POLLEN COMPETITION IN *POPULUS NIGRA* FEMALES REVEALED BY MICROSATELLITE MARKERS

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ABSTRACT

The hypothesis of pollen competition between pollen of *Populus nigra* L. and of *Populus* × *canadensis* Moench (syn. *Populus* × *euramericana* (Dode) Guinier) in fertilising *P. nigra* ovules was tested by analysing the offspring of a series of controlled species mixed crosses. Exclusion analysis was performed on nuclear microsatellite data to identify the paternal genotype of each seedling for each controlled cross. Evidence is presented for non-random mating between *P. nigra* and *P.* × *canadensis* pollen in fertilising *P. nigra* ovules; pollen of *P. nigra* was much more successful in fertilising *P. nigra* females than pollen of *P.* × *canadensis*. The results of this study help explain the low levels of introgression reported in open pollinated offspring of *P. nigra* in natural populations.

Key words: *Populus nigra, Populus* × *canadensis,* introgression, pollen competition, introgressive hybridisation, microsatellite markers.

INTRODUCTION

Within the framework of the domestication of the genus *Populus*, the European black poplar (*Populus nigra* L.) has been largely used in intrasectional controlled crosses with the North American cottonwood *Populus deltoides* Marsh. resulting in well-performing, fast-growing F_1-hybrids, classified under the taxon *P.* × *canadensis* Moench (syn. *Populus* × *euramericana* (Dode) Guinier). Clones of *P.* × *canadensis* are an important source of wood. In many countries of temperate Eurasia, they were massively introduced and planted on alluvial floodplains to replace the autochthonous black poplar resources since the 18th century. This has resulted in a severe reduction in population size of the European black poplar all over Europe. Moreover, there is the potential threat of introgression of genes of *P.* × *canadensis* into the native *P. nigra* (e.g. CAGELLI & LEFÈVRE 1995, FRISON *et al.* 1995, HEINZE 1997, VANDEN BROECK *et al.* 2002, VANDEN BROECK *et al.*, in press) which could make the native species susceptible to extinction. Although gene exchange between species is a major event for evolution, the threat in this case is that the genetic "polluter" represents a very narrow genetic base spread on a very wide scale (CAGELLI & LEFÈVRE 1995). The main concern is that massive introgression would lower the effective population size in an already endangered species. Furthermore, a high level of introgression could lower overall seedling fitness (HEINZE & LEFÈVRE 2001).

However, most studies reporting on the genetic origin of open pollinated (OP) offspring of *P. nigra*, detected no introgression of *P.* × *canadensis*, even if flowering *P.* × *canadensis* males were present in the vicinity (RAJORA 1986, HEINZE 1997, JANSSEN 1998, BENETKA *et al.* 1999, VANDEN BROECK *et al.* 2002, TABBENER & COTTRELL 2003). Given the facts that there are no phenological barriers (VANDEN BROECK *et al.* 2002) and that introgression can occur (VANDEN BROECK *et al.*, 2002; VANDEN BROECK *et al.*, in press) it is likely that competitive interactions between conspecific pollen and interspecific pollen affect the probability of hybridisation in nature. The hypothesis of pollen competition between *P. nigra* and *P.* × *canadensis* is discussed by VANDEN BROECK *et al.* (2002) and by TABBENER & COTTRELL (2003). Also, RAJORA (1986) found that the mating system and fertilising pollen gene pools of *P. deltoides* and *P. nigra* depended upon the presence of different compatible species in the neighbourhood. In a mixed pollen cloud, pollen of *P. nigra* may be more successful than that from *P.* × *canadensis* in pollinating female black poplar (RAJORA 1986, VANDEN BROECK *et al.* 2002, TABBENER & COTTRELL, 2003). However, if no pollen of the own species is present, *P. nigra* females are pollinated

successfully by pollen of *P.* × *canadensis* (VANDEN BROECK *et al.* in press). Similar results have also been obtained by BENETKA *et al.* (1999, 2002) with isozyme markers suggesting that pollen of *P. nigra* is more successful in fertilising ovules of *P. nigra* than pollen of *P.* × *canadensis*. Also, the fact that hybridisation in controlled conditions between *P. deltoides* and *P. nigra* is only possible when *P. deltoides* is the female parent (ZSUFFA 1974) is often discussed as another possible reason for the lack of introgression of genes of *P.* × *canadensis* in OP progenies of *P. nigra* (RAJORA 1986, BENETKA *et al.*1999). In this study, the hypothesis of pollen competition between *P. nigra* pollen and *P.* × *canadensis* pollen in fertilising *P. nigra* ovules is tested by analysing the genotypes of seeds produced in a series of controlled hand-pollinated crosses in which pollen of the two species were mixed (heterospecific cross). In contrast with the study of BENETKA *et al.* (2002), pollen viability was tested *in vitro* before pollination. In addition, control crosses were performed with conspecific (pollen of *P. nigra* only) and interspecific pollen (pollen of *P.* × *canadensis* only) on each female parent. This enables the comparison of conspecific and interspecific pollen fertilisation with heterospecific pollen competition. Pollen competition is understood here as all processes involved in the attempt of pollen to fertilise a finite number of ovules and to produce viable seeds. Microsatellite markers were used in this study for paternity analysis. Microsatellite markers or simple sequence repeats are, in contrast to isozyme markers, highly variable markers that have proven to be effective in paternity analysis (*e.g.* TABBENER & COTTRELL 2003).

The main objective of this study was to investigate if the frequency of seedlings sired by *P.* × *canadensis* in pollen-mix crosses differed significantly from the ratio expected if pollen selection was random. Other objectives were to test (i) if there was a difference in the percentage of hybrid seedlings due to different ratios of pollen proportions, (ii) the effect of female genotypes on the hybrid frequencies and (iii) the influence of pollen proportions on seed germination percentages.

MATERIALS AND METHOD

Controlled crosses

A total of 19 controlled crosses was performed in 2001 and 2002. Three female genotypes of *Populus nigra* and four and two male parents of *P. nigra* and *P.* × *canadensis*, respectively, were used for the controlled crosses (Table 1a). The parents were selected randomly from the poplar breeding material of the Institute for Forestry and Game Management (Geraardsbergen, Belgium). The multilocus genotypes of the parents is described in Table 1b. The composition of pollen mixtures used is given in Table 2 and the controlled crosses performed are represented in Table 3. Each heterospecific pollen mixture experiment (pollen mix 2 and 3) was accompanied by its correspondending conspecific (pollen mix 4) and interspecific pollen cross (pollen mix 1) on the same female parent. In 2002, for each female parent, an additional controlled conspecific cross was also performed by using four different pollen parents of *P. nigra*; N_1, N_2, N_3 and N_4 (pollen mix 5). Pollen germination was tested *in vitro* within 24 h. before pollination for each male genotype on two different pollen lots (RAJORA & ZSUFFA 1986). In 2001, the pollen proportions were prepared according to the weight ratios of the pollen. In 2002, the pollen of different males was mixed in proportion to their weight ratios, corrected for the *in vitro* germination percentage. In both years, the controlled crosses were carried out from January until April. Pollen was collected by forcing dormant male floral buds in the greenhouse (temperature: 18 ± 5 °C) and was stored at 7 °C for 1 to 21 days before pollination. Pistillate inflorescences were isolated before separation of the bud scales to prevent unintentional open pollination. Controlled pollination was carried out according to the bottle grafting-technique (STANTON & VILLAR 1996). Six and two bottle-grafts per cross were used for the crosses performed in 2001 and 2002, respectively. Pollination was carried out with a hairbrush twice a day during 2 to 4 days, as described by STANTON & VILLAR (1996). During the development of the seed capsules, the catkins were reduced to about 20 per bottle graft in order to insure full ripening (RONALD 1982).

Seeds were collected from April to May, separated from the cellulose fibres and sown in flat trays in the greenhouse (50 % white peat / 50 % black peat) within 24 h. of collection. Three to four weeks after sowing, seedlings were transplanted to containers in order to avoid seedling competition and to conserve a maximum number of progeny. During transplantation, the proportion of seedlings produced was recorded for each cross and this was considered as the seed germination percentage.

Determination of paternity

In August, when seedlings were large enough for non-destructive sampling, about 30 to 60 seedlings per cross were selected at random for analysis. When less than 30 seedlings were available for a specific cross, all seed-

Table 1. Plant material used for the controlled crosses. For the ortets that were originated from a natural setting, the river system where the ortet was located is given. For ortets originated from a controlled cross, the parents are specified. The seed orchard was located at 50°40'30"N/4°00'00"E, 30 km Southwest of Brussels, Belgium.

Sex	Species	Code	Clone name	Origin ortet	Collecting place
Female	*P. nigra*	FN1	Lessine-Wannebecq	Natural setting (River Dender, Belgium)	Seed orchard
	P. nigra	FN2	Oosterzele	Natural setting (River Scheldt, Belgium)	Natural setting
	P. nigra	FN3	Grandmetz x Gibecq/12	Controlled cross	Seed orchard
Male	*P. nigra*	N1	Ogy	Natural setting (River Dender, Belgium)	Seed orchard
	P. nigra	N2	V497	Natural setting (France)	Seed orchard
	P. nigra	N3	Elst	Natural setting (River Scheldt, Belgium)	Natural setting
	P. nigra	N4	Terwolde x Isière	Controlled cross	Seed orchard
	P. × *canadensis*	DN1	S.812-8	Controlled cross (*P. deltoides* cv. "S.4-89" x *P. nigra* cv. "V497")	Seed orchard
	P. × *canadensis*	DN2	S.814-3	Controlled cross (*P. deltoides* cv. "S. 4-94" x *P. nigra* cv. "Ollignies")	Seed orchard

Table 1b. Genotypes of the plant material used in the controlled crosses, based on 7 SSR loci. For each locus, the fragment lengths of the alleles (in basepares) are given. (– : missing data). The mean number of alleles per locus was 5.0 and 6.7 for the crosses performed in 2001 and 2002, respectively.

	PMGC 14		WPMS09		WPMS12		WPMS14		WPMS15		WPMS16		WPMS18	
FN1	208	211	256	292	161	169	247	247	213	213	151	157	223	232
FN2	205	208	250	292	161	163	263	259	213	213	151	157	223	232
FN3	205	208	250	292	168	177	232	253	195	210	151	157	232	232
N1	205	208	252	264	167	161	253	253	213	213	151	157	232	232
N2	202	205	246	250	161	169	275	278	210	213	154	151	226	226
N3	208	208	294	252	167	161	232	253	204	213	151	157	229	232
N4	199	205	266	294	167	177	–	–	210	213	151	157	232	232
DN1	193	208	234	292	153	161	247	259	195	213	142	151	217	232
DN2	190	208	234	252	167	161	253	265	195	213	131	151	217	232

lings were sampled. Young leaves were collected on each selected seedling and freeze-dried prior to DNA-extraction. DNA was extracted using Dneasy Plant Miniprep Kit (Qiagen, Helden, Germany). Microsatellite analysis was carried out to determine the paternal genotype of each seedling. Parent and offspring samples were analysed with seven primer pairs (PMGC14, WPMS09, WPMS12, WPMS14, WPMS15, WPMS16, WPMS18) to provide a multilocus genotype for each individual. The microsatellite PMGC14 (forward: 5'-TTCAGAATGTGCATGATGG-3' / reverse: 5'-GTGATGATCTCACCGTTTG-3') was developed for the detection of polymorphisms in *Populus trichocarpa* and is listed in the PMGC database (*http://poplar2.cfr.washington.edu/pmgc*). The other 4 microsatellites analysed were developed for *P. nigra* (VAN DER SCHOOT *et al.* 2000, SMULDERS *et al.* 2001). Primer sequences and PCR-profiles were as described by VAN DER SCHOOT *et al.* (2000) and SMULDERS *et al.* (2001). Fragment separation was performed on an ABI

Table 2. Composition of the 5 pollen mixtures used in the controlled crosses performed in 2001 and 2002. In 2001, pollen ratios were prepared based on pollen weight ratios. In 2002, pollen weight ratios were corrected for their in vitro pollen germination percentages.

Cross type	Pollen mix	Pollen (%)					
		DN1	DN2	N1	N2	N3	N4
Heterospecifi	1	50	50	0	0	0	0
c	2	33	33	16	16	0	0
Interspecific	3	25	25	25	25	0	0
Interspecific	4	0	0	50	50	0	0
Conspecific	5	0	0	25	25	25	25
Conspecific							

Table 3. The number of seeds, the seed germination percentage one month after sowing, the number of seedlings analysed and the results of the paternity analysis for each of the 19 controlled crosses performed.

Year	Cross type	Female parent	Pollen mix	Number of seeds	Seed germination (%)	# Seedlings analysed	Paternity					
							N1	N2	N3	N4	DN1	DN2
2001	interspecific	FN1	1	2401	1	1*	-	-	-	-	0	1
2001	heterospecific	FN1	2	1402	14	33	16	14	-	-	0	3
2001	interspecific	FN1	3	2000	22	62	44	15	-	-	1	2
2001	conspecific	FN1	4	1055	10	29	17	12	-	-	-	-
2002	interspecific	FN1	1	0	-	-	-	-	-	-	-	-
2002	heterospecific	FN1	2	0	-	-	-	-	-	-	-	-
2002	heterospecific	FN1	3	0	-	-	-	-	-	-	-	-
2002	conspecific	FN1	4	0	-	-	-	-	-	-	-	-
2002	conspecific	FN1	5	201	12	19	7	5	5	2	-	-
2002	interspecific	FN2	1	94	20	16	-	-	-	-	10	6
2002	heterospecific	FN2	2	125	0	-	-	-	-	-	-	-
2002	heterospecific	FN2	3	1093	23	49	23	24	-	-	1	1
2002	conspecific	FN2	4	496	25	48	17	31	-	-	-	-
2002	conspecific	FN2	5	2125	35	50	24	11	10	5	-	-
2002	interspecific	FN3	1	0	-	-	-	-	-	-	-	-
2002	heterospecific	FN3	2	1114	42	50	18	30	-	-	2	0
2002	heterospecific	FN3	3	250	48	48	17	30	-	-	1	0
2002	conspecific	FN3	4	1184	54	49	26	23	-	-	-	-
2002	conspecific	FN3	5	0	-	-	-	-	-	-	-	-

* For the cross FN1 × pollen mix 1, only one seedling survived at the time of sampling for DNA-analysis.

310 Prism Genetic Analyser (Perkin Elmer - Applied Biosystems) and the software Genescan and Genotyper 2.5 (PE-Applied Biosystems, Foster City, CA) were used to process and score the SSR data.

Paternity was assigned by exclusion analysis by using the software CERVUS 1.0 (MARSHALL *et al.* 1998), manually corrected for null alleles, assuming simple codominant inheritance of alleles. Null alleles were expressed by many repeatable homozygote-homozygote mismatches between the known female parent and their offspring and by relative strong deviations from Hardy-Weinberg equilibrium. The maternal allele at each locus was subtracted from the genotype of each sampled seedling, and this process of maternal exclusion revealed the multilocus haplotype of the paternal contribution. The results of the paternity assignment carried out by the program CERVUS 1.0, were manually checked; for each locus and for each seedling putative fathers were excluded based on credible mismatches (*i.e.* those unlikely to result from null alleles or failed amplification) with the paternal haplotype of the seedling. The average polymorphic

information content (PIC) over all loci and an estimate of null allele frequency for each locus was calculated by using the software CERVUS 1.0 (MARSHALL *et al.* 1998).

Statistical analysis

The software S-PLUS® 6.1 (Lucent Technologies, Inc.) was used for the statistical analysis. Under the null hypothesis (no pollen competition), the frequency of the seedlings fathered by *P.* × *canadensis* in heterospecific pollen-mix crosses should not differ significantly from the ratio expected if pollen selection was random. Proportion tests (FLEISS 1981) were used to compare the frequency of hybrid seedlings with the expected frequency under the null-hypothesis (Table 4). Proportion tests were also used to test different fertilisation success between *P. nigra* male N_1 and N_2 (data pooled over all crosses).

Chi-square statistics (FLEISS 1981) were used to test the effect of the pollen proportion and the effect of the female genotype on the frequency of hybrid seedlings. The data of the crosses performed in 2001 and 2002 were in good agreement and were, therefore, pooled together per pollen proportion (pollen mix 2 and 3) or per female, according to the test.

Analysis of variance (ANOVA) and a linear regression analysis were carried out to test the effect of the pollen proportion on the seed germination percentage. An angular transformation (p_a) (SOKAL & ROHLF 1995) of the seed germination percentages (p) was performed to stabilise the variance of the proportions. In order to obtain values between 0 and 100, the transformed variable was multiplied by $100*2/\pi$

RESULTS

From the 19 crosses performed, 13 were successful (Table 3). Crosses that failed were due to the dropping of twigs with either fruiting catkins or catkins or capsules with immature seeds.

The combined power of the set of loci to exclude a randomly-selected unrelated candidate parent from

$$p_a = \left[100\ \frac{2}{\pi}\right] \arcsin\left(\sqrt{\frac{p}{100}}\right)$$

parentage of an arbitrary offspring, given the genotype of the offspring and of a female parent (*i.e.* the total exclusionary power) was 0.965 and 0.982 for 2001 and 2002, respectively. The average polymorphic information content over all SSR loci and all crosses was 0.55.

By using the combined data of seven SSR loci, paternity could be assigned unequivocally for all except two seedlings. The two seedlings with more than one putative non-excluded father originated from conspecific crosses (pollen mix 5) and were removed from further analysis.

The microsatellite analysis revealed strong deviations from Hardy-Weinberg equilibrium and a large (relative to other loci) positive estimate of null allele frequency due to null alleles at locus WPMS15 for *P. nigra* female FN_2 and at locus WPMS18 for *P. nigra* female FN_3. Also, manually excluding putative parents

Table 4. For each interspecific controlled cross (pollen mix 2 and 3), the number of seedlings analysed, the percentage of hybrid seedlings and the Chi-square statistics and 95 % confidence limits, testing for significant deviations of the observed frequencies of hybrids and non-hybrids from theoretical expectations under the null hypothesis that there is no interspecific pollen competition among *P.* x *canadensis* and *P. nigra*.

Pollen-mix interspecific cross	Year	Seedlings	Hybrid seedlings (%)	Chi-square	P-value	95 % Confidence limits
Pollen mix2: $p(H_0) = 2/3$						
FN1	2001	33	3 (9.1 %)	46.7	0.000	2.4-25.5
FN3	2002	50	2 (4.0 %)	85.6	0.000	0.7-14.9
FN1+FN3		83	5 (6.0 %)	134.6	0.000	2.2-14.1
Pollen mix 3: $p(H_0) = 1/2$						
FN1	2001	62	3 (4.8 %)	48.8	0.000	1.3-14.4
FN2	2002	49	2 (4.1 %)	39.5	0.000	0.7-15.1
FN3	2002	48	1 (2.1 %)	42.2	0.000	0.1-12.5
FN1+FN2+FN3		159	6 (3.8 %)	134.1	0.000	1.5- 8.4

$p(H_0)$ = expected frequency of hybrid offspring under the null hypothesis (H_0) of no competition among pollen of the two species studied.

based on credible mismatches suggested that a null allele might be present at locus WPMS18 for the *P. nigra* candidate male parents N_1 and N_3.

When *P.* × *canadensis* pollen was used in mixtures with *P. nigra* pollen to pollinate *P. nigra* females, most of the seedlings were fathered by *P. nigra*. The results of the paternity analysis are presented in Table 3. The percentages of hybrid seedlings in the heterospecific crosses varied from 2 % to 9 %, with a mean of 4.6 % (Table 4). The departures from identical fertilisation success (random mating) between pollen of *P.* × *canadensis* and *P. nigra* were highly significant ($p < 0.0001$) both for individual crosses and for the data pooled according to pollen mixture (Table 4). In 2001, when pollen mixtures were prepared according to equal weight, the mean *in vitro* pollen germination percentage of N_1, N_2, DN_1, DN_2 was 54, 55, 54, 32, respectively. The effect of the proportion of hybrid pollen in a pollen mixture on the resulting frequencies of hybrid seedlings was statistically not significant ($\chi^2 = 0.22$, p = 0.64). There was also no significant difference in the frequency of hybrid seedlings due to the female genotypes for pollen mix 2 ($\chi^2 = 0.23$, $p = 0.63$), nor for pollen mix 3 ($\chi^2 = 0.58$, $p = 0.75$). Also, no significant intraspecific paternal effect was detected ($\chi^2 = 0.4183$, $p = 0.5178$). *P. nigra* male N_1 fathered a total of 209 seedlings (52 % of the total *P. nigra* seedlings) while the male N_2 fathered 195 seedlings (48 % of *P. nigra* seedlings).

The number of seeds and the seed germination percentages obtained for each cross are presented in Table 3 and Figure 1. Mean seed germination percentage increased when more pollen of *P. nigra* were present in the pollen mixture, although the observed differences between the pollen mixes were not significant (F-statistic (ANOVA): 1.04, $p = 0.42$). The results of the ANOVA-analysis were confirmed by the regression analysis: the regression coefficient was not significant (F-statistic: 1.62, $p = 0.23$) (Figure 1). No clear relationship seems to exist either between number of seeds produced and seed germination percentage.

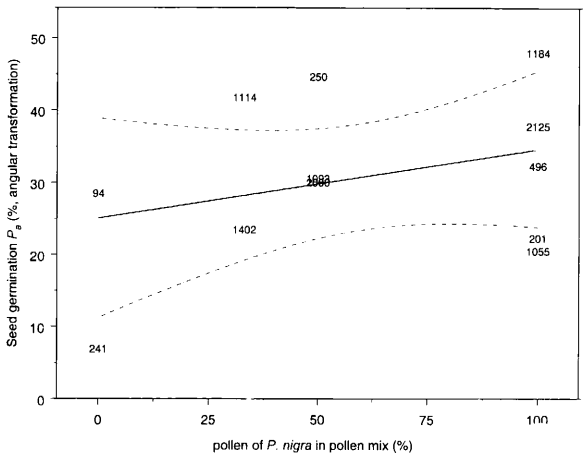

Figure 1. The seed germination percentages p_a (angular transformation) versus % pollen of *P. nigra* included in the pollen mix. Data points represent the number of seed obtained for each successful cross.

DISCUSSION

Evidence for non-random mating

The results of this study clearly show evidence for non-random mating between *P. nigra* and *P.* × *canadensis* pollen in fertilising *P. nigra* ovules. The frequency of hybrid production in heterospecific pollen-mix crosses was highly significantly lower than the frequency of conspecific progeny, even when higher proportions of pollen from *P.* × *canadensis* were present in the pollen mix. These results confirm the results of BENETKA *et al.* (2002) who obtained comparable percentages of hybrid seedlings (7.14 %) by using isozyme analysis on seedlings from three heterospecific pollen-mix crosses. However, BENETKA *et al.* (2002) did not test the *in vitro* viability of the pollen used in the pollen-mix crosses and therefore the low percentage of hybrids obtained in that study could also be due to a low fertility of the specific *P.* × *canadensis* trees used as male parents relative to the *P. nigra* males used in the pollen-mix crosses. Poplar hybrids are generally characterised by reduced fertility relative to parental species; pollen and seed viability is significantly lower in F1 hybrids (PREGITZER & BARNES 1980, STETTLER *et al.* 1996, U.S. ENVIRONMENTAL PROTECTION AGENCY 1999).

In this study comparable *in vitro* pollen germination percentages were obtained for the pollen of *P.* × *canadensis* and *P. nigra* (crosses performed in 2001). However, in 2002 pollen mixtures were corrected for differences in germination percentages, to eliminate the possibility that the differences found were just due to differences in pollen viability among clones. Therefore, it is clear from the results of this study that other factors than individual clonal fertility must have influenced the fertilisation success of conspecific male gametes. The results obtained explain the low levels of introgressive hybridisation reported in OP offspring of *P. nigra females* surrounded by conspecific as well as interspecific males (RAJORA 1986, HEINZE 1997, JANSSEN 1998, BENETKA *et al.* 1999, VANDEN BROECK *et al.* 2002, TABBENER & COTTRELL 2003).

Factors determining fertilisation success

The vigour of the pollen or the pollen tube growth rate

is often mentioned as an important factor in determining fertilisation success predicting hybrid formation in plants (e.g. SARI GORLA *et al.* 1976, MULCAHY & MULCAHY 1987, ARNOLD 1997, DIAZ & MACNAIR 1999, ZHIPING *et al.* 2002). Pollen vigour basically determines the capacity of an individual pollen grain to germinate, grow and fertilise (STEINER & GREGORIUS 1999). Genetic characteristics of the pollen or its producer and pollen/stigma or pollen/style interactions may affect this capacity (e.g. SARIGORLA 1976, STETTLER *et al.* 1980, GAGET *et al.* 1984). In the interspecific pollen mixture situations, these mechanisms might favour union of gametes with matching genetic systems (RAJORA 1989). This phenomenon may be universal and it has also been observed in *Populus* (RAJORA 1989) and in *Pinus* (TOBOLSKI & CONKLE 1977). RAJORA (1989) studied interspecific pollen competition among *P. deltoides*, *P. nigra* and *P. maximowiczii* in fertilising *P. deltoides* ovules by using a pollen mixture technique, allozymes and leaf morphology parameters, and found that the frequencies of F^1 seedlings displayed highly significant departures from the ratio expected if pollen selection was random. *Populus* has been considered to have a surface (sporophytic) type of interspecific incompatibility system (HAMILTON 1976) but GAGET *et al.* (1984) demonstrated that pollen tube arrest in *P. nigra* × *P. alba* occurs in the style and that the stigmatic surface does not represent a strong sexual barrier even for intergeneric crosses (VILLAR *et al.* 1986).

In both pre- and postzygotic barriers, natural variation among parents, especially in female parents, is expected (STETTLER *et al.* 1996). It is very likely, that no significant maternal effect on the hybrid frequencies was found because of the small number of females used in this study. Further research is needed to investigate the variation in female receptivity and the maternal effect on the frequency of hybrid progeny

Pollen load size and the proportion of interspecific pollen in the pollen load may also determine reproductive success (*e.g.* ALARCÓN & CAMPBELL 2000, QUESADA *et al.* 2001). In this limited study including only two heterospecific pollen ratios, frequency of hybrid formation indeed increased (al-though not significantly) when a higher proportion of heterospecific pollen were used in the pollen mixture. These findings agree with the suggestion that the frequency of hybrid formation in angiosperms depends on how often interspecific pollen is transferred to the stigma (ALARCÓN & CAMPBELL 2000).

In contrast with the natural hybridisation process, in this experimental study hand-pollination was carried out twice a day. In natural situations, the continued presence of interspecific pollen may increase the likelihood of hybrid progeny being formed, especially if no source of conspecific pollen is available. This could explain the huge amounts of viable seeds that were harvested on isolated *P. nigra* females surrounded by many flowering hybrid males and in the absence of conspecific males (VANDEN BROECK, unpublished data).

The low frequencies of hybrid progeny observed in the heterospecific crosses could also be due to preferential embryo abortion, with hybrid genotypes aborted more frequently than conspecific seeds. Early abortion of the embryo results in empty seed, or in the disruption of embryo maturation through premature capsule dehiscence (MELCHIOR & SEITZ 1968). Information on controlled crosses with *P. nigra* as the female parent and *P.* × *canadensis* as the male parent is sparse. These crosses are not frequently used in poplar breeding programmes because they result in heterogeneous progenies with decreased growth capacity (STETTLER *et al.* 1996). In frame of interspecific crossability studies within *Populus*, MELCHIOR & SEITZ (1968) report on 6 successful crosses of *P. nigra* females and *P.* × *canadensis* males of which 2 crosses rendered more then 500 seedlings. JANSSEN (1998) was not successful in performing similar crosses. JANSSEN (1998) reports on two crosses that failed probably due to the limited amount of hybrid pollen that was available for the crosses. Although they might be less compatible relative to conspecific crosses, the results of this study confirm that crosses between *P. nigra* females and *P.* × *canadensis* males can produce viable seeds. This is consistent with the results of VANDEN BROECK *et al.* (in press) where evidence was presented for natural hybridisation between *P.* × *canadensis* males and *P. nigra* females.

CONCLUSION

The results of this study suggest that low levels of introgressive hybridisation can be expected in natural populations of black poplar. This finding has practical implications for *in situ* conservation of black poplar and for the restoration of floodplain forests. Further research is needed to identify the processes responsible for the high restrictions of hybrid formation in the offspring of black poplar.

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