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# Paternity analysis of *Populus nigra* L. offspring in a Belgian plantation of native and exotic poplars

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**Abstract** – Gene flow from cultivated poplar plantations into wild populations of *Populus nigra* L. is considered to represent a potential threat to the survival of *P. nigra*. In this study we investigated if pollen competition of *Populus nigra* L. and *Populus × canadensis* Moench in fertilising *P. nigra* ovules detected in a greenhouse experiment also occurs under field conditions. The results confirm non-random mating between males of *P. × canadensis* and *P. nigra* in fertilising *P. nigra* females in the artificial species-mixed Belgian poplar stand. A paternity analysis also revealed non-random intra-specific mating patterns within *P. nigra* in the stand. No paternities were assigned to the male cultivar *P. nigra* cv. Italica. Practical implications for the conservation of wild *P. nigra* populations are discussed.

*Populus* / intra- and inter-specific crossing / introgression / conservation / paternity analysis

**Résumé** – Analyse de paternité dans des descendance de *Populus nigra* L. issues d'une plantation belge de peupliers indigènes et exotiques. Les flux de gènes à partir des plantations de peupliers cultivés vers les populations sauvages de *Populus nigra* L. peut représenter une menace potentielle pour la survie de *P. nigra*. Dans cette étude, nous avons voulu vérifier si la compétition pollinique entre pollen de *P. nigra* et de *P. × canadensis* Moench, observée en croisements artificiels sur *P. nigra* se produit également au sein d'une plantation in situ. Les résultats de cette étude confirment qu'une fertilisation non aléatoire entre les parents mâles de *P. × canadensis* et *P. nigra* d'une part et les parents femelles de *P. nigra* d'autre part a bien lieu sur le terrain. Une analyse de paternité indique également la présence d'une fertilisation intraspécifique non aléatoire au sein des *P. nigra*. Aucun lien de paternité n'a pu être attribué au cultivar mâle *P. nigra* cv. Italica. Les implications pratiques pour la conservation de populations sauvages de *P. nigra* sont discutées.

*Populus* / croisement intra- et interspécifique / introgression / conservation / analyse de paternité

## 1. INTRODUCTION

Gene flow from cultivated poplar plantations into wild European black poplar (*Populus nigra* L.) populations (i.e. introgression) may represent a potential threat for black poplar [4, 9, 11, 18, 30]. In view of the fact that habitat reduction followed by introgressive hybridisation can lead to the extinction of rare plant species, native poplars are believed to be the most threatened forest tree species of old, natural floodplain forests in the temperate zones [18]. Not only introgression of genes from exotic species (mainly *Populus deltoides* Bart. ex-Marsh. and *P. trichocarpa* Torr. Ex Gray) via genetically narrow based hybrid cultivars constitutes a potential threat but also from non-hybrid *P. nigra* cultivars like the Lombardy poplar (*P. nigra* cv. Italica). This cultivar is present as large numbers of individuals with identical genotype which may, under certain circumstances, swamp the gene pool of native black poplar, thereby reducing genetic diversity of the native poplars [4].

Recently in Belgium, evidence was found for introgression of genes of *P. deltoides* in the offspring of an open pollinated (OP) *P. nigra* female [30]. This was the first study to present evidence for introgression of foreign genes into OP offspring of a wild *P. nigra* tree. In addition, it was shown that crosses between *P. nigra* females and *P. × canadensis* males are compatible and can produce viable seeds under field conditions [31]. However, most studies that have investigated the genetic origin of open pollinated offspring of *P. nigra* detected no introgression of foreign genes. This is true even in those circumstances in which flowering males of *P. × canadensis* Moench (syn. *P. × euramericana* (Dode) Guinier, cross between *P. deltoides* and *P. nigra*) were present in the vicinity [2, 9, 14, 22, 26]. These contrasting results might be explained, at least partly, by the influence of competition between pollen of different sources. Pollen competition is understood here as all processes involved in the ability of pollen to fertilise a finite number of ovules and to produce viable seeds. Artificial controlled crosses in greenhouse conditions confirmed that

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non-random mating occurs when pollen mixtures containing *P. nigra* and *P. × canadensis* pollen are applied to female *P. nigra* flowers [31]. In a mixed pollen cloud applied in a controlled cross, pollen of *P. nigra* was more successful than that of *P. × canadensis* in pollinating female black poplars [3, 26, 31]. However, it is generally assumed that greenhouse conditions can differ significantly from field conditions. Field populations are likely to experience smaller and more variable pollen load sizes than those used in greenhouse experiments [19].

In this study, we investigate whether non-random mating occurs under field conditions in an artificial species-mixed poplar stand located in Belgium when the pollen cloud around *P. nigra* ovules contains pollen from both *P. nigra* and *P. × canadensis* trees. This study differs from former studies (e.g. [2, 7, 9, 14, 22, 26]) in that a higher number of *P. × canadensis* males surround the *P. nigra* females investigated. Furthermore, we combined a greater number of diagnostic molecular markers than in former studies thereby increasing the probability of being able to detect mating events of *P. nigra* females and *P. × canadensis* males. The SSR marker WPMS09 [7], the isozyme systems LAP, PGM, PGI [24] and the nuclear co-dominant STS marker win3 [9] were found to be diagnostic in discriminating between parent *P. nigra*, *P. deltoides* and the F1 hybrid, *P. × canadensis*. While it is possible to differentiate between first generation hybrid and non-hybrids using any one of these markers, further generations of hybrids and B1-hybrids from backcrossing cannot always be accurately assigned using a single marker [10]. However, it is possible to increase the probability of detecting hybrids from backcrossing by combining the data from different enzyme and/or nuclear markers.

Besides the inter-specific mating events between black poplar females and hybrid males, we also investigated intra-specific mating events of black poplar based on microsatellite markers (SSRs). Paternity analysis based on SSRs has been used successfully in the study of intra-specific and inter-specific mating patterns in *Populus* [17, 26]. To our knowledge, this is the first study to present data for intra-specific pollen gene flow in a *P. nigra* stand.

The main objectives of this study were: (i) to quantify the frequency of mating events of *P. nigra* females with *P. × canadensis* hybrids, and (ii) to assess intra-specific mating events of black poplar in the artificial species-mixed poplar stand on an individual clone and tree basis. Furthermore, we investigated whether gene flow occurred between the widely planted male cultivar *P. nigra* cv. *Italica* growing outside the stand and the *P. nigra* females inside the stand.

## 2. MATERIAL AND METHODS

### 2.1. Study site

The study site consists of an artificial stand of native and exotic poplars located at Marcq (Edingen), 50° 40' 30" N/ 4° 00' 00" E, 30 km Southwest of Brussels (Belgium) and composed of 11, 56 (including 14 flowering male) and 19 (including 14 flowering male) trees of *P. deltoides*, *P. nigra* and *P. × canadensis*, respectively (Fig. 1). The study stand covers an area of 0.4 ha (200 m × 20 m)

and is located at the edge of a large poplar plantation covering a total area of 5.25 ha. This large plantation is composed of clones of *P. deltoides*, *P. trichocarpa*, *P. × canadensis*, *P. × generosa*, *P. deltoides × P. maximowiczii*, *P. trichocarpa × P. lasiocarpa* and *P. trichocarpa × P. yunnanensis*. A more detailed description of the study site and the plant material is given in Vanden Broeck et al. [29]. A row plantation of mature trees of *P. nigra* cv. *Italica* is located outside the study stand at a distance of about 500 m to the North. The study plot is situated in an agricultural landscape in which there are many poplar plantations of *P. × canadensis* but no stands of *P. nigra* (except plantations of *P. nigra* cv. *Italica*). The nearest known *P. nigra* stand (except *P. nigra* cv. *Italica*) is an artificial stand located 20 km away.

### 2.2. Plant material

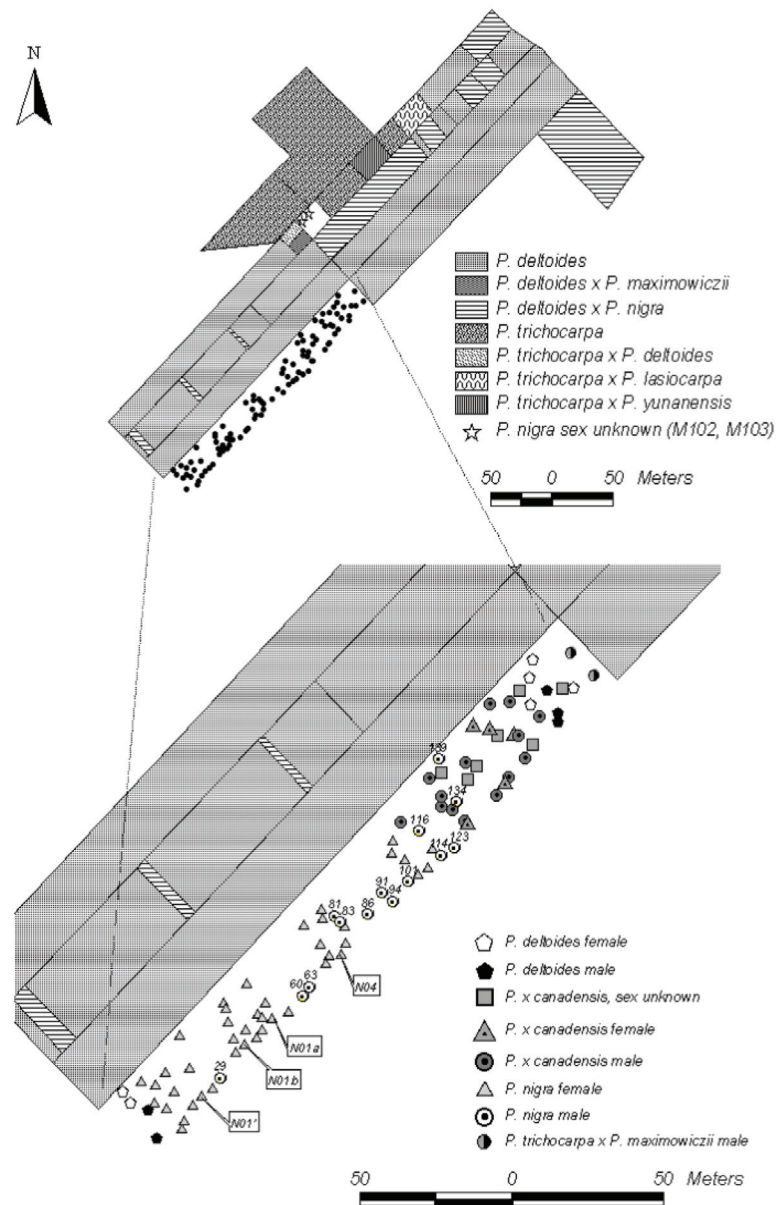
In 1999, seeds were collected in the study stand from the lower branches on the southern side of the crown of two open pollinated (OP) black poplar females named N01a and N04. N01a is a ramet collected from an autochthonous tree and N04 is a ramet collected from a black poplar growing in Hungary. In 2000, seeds were collected again from the same tree N01a, from another ramet of the same genet N01b and from a third female N01' which was a different genet. In total 5 OP progenies (2 in 1999 and 3 in 2000) from three different genets (N01, N01', N04) were harvested (Tab. I). The relative positions of the mother trees are given in Figure 1. Seeds were extracted from the catkins and surface sown in trays in the greenhouse on substrate (50% white peat/50% black peat) within 24 h of collection. From each progeny, young leaves were harvested from 30 to 35 seedlings selected at random (Tab. I). A proportion of these leaves was used within 24 h for isozyme analysis, while the remainder was lyophilised prior to DNA-extraction. DNA-extraction was carried out using the DNeasy Plant Miniprep Kit (Qiagen, Helden, Germany). Leaves were also collected for DNA-extraction and paternity analysis from each male black poplar tree, two black poplar trees of undetermined sex (M102 and M103) (Fig. 1), and *P. nigra* cv. *Italica*.

In order to have some indication of the relative crown volumes of the *P. nigra* males, stem diameter, which is related to crown volume [8] was measured at breast height for all the candidate fathers of *P. nigra* in the poplar plantation.

### 2.3. Identification of hybrids

Morphological observations were used for a first screening of putative hybrids with hybrid and non-hybrid seedlings from controlled crosses as standards. Visual observations of the leaf morphology of the 1-year old seedlings were performed. Other characters, including the general shape of the leaf, the angle between the midrib and the first lower lateral vein [16], the growth habit and the stem form (circular/rectangular) [12] were also assessed in this first screening. Seedlings from the following controlled crosses were used as standards: *P. nigra × P. nigra*, *P. deltoides × P. nigra*, *P. nigra × P. × canadensis* and *P. trichocarpa × P. trichocarpa*.

The molecular analysis included five diagnostic markers that had proved to be useful for the detection of *P. deltoides*-specific alleles in hybrids with *P. nigra* [24]; three diagnostic enzyme systems [24], the SSR locus WPMS09 [24] and the nuclear STS marker win3 [9]. The analysis of the diagnostic enzyme systems PGI, PGM and LAP, and of the nuclear STS marker win3 was performed as described by Fady



**Figure 1.** Location and species composition of the study site in the poplar plantation ‘Marcq’. The location of the four *P. nigra* mother trees N01a, N01b, N01’ and N04 sampled are labelled, the male black poplar trees are indicated by a number.

and Hochu [6] and Heinze [9], respectively. The analysis of the SSR locus WPMS09 is described below. Based on the combined molecular marker data the level of introgression of genes from *P. deltoides* in the offspring of *P. nigra* was estimated. The following reference samples were included in the molecular analysis: *P. nigra* cv. Wolterson, *P. deltoides* cvs. Harvard, Peoria and *P. x canadensis* cvs. Gaver, Ghoy, Robusta.

#### 2.4. Intra-specific mating patterns

Intra-specific mating patterns were studied by conducting a paternity analysis on the 5 OP progenies. Samples of the 5 OP progenies (155 seedlings, Tab. I) and of all candidate father trees of *P. nigra* lo-

cated in the total poplar plantation (14 *P. nigra* males from the study stand + 2 *P. nigra* trees with unknown sex located in the total plantation) were analysed using 14 SSR loci (Tab. II). Furthermore, *P. nigra* cv. Italica, located outside the poplar plantation was also included as a candidate father and was analysed for 5 SSR loci; PMGC14, WPMS09, WPMS14, WPMS16 and WPMS20 (Tab. II). Primer sequences and PCR-profiles were as described by van der Schoot et al. [27] and Smulders et al. [25]. Seven SSR loci used in this study have been mapped by Cervera et al. [5] (Tab. II) and can be considered to be unlinked on the basis of their map position. Fragment analyses were performed on an ABI 310 Prism Genetic Analyser (Perkin Elmer – Applied Biosystems, Forster City, CA). The software programs Genescan and Genotyper 2.5 (PE – Applied Biosystems) were used to process and score the SSR data.

**Table I.** Number of seeds used for paternity analyses.

Collection year	Code genet	Ramet ID	Progeny label	No. seeds analysed
1999	N01	N01a	N1a99	30
	N04	N04	N04	35
2000	N01	N01a	N01a00	30
	N01	N01b	N01b	30
	N01'	N01'	N01'	30
TOTAL				155

**Table II.** SSR loci used for paternity analysis.

Locus	Repeat motif <sup>f</sup>	Linkage group <sup>d</sup>
PMGC14 <sup>a</sup>	CTT	XIII
WPMS03 <sup>b</sup>	(GT) <sub>26-1</sub>	–
WPMS04 <sup>b</sup>	(GT) <sub>25</sub>	–
WPMS05 <sup>b</sup>	(GT) <sub>27</sub>	XII
WPMS09 <sup>b</sup>	(GT) <sub>21</sub> (GA) <sub>24</sub>	VI
WPMS10 <sup>b</sup>	(GT) <sub>23</sub>	III
WPMS12 <sup>c</sup>	(GT) <sub>19</sub>	VI
WPMS13 <sup>c</sup>	(GT) <sub>22</sub>	VIII
WPMS14 <sup>c</sup>	(CGT) <sub>28-3</sub>	–
WPMS15 <sup>c</sup>	(CCT) <sub>14-3</sub>	–
WPMS16 <sup>c</sup>	(GTC) <sub>8</sub> (ATCCTC) <sub>5</sub>	VII
WPMS18 <sup>c</sup>	(GTC) <sub>13</sub>	–
WPMS19 <sup>c</sup>	(CAG) <sub>28-3</sub>	–
WPMS20 <sup>c</sup>	(TTCTGG) <sub>8</sub>	–

–: Not mapped due to absence of polymorphism in the mapping population. <sup>a</sup> From Poplar Molecular Genetics Cooperative (<http://www.ornl.gov/sci/ipgc>); <sup>b</sup> from van der Schoot et al. (2000); <sup>c</sup> from Smulders et al. (2001); <sup>d</sup> mapped by Cervera et al. (2001).

In order to characterize the SSR loci, expected heterozygosity (He) and number of alleles (N) were calculated at the progeny level (total offspring) and at the parental level (adult black poplars in the stand) using the software POPGENE 3.31 (<http://www.ualberta.ca/~fyeh/index.htm>).

Paternity analysis was carried out using a likelihood-based approach and the program CERVUS 2.0 [20]. Taking into account that no other indigenous black poplar trees or black poplar plantations were found within a distance of 20 km from the poplar plantation 'Marcq' (except plantations of the male cultivar *P. nigra* cv. Italica), we assumed that the majority of the potential fathers (85%) were sampled. A 1% error rate (genotyping errors, mutations and null alleles) [20] was also assumed. The analysis is based on paternity assigned at 95% and 80% statistical confidence levels (C.L.) and 10 000 simulated offspring [20]. Identical SSR genotypes of potential males were treated as one potential father in the paternity analysis. Repeated mother-offspring mismatches and estimated null allele frequencies calculated by CERVUS 2.0 were used to detect null alleles. Based on the results of the paternity analysis, the contribution of each

male clone as a pollen donor was evaluated by using  $\chi$ -square tests of goodness-of-fit against the null hypothesis of random mating.

### 3. RESULTS

#### 3.1. Identification of hybrids

No typical morphological leaf or seedling characteristics of *P. × canadensis* (or of *P. nigra × P. trichocarpa*) hybrids were found in the total *P. nigra* offspring analysed. The molecular analysis confirmed the morphological observations. Based on the combined dataset of the three isozymes and two DNA markers (WPMS09 and win3), no genes of *P. deltoides* were detected in the total offspring analysed. Isozyme analysis of the 5 progenies resulted in a clear banding pattern characteristic of *P. nigra* for all seedlings analysed (155/155). No specific alleles of *P. deltoides* were detected at any of the three isozyme loci investigated (*pgi-2*, *pgm-2*, *lap-1*). Analysis of the locus win3 resulted in a clear banding pattern for 151/155 seedlings investigated. Data for 4/155 seedlings are lacking due to death of seedlings. For all the seedlings that were analysed successfully, the amplification products consisted of one band at approx. 150–180 bp. According to Heinze [9], this banding pattern can be considered to be specific for *P. nigra*. The amplification products consisted of one band at approximately 260 bp for all the reference samples of *P. deltoides* and a combination of the two bands (150–180 bp and 260 bp) for the references of *P. × canadensis*. For the diagnostic SSR marker WPMS09 [7], the allele of 234 bp that is specific for *P. deltoides*, was absent in the total progeny as well as in the adult *P. nigra* trees sampled.

#### 3.2. Intra-specific mating patterns

Table III summarises the genetic analysis based on the 14 SSR loci for the 19 *P. nigra* adults (3 sampled females + 14 males from the study stand + 2 *P. nigra* trees of unknown sex located in the total plantation) and the total offspring (5 progenies). The observed variability at the 14 loci was rather small with the number of alleles per locus ranging from 4 to 11 and a total number of 97 and 89 alleles for the adults and the total offspring, respectively (Tab. III). The average number of alleles was similar in the parent generation ( $N = 6.9$ ) and the total offspring ( $N = 6.3$ ). The expected heterozygosity (He) in the parent generation and the total offspring was 0.72 and 0.61, respectively. For all 14 loci, 23/97 alleles (23%) that were present in the parents but did not appear in the offspring, while 13/89 alleles (14%) occurred in the offspring but were not found in the parents. The loci WPMS14 and WPMS18 showed a large positive estimate of null allele frequency (relative to other loci in the analysis) (Tab. III) and could therefore potentially involve the presence of a null allele [21]. It was possible to confirm the presence of a null allele for locus WPMS18 and the progeny harvested on N04; 12 seedlings appeared to be homozygous 213 bp/213 bp while the mother N04 had genotype 223 bp/223 bp. The loci

**Table III.** Parameters of genetic diversity for the adult *P. nigra* trees and the total progeny of the four mother trees for each SSR locus analysed. Expected heterozygosity (He), number of amplified alleles (N) and null allele frequency estimate (negative values imply an excess of observed heterozygote genotypes).

Locus	Adults		Progeny		Null allele frequency estimate
	He	N	He	N	
PMGC14	0.76	6	0.71	6	-0.2241
WPMS03	0.86	7	0.79	7	-0.0895
WPMS04	0.81	8	0.68	8	-0.0238
WPMS05	0.72	7	0.69	7	-0.0745
WPMS09	0.89	11	0.80	9	-0.1032
WPMS10	0.89	11	0.79	8	-0.0809
WPMS12	0.67	7	0.64	7	-0.0674
WPMS13	0.83	9	0.75	9	-0.0555
WPMS14	0.70	6	0.47	5	0.2241
WPMS15	0.28	4	0.045	5	-0.0043
WPMS16	0.64	4	0.59	4	-0.0336
WPMS18	0.62	4	0.51	4	0.2840
WPMS19	0.76	8	0.60	6	-0.0762
WPMS20	0.63	5	0.48	4	-0.0852

WPMS14 and WPMS18 were therefore eliminated for further paternity analysis. Successive amplification of a seedling of mother tree N01a at locus WPMS04 revealed that the heterozygous seedling mismatched for both alleles with the mother. This seedling showed the genotype 251 bp/271 bp for locus WPMS04 while its female parent had the heterozygous genotype 249 bp/269 bp. This could be due to a mutation or to mislabelling of the seedling.

Microsatellite analysis of the remaining 12 SSR loci revealed 11 distinct genotypes in the 16 *P. nigra* candidate fathers in the total poplar stand (Tab. IV) and three different genotypes (N01, N01', N04) in the mother trees investigated. Generally, the paternal genotypes differed by several alleles on several SSR loci. However, there were two exceptions: N01' differed from N01 by only one out of the 28 alleles (at locus WPMS03) while the male tree M091 also differed by only one out of 28 alleles (at locus WPMS14) from the multilocus genotype represented by 6 males (Tab. IV). This latter difference could be confirmed by a controlled cross performed in the greenhouse between *P. nigra* female "N01" and *P. nigra* male "M091" (results not shown). No duplicate genotypes were found among the progeny plants analysed.

Paternity was determined with 95% confidence for 133/155 (86%) seedlings. When using a more relaxed confidence level of 80%, paternity was assigned to 152/155 or 98% of the seedlings. For 3 (2%) seedlings, paternity was unresolved at the 80% confidence level. Six of the 11 candidate male genets (based on 12 SSR loci) of *P. nigra* in the poplar stands, contributed to the paternity of the offspring (Tab. IV). A highly significant difference was found between the observed paternities and the expected paternities under random mating, taking into account the number of ramets of each genet in the stand

( $P = 0$ ,  $\chi$ -square = 146.36,  $df = 10$ ). No paternities were assigned to the male cultivar *P. nigra* cv. Italica located outside the stand.

## 4. DISCUSSION

### 4.1. Interspecific matings

Two studies report on natural mating of *P. nigra* females in artificial stands which are surrounded by a range of compatible *Populus* species, including male trees of *P. × canadensis* [22, 26]. In these observational studies and in common with former studies conducted in natural populations, no evidence for introgression of genes of *P. deltoides* was found in the offspring of *P. nigra* females. However, in all these studies the relative proportion of *P. × canadensis* males in or surrounding the study area was low [2, 7, 9, 13, 22, 26]. It is suggested that, besides the hypothesis of pollen competition, also the relatively low proportion of interspecific pollen in the total pollen cloud could have been one possible reason for the lack of *P. deltoides* parentage detected in OP progenies of the *P. nigra* clones [22, 26]. In this study, flowering *P. × canadensis* males (14) were present in the same abundance as *P. nigra* males (14) at the study site and asynchronous flowering was not a factor in preventing hybridisation between *P. nigra* and *P. × canadensis* [28, 29]. When considering the poplar plantation as a whole, the area covered by *P. × canadensis* (0.80 ha) was about double that occupied by *P. nigra* (0.44 ha). Moreover, the poplar plantation was located in an agricultural landscape in which there were many other hybrid poplar plantations composed of *P. × canadensis* and *P. × interamericana* (cross between *P. trichocarpa* and *P. deltoides*). Despite the abundance of *P. × canadensis* hybrids, no evidence of introgression of genes of *P. deltoides* in the offspring of *P. nigra* was detected. Therefore, the results of this study suggest that under these field conditions pollen of *P. nigra* may be more successful than that from *P. × canadensis* in pollinating female black poplar. However, in a previous study it was shown that when native black poplar stands become very small compared to the abundant hybrid poplar plantations, gene flow from cultivated poplar into native black poplar can occur and the black poplar populations may therefore be in danger of being lost through genetic assimilation [30]. In the latter case, efforts should focus on maintaining and expanding the remaining non-hybrid native populations. Reforestation programmes combined with habitat restoration may limit the potential risk of gene flow with cultivated hybrid *P. × canadensis* poplars.

While our observations in this artificial poplar stand add to our understanding of the reasons behind the low levels of introgression found in natural black poplar populations, the results of this study leave several questions unanswered. Male reproductive success may be influenced by numerous factors such as the spatial distance to the mother tree, wind direction and velocity, stand density and canopy configuration. Individual male fecundity differences like size of the individual pollen load, pollen viability and duration of pollen release were not

**Table IV.** For each candidate male tree (CM) of *P. nigra* in the study stand, the diameter at breast height (DBH) and the distance to the mother trees is given. For each male *P. nigra* genet in the stand, the frequency in the stand (F), the assigned paternities per mother tree (CERVUS, 95% C. L.), the expected paternity frequency (P. exp.) under random mating and the observed paternity frequency (P. obs.) are given.

CM	F	DBH (cm)	Distance to mother tree (m)				Assigned paternities (95% C.L.)						P. exp.	P. obs.
			N01a	N01b	N04	N01'	N01a 1999	N01a 2000	N01b	N04	N01'	Total		
M029		72	24	8	54	7.5								
M060		74	13	28	19	44								
M063	6	81	16	31	15	47	15	30	22	35	13	115	0.375	0.864
M083		51	37	52	8	69								
M094		79	53	68	22	85								
M091		70	52	67	19	84								
M081	1	31	38	52	12	69	1	0	0	0	1	2	0.062	0.015
M086	1	82	45	59	14	76	8	0	0	0	2	10	0.062	0.075
M101	1	45	61	76	29	93	0	0	1	0	2	3	0.062	0.022
M102	1	43	176	191	152	205	0	0	0	0	0	0	0.062	0.000
M103	1	51	169	183	144	200	0	0	0	0	2	2	0.062	0.015
M114	1	76	74	89	43	105	1	0	0	0	0	1	0.062	0.007
M116	1	62	74	89	43	106	0	0	0	0	0	0	0.062	0.000
M123	1	60	79	94	48	111	0	0	0	0	0	0	0.062	0.000
M134	1	34	88	103	57	120	0	0	0	0	0	0	0.062	0.000
M139	1	29	94	109	65	126	0	0	0	0	0	0	0.062	0.000

studied. [23,26,31]. The fact that the black poplar males compared to those of the *P. × canadensis* were closer and in a better position in terms of wind direction in relation to the females investigated, may also explain why the *P. × canadensis* males did not contribute to the seedling crops analysed. In order to test the hypothesis of inter-specific pollen competition under field conditions larger studies need to be conducted over several years in a range of environments in which more mother trees are sampled.

#### 4.2. Intra-specific matings

In this study, we twice found two genotypes that differed only for one out of 28 alleles. Continuous human mediated vegetative propagation has probably extended the natural lifetime of many *P. nigra* genotypes in Belgium. For the heterozygous genotypes of N01 and N01' at WPMS03, it is therefore plausible to suggest that once a single somatic mutation has occurred at locus WPMS03 in a meristem which produced a shoot that was selected for further vegetative propagation. Although further research is needed to investigate the origin of these small genotypic differences, the results of this study confirm that it is important for parentage assignments based on large datasets, to take into account the possible existence of undetectable alleles, scoring errors and mutations [15, 20, 21].

Mating patterns in a stand influence the levels of inbreeding, effective population size and the degree of genetic structuring resulting from selection or drift [1]. Knowledge of mating patterns in seed orchards is also of value to plant breeders concerned with promoting cross-fertilization among cultivars or assessing the validity of OP progenies for use in genetic

testing. The results of this study suggest non-random mating within *P. nigra* (Tab. IV) and indicate that female trees mate with a restricted number of males. The majority of the paternities (115/133 or 86%) were assigned to the genet represented by six ramets and 5 potential fathers did not contribute to the offspring (Tab. IV). For 3 seedlings (2%) paternity was unresolved at the 80% confidence level. This is likely to be due to the fact that these seedlings and multiple equally-likely fathers share very common alleles at each locus which occur very frequently in the plantation. However, although the majority of the paternities were resolved, we cannot completely rule out that there may be gene flow from outside the study stand. The programme CERVUS provides no mechanism for estimating gene flow from outside the sampled population; no distinction is made between multiple, equally-likely fathers and no fathers within the sampled population.

The six ramets representing the genet that contributed to the majority of the paternities were generally trees with a well developed canopy relative to the other candidate fathers. Unfortunately, due to the clonal constitution of the study stand where several trees represented the same genet, we were not able to analyze relationships between distance to the mother trees and DBH versus paternity. *P. nigra* cv. *Italica* did not father any of the 155 seedlings investigated. Trees of *P. nigra* cv. *Italica* were located outside the stand at a distance of about 500 m. This distance to the mother trees, combined with the early flowering phenology [29] might explain why this cultivar did not father any of the seedlings. These results agree with the findings of Tabbener and Cottrell [26] that there was asynchronous flowering and no evidence of mating between a female *P. nigra* and a male *P. nigra* cv. *Italica* growing about

350 m apart in Scotland. However, flowering of *P. nigra* cv. *Italica* may be more synchronous with wild black poplar resources in more Southern European regions such as France (Villar, pers. communication).

A small reduction in expected heterozygosity ( $H_e$ ) in the offspring compared to that in the parent population was observed and 23 alleles detected in the parents were not found in the offspring. Also, 13 alleles in the offspring were not found in the parents. The frequency of these latter alleles was usually very low and generally observed only once in the total progeny. Although amplification errors, scoring errors and mutations could partly explain the presence of these unique alleles of the progeny population, we cannot exclude gene flow from outside the study stand as a possible reason for the presence of these alleles in the offspring.

It is important for restoration projects of black poplar populations to take into account that the number of trees that effectively contributed to the offspring (i.e. effective population size) is likely to be smaller than the number of trees present in a restored population. Therefore, measures should be taken to maximise the effective population size in order to reduce genetic drift, inbreeding and subsequent reduction in diversity. The results of this study also indicate that half-sib progenies from seed orchards might not be a good source of reproductive material for restoration projects of black poplar populations because their genetic relatedness due to the effect of preferential mating.

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