derived from a seed orchard should reflect both the genetic quality of the traits and the genetic diversity of the reproductive material in the orchard. In general, the seed orchard is designed in such a way that the following conditions are met: (1) self fertilization is avoided as much as possible; (2) equal opportunity to all cross combinations are offered to occur with approximately the same frequency (LANGNER & STERN 1955; HUNT 1962). Moreover, it is assumed that (1) pollen contamination from outside the orchard is negligible and (2) all possible crosses are equally compatible (ERIKSSON *et al.* 1973; WOESSNER & FRANKLIN 1973).

In practice it appears that seed orchards are rarely ideal panmictic populations. Many studies have shown that a large proportion of fertilizations in seed orchards results from gene flow from surrounding pollen sources, also known as background pollination (ADAMS *et al.* 1992; SAVOLAINEN, 1991). Most background pollination studies are conducted in conifer species. In *Pinus* species and *Pseudotsuga menziesii* contamination rates of 22 till 89 % are reported (for a review see SAVOLAINEN 1991; WHEELER & JECH 1992).

Here we investigate the amount of pollen contamination in an oak clonal seed orchard. In The Netherlands only one seed orchard of oak is in operation. The clones in this seed orchard are selected phenotypically as plus-trees in provenances. The oak seed orchard in The Netherlands has been established to produce seeds with genetic gain for characteristics such as growth (production capacity), form (stem straightness, branching habits), disease resistance (mildew) and phenology (DE VRIES & VAN DAM 1999).

The amount of background pollination will be estimated by evaluating the parental contribution to the offspring using microsatellite markers. Microsatellites are highly polymorphic markers and therefore very suitable to determine parentage by exclusion (CHAKRABORTY *et al.* 1988). Pollen dispersal studies in natural stands of temperate oak species are performed using four to six microsatellites (DOW & ASHLEY 1996; STREIFF *et al.* 1998). Exclusion probabilities as high as 99 % can be obtained using only six loci (STREIFF *et al.* 1999). Seed orchards are ideal situations to investigate mating patterns. Knowledge regarding the mating pattern is of interest to determine to what extent paternal trees contribute to the offspring. Therefore, mating patterns between the three mother trees and the pollen fathers in the seed orchard was assessed. The level of genetic diversity in a clonal seed orchard might be

reduced as compared to original seed stands of oak, particularly if low numbers of clones are used. Therefore, in addition, the genetic diversity of the seed orchard is compared with two Dutch indigenous oak stands.

MATERIALS AND METHODS

Seed orchard

The seed orchard with provenance name 'Bremerberg-01' used in this study is situated in Flevoland, Forest-district 'Spijk-Bremerberg' in the center of The Netherlands. This particular seed orchard, covers 4.5 ha and consists of 57 clones, which were planted in 1978 as 10-year old grafts at a spacing of 8 × 10 m. The plus-trees were selected phenotypically for stem straightness, growth and mildew resistance on 17 localities. Six of these are registered Dutch provenances, most of which are roadside plantations. The origin of these provenances is unknown. At the time of analysis the number of ramets per clone varied between 1 and 15. This large variation in numbers is caused by a low survival rate of some clones due to grafting incompatibility. Ramets were randomly distributed in the orchard, for design of the seed orchard see Figure 1. Within the seed orchard also *Fraxinus excelsior* trees were planted for nursing purposes.

From all 57 clones leaves were harvested for genotyping. In autumn 1999 60 acorns each of the three trees (1 ramet of clone number 23 and 2 ramets of clone number 99) were collected for paternity analysis. The acorns were harvested directly from the trees. They were harvested from all four sides of the crown as well as from the upper, middle and lower part of the crown. Locations of the mother trees are given in Figure 1.

Microsatellite analysis

DNA was isolated from fresh leaves of the 57 clones and the 180 acorns with a DNA isolation kit (Puregene®) according to the protocol as described by the supplier (Gentra Systems, Minneapolis, USA). Six microsatellite markers were used for genotyping the clones and acorns. The four loci ssrQpZAG104, ssrQpZAG9, ssrQpZAG1/5, ssrQpZAG36 were developed for *Q. petraea* by STEINKELLNER *et al.* (1997a/b). Two other microsatellite loci (MSQ4 and MSQ13) were developed in bur oak (*Quercus macrocarpa*) and described by DOW *et al.* (1995). All six markers showed mendelian inheritance as was observed in controlled crosses (STEINKELLNER et al. 1997a) or by maternity

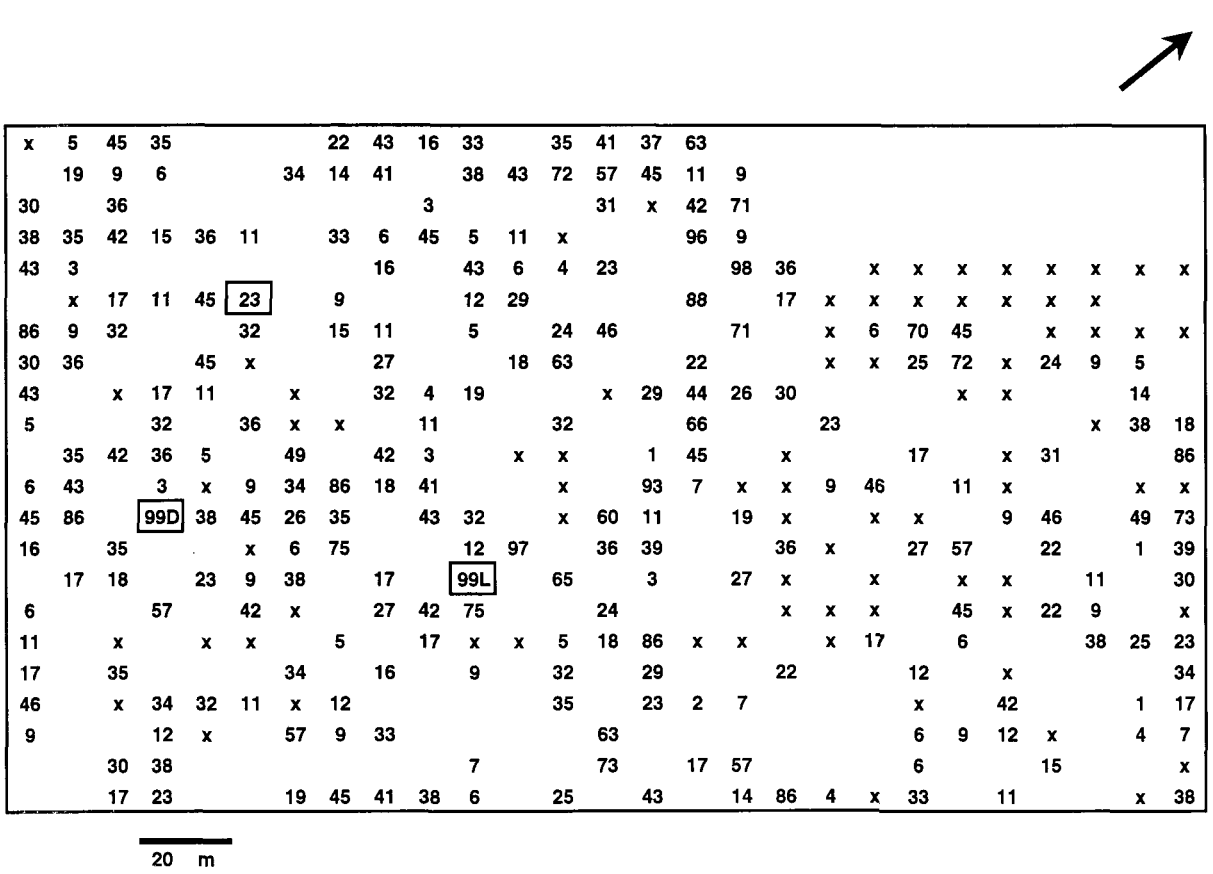

Figure 1. Map of the seed orchard. The trees sampled for paternity analysis are highlighted, x = *Fraxinus excelsior*.

testing (DOW et al. 1995). PCR amplification, electrophoresis on 6% standard denaturing polyacrylamide gels and detection was performed according to the procedures described in STREIFF *et al.* (1998).

Data analysis

General estimates of diversity were calculated in the populations at the progeny level and at the parental level: Expected heterozygosity (H_e), number of alleles (N), effective allele number ($N_e = 1/(1-H_e)$ and fixation index ($F_{IS} = 1 - (H_o/H_e)$) were calculated after NEI (1987). F_{ST} values were estimated following WEIR (1990). Genetic differentiation (G_{ST}) between different pollen clouds was estimated according to NEI (1973). Pollen clouds that fertilized the three mother trees were compared with each other. Furthermore the pollen cloud derived from outside the seed orchard was compared with the pollen cloud derived from the seed orchard itself. The pollen genotypes were deduced from the genotypes of the offspring after subtracting the female contribution. Parameters were obtained using the computer program POPGENE 1.31 (YEH & BOYLE 1997). If a substantial genetic differentiation between the pollen clouds was found, a Fisher exact test was performed to test the allelic distribution across pollen populations for pairs of populations using the GENEPOP program version 3.2a (RAYMOND & ROUSSET 1995).

The parentage assignments were calculated using NEWPAT 1.6 (written by W. Amos, email: w.amos@zoo.cam.ac.uk) (WORTHINGTON WILMER *et al.* 1999). NEWPAT 1.6 is a generalized paternity analysis program, which searches for parent-offspring relationships according to user-defined criteria and then uses a randomization approach to assess the significance of any matches found. User-defined parameters were needed for paternity matching such as: (1) the maximum number of unscored loci allowed; (2) the maximum number of allowable mismatches (allows imperfect matches when set to 1 or more; (3) the minimum acceptable probability for a match requiring null alleles. Each father-offspring, which fulfils the match criteria, is then assessed by randomization. In addition, relatedness values were calculated for all father-offspring matches, following QUELLER & GOODNIGHT (1989). In our analyses, the number of allowable mismatches was set to zero, a low acceptable probability of null alleles ($p = 0.03$) was used and no unscored loci were allowed. The randomization number was set at 100 times the size of the father data set. A paternity was assigned if: (1) it was the only father (clone) found to match an offspring (acorn); (2) it was the father with the highest relatedness (r) -value and lowest randomization number among multiple candidates. In addition, a genotype identity check was performed by NEWPAT 1.6 for all individuals within a file. The number of mismatches allowed for genotype identity analysis was set to zero.

Differences in background pollination between the three trees and within the crown of the trees were analyzed by means of a Chi-square goodness-of-fit test.

In order to test if acorns could be the result of random mating between clones inside the orchard, a Monte-Carlo test was performed. Under the assumption of mating probabilities proportional to the number of ramets of a particular clone in the orchard and assuming the multinomial probability distribution, a simulation of 1000 runs of random experimental outcomes was performed using Genstat 5 (Release 3; Lawes Agricultural Trust, Rothamsted, UK). For each outcome the log likelihood for the multinomial distribution was calculated (COX & HINKLEY, 1982). The log likelihood for the real outcome (under the above assumptions) is then compared with the outcomes of the 1000 runs.

RESULTS

Genetic diversity

The expected heterozygosity (H_e) in the clones of the seed orchard was 0.84 (Table 1). The expected heterozygosity in the progeny had decreased with 15 % compared to the adults in the seed orchard ($H_e = 0.72$). The average number of alleles was similar in the clones and the progeny. However, for all loci 7 alleles that were present in the progeny, did not appear in the clones, while 12 alleles in the clones were not found in the progeny. The average number of effective alleles was lower in the progeny population than in the adult population. Deviations from Hardy-Weinberg expectations were measured using the fixation index (F_{IS}). For both the adults and the progeny populations the F_{IS} values were close to zero (Table 1). Small differences were observed between the progeny population and seed orchard adult population ($F_{ST} = 0.0231$).

In addition, the genetic diversity of the seed orchard was compared with two indigenous oak stands in The Netherlands ('De Meinweg' and 'De Stompert'). In both stands 50 trees were characterized with the same six microsatellites (BAKKER 2001).

The seed orchard (adults) exhibited a similar genetic diversity as compared to the two indigenous oak stands (Table 2). Expected heterozygosities, number of alleles as well as effective number of alleles were more or less the same. Genetic differentiation among the two stands and the seed orchard was very low (F_{ST} = 0.0134).

Table 1. Parameters of genetic diversity (expected heterozygosity (H_e), number of alleles per locus (N) and number of effective alleles (N_e)), fixation index (F_{IS}) and F_{ST} value for the 57 adults and the progenies of three trees in the seed orchard.

Locus	Adults				Tree 99D			Tree 99L		
	H_e	N	N_e	F_{IS}	H_e	N	N_e	H_e	N	N_e
MSQ13	0.74	12	3.8	–0.124	0.40	7	1.7	0.50	8	2.0
MSQ4	0.79	12	4.7	0.167	0.73	10	3.8	0.72	8	3.5
AG9	0.86	12	7.1	0.076	0.72	8	3.5	070	11	3.3
AG36	0.88	12	8.6	0.007	0.75	9	4.0	0.74	10	3.9
AG1/5	0.85	12	6.5	0.108	0.57	8	2.3	0.61	9	2.6
AG104	0.93	24	13.3	–0.005	0.69	14	3.2	0.73	15	3.7
Mean	0.84	14	7.3	0.038	0.64	9.3	3.1	0.66	10.2	3.2

	Tree 23			Total progeny				F_{ST}*
	H_e	N	N_e	H_e	N	N_e	F_{IS}	
MSQ13	0.61	8	2.6	0.53	10	2.1	–0.026	
MSQ4	0.73	11	3.7	0.75	12	4.0	–0.087	
AG9	0.80	12	5.0	0.80	13	5.0	0.015	
AG36	0.77	12	4.4	0.83	13	5.8	0.030	
AG1/5	0.67	8	3.1	0.65	9	2.9	–0.164	
AG104	0.80	16	5.1	0.78	21	4.4	–0.060	
Mean	0.73	11.2	4.0	0.72	13	4.0	–0.049	0.0231

* F-statistics indicating differences among the adult and total progeny population.

Table 2. Genetic diversity parameters (expected heterozygosity (H_e), number of alleles (N) and effective number of alleles (N_e)) and F_{ST} value for the seed orchard and two indigenous populations in The Netherlands ('De Meinweg' and 'De Stompert').

Locus	Seed orchard			De Stompert			De Meinweg			F_{ST}*
	H_e	N	N_e	H_e	N	N_e	H_e	N	N_e	
MSQ13	0.74	12	3.8	0.80	11	4.9	0.80	13	5.1	
MSQ4	0.79	12	4.7	0.84	12	6.2	0.89	15	8.9	
AG9	0.86	12	7.1	0.85	10	6.5	0.83	10	5.8	
AG36	0.88	12	8.6	0.85	13	6.6	0.86	11	7.1	
AG1/5	0.85	12	6.5	0.79	13	4.7	0.85	14	6.8	
AG104	0.93	24	13.3	0.91	25	11.4	0.93	24	13.8	
Mean	0.84	14	7.3	0.84	14	6.7	0.86	14.5	7.9	0.0134

* F-statistics indicating differences among the seed orchard and two indigenous stands.

Comparison of pollen clouds

There was a very weak genetic differentiation between the pollen cloud derived from outside the seed orchard and the pollen cloud derived from the clones in the seed orchard ($G_{ST} = 0.013$). A G_{ST} value of 0.043 was calculated for the differentiation between the pollen clouds that have fertilized the 3 mother trees 23, 99D and 99L. Subsequently, a test of genic differentiation between the pairs of populations was performed. This test revealed that the allelic distribution between the pollen clouds that fertilized tree 99D and 99L were identical for all loci ($p>0.05$) except for AG104. A significant differentiation between the pollen cloud that fertilized

tree 99D and the one that fertilized tree 23 was shown for locus MSQ13, MSQ4, AG9, AG1/5 and AG104 ($p < 0.05$). Similar results were obtained for pollen clouds of tree 99L and tree 23 for locus MSQ4, AG36, AG1/5 and AG104 ($p < 0.05$).

Parentage assignments

The genotype identity analysis performed by NEWPAT 1.6 revealed that within the seed orchard three pairs of clones have identical genotypes for the six microsatellite loci. Identical genotypes were found for clone 7 and 29, clone 23 and 43 and for clone 72 and 46. Also two pairs of acorns had an identical genotype based on the six microsatellites loci.

Paternity analysis with NEWPAT 1.6 showed that of the 180 acorns 65 paternities could be assigned. This implies that for 115 acorns (64 %) no matching genotypes within the seed orchard could be found and that these acorns are most likely pollinated by trees from outside the seed orchard. Ten paternities were doubtful based on a low *r*-value (relatedness) and high randomization value. For three of these, more than one father was assigned, partly due to the fact that these fathers had the same genotype (clone 23 and 43). If corrected for these paternities 70 % of the acorns were pollinated by fathers from outside the seed orchard.

When background pollination is associated to the mother trees: tree 23 obtained 62 % of the fertilizations from outside the seed orchard, tree 99D 82 % and tree 99L 65 %. The number of outside pollinations was significantly different between the three mother trees ($\chi^2 = 6.49, p < 0.05$). No significant differences between the contamination rate in the lower, middle and upper part of the crown (data added up for the 3 trees) were found ($\chi^2 = 4.18, p > 0.05$) nor between the four different sides of the crown ($\chi^2 = 2.3, p > 0.05$).

In figure 2 the clone numbers (pollen donors) in the seed orchard that have fertilized the three mother trees are given. The results show that not all trees contributed to the fertilizations. In total 19 out of the 57 clones contributed to the 55 paternities. The number of fathers contributing to the fertilizations varied between 5 and 13 per mother tree. Both ramets of clone 99 obtained a remarkably high number of fertilizations by clone 17. Subsequently, a test for the occurrence of random mating was done in order to test if there was a disproportional high number of fertilizations by some clones. Using the Monte-Carlo test it appeared that in 562 of the 1000 runs a value smaller or equal to the 'real outcome likelihood' existed ($p = 0.562$). This implies that random mating between the mother trees and clones inside the orchard must be accepted against non-random mating schemes.

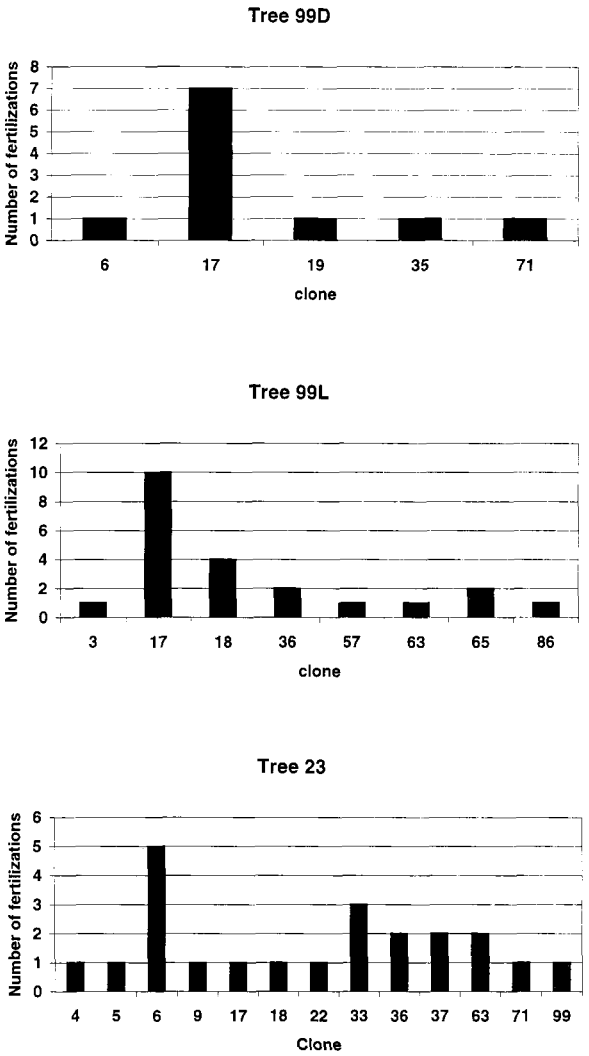

Figure 2. Number of fertilizations per seed orchard pollen donor specified for the three maternal trees (23, 99L and 99D).

None of the 180 acorns was the product of a self-fertilization.

DISCUSSION

The genetic diversity observed in the seed orchard is similar to that of the two indigenous oak stands in The Netherlands (BAKKER 2001). STREIFF *et al.* (1998) measured an expected heterozygosity of 0.87 in a *Q. robur* stand in France using the same six microsatellite loci as in our study. A similar genetic diversity based on four microsatellite loci (AG36, AG1/5, AG9 and AG104) was reported for two North German stands (DEGEN *et al.* 1999). The effective number of alleles of the French and German stands was comparable to the Dutch seed orchard. Accordingly, the Dutch seed orchard represents more or less the same genetic

diversity found in other Northwest European stands.

A small reduction in expected heterozygosity (H_e) in the progeny of the seed orchard clones compared to the adult population can be seen. Twelve alleles present in the adult population could not be found in the progeny. When taking into account the number of ramets, which reflects the real status of the mating population and its allele frequencies, the frequency of these alleles varied between 0.06 and 0.002. Four of these had a frequency of less than 0.005 and can be considered as rare alleles (HARTL & CLARK 1997). Thus, alleles with a frequency lower than 0.06 and not exclusively rare alleles had some chance to disappear from the progeny population of the seed orchard.

Background pollination in the seed orchard was very high. There is a chance that some ramets of clones are mislabeled during grafting or establishment of the orchard and that these have contributed to the background pollination. Of the original 64 clones 7 have died. As not all ramets of a clone were genotyped, some of these 7 clones could in theory still exist in the seed orchard, due to mislabeling. These mislabeled ramets will not be detected as potential seed orchard fathers, but as fathers from outside the orchard. However it is very unlikely that they are responsible for all background pollinations.

The high percentage of background pollinations is in accordance with observations made in natural stands of oak by others. STREIFF *et al.* (1999) found in a mixed stand of *Quercus petraea* and *Quercus robur* that on average 67 % of the offspring (65 % for *Q. robur* and 69 % for *Q. petraea*) was pollinated by male parents from outside the study site. Paternity analysis in a bur oak (*Quercus macrocarpa*) stand revealed that 57 % of the acorns were pollinated by trees outside the stand (DOW & ASHLEY 1996). Moreover, the nearest distance to trees outside the stand was at least 100 m. This suggests that pollen flow from distant sources is an important phenomenon in bur oak. Our observations are in agreement with these results. No oaks are present within a distant of 400 m to the seed orchard. The seed orchard is located in an area with predominantly agricultural land (grass- and farmland). Within a radius of 5 km of the seed orchard only some single oak trees and some small groups of young, not fully matured oaks were found, but no large oak stands. Therefore, it is likely that long-distance gene flow from more distant oak stands is responsible for the background pollination in our seed orchard. Pollen dispersal parameters show that *Q. robur* has a high pollen-dispersal potential. Distances of 199 km have been predicted by models for pollen dispersal (DYAKOWSKA & ZURZYCKI 1959). This could even mean that the large oak stands at The Veluwe, a large forest area about 10 km further could have contributed to the pollinations.

No clear explanation can be found for the high percentage of background pollination. The amount of background pollination differed slightly between the three mother trees. The variation between the trees can not be explained by differences in genetic background of the trees, which indicates that the genotype of the clone does not affect the amount of fertilizations by fathers from outside the orchard. The seed orchard has an open design with spacing of 8 by 10 m, in order to stimulate flower production. So it is unlikely that there is a low pollen production favoring the pollen from outside the orchard. Furthermore, studies in other species show that internal pollen production is not always correlated to contamination rate (SAVOLAINEN 1991).

Unfortunately, due to the relatively low number of fertilizations by donors from within the seed orchard, it was impossible to test the occurrence of preferential matings between the clones. For all three mother trees it was found that the number of fertilizations by the clones corresponded more or less with the number of ramets of the clones. This means that there is no direct evidence for a non-random mating between the clones. These results show also that there is no indication for differences in reproductive success between the fathers in the seed orchard. DOW and ASHLEY (1998) examined the influence of several factors (distance, genetic relatedness, direction, crown volume of pollen donor and DBH of pollen donor) that could affect the male mating success in bur oak. They found a slight influence on the fertilization success only for crown volume and distance. This is more or less in agreement with our observation that the number of fertilizations of a pollen donor corresponds to the number of ramets of a pollen donor in the seed orchard. As the number of crowns and thus the total crown volume increases with the ramet size this might positively affect the pollen production or dispersal of pollen and result in more fertilizations.

On the other hand a weak genetic differentiation between the pollen clouds received by the three maternal trees was found. When the total pollen cloud (derived from fathers within and outside orchard) is considered, both tree 99D and 99L differed slightly from tree 23 in allelic distribution of the received pollen cloud. This might indicate that clone 9 differs from clone 23 as a recipient of different pollen.

Not for all aspects the function of the oak seed orchard is fulfilled. The genetic gain might be lower than expected, because of the high contamination rate. On the other hand, the genetic variation is comparable to *Q. robur* stands in The Netherlands and in other European countries. Unfortunately, there is no real

solution for the high level of contamination in the oak seed orchard. It seems almost impossible to locate the seed orchard in remote distance of other oak stands and thereby isolate the orchard from external pollen sources. If pollen production does influence the number of fertilizations it might be advisable to enlarge the seed orchard by planting many repetitions per clone.

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