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Natural hybridization and potential seed set of sympatric *Populus nigra* and *Populus* × *canadensis* along the river IJzer in Flanders (Belgium)

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Background and aims – In Europe, native *Populus nigra* generally grows sympatric with related exotic cultivated poplars. Hybridization followed by introgression and genetic swamping is often mentioned as a potential threat for the conservation of *P. nigra*. This potential threat is supposed to be higher when the cultivated poplars are reproductively more effective compared to *P. nigra*. We investigated hybridization events of *P. nigra* females with cultivated *P.* × *canadensis* and *P. nigra* ‘Italica’ along the IJzer river in Flanders (Belgium). We also compared the reproductive potential of *P. nigra* and *P.* × *canadensis*.

Methods – After first determining the genotypic diversity within *P. nigra*, we looked for diagnostic microsatellite alleles of *P. deltoides* in the offspring of open-pollinated *P. nigra*. We also studied the possible paternity by the cultivar *P. nigra* ‘Italica’. The reproductive potential of *P.* × *canadensis* and *P. nigra* was compared in terms of potential seed set.

Key results – This study provides evidence for natural hybridization between *P. nigra* and *P.* × *canadensis* in West Flanders. In 58% of the seedlings from the open pollinated *P. nigra* trees, at least one diagnostic allele of *P. deltoides* was detected. *P. nigra* ‘Italica’ was likely the father of only one seedling. The exotic *P.* × *canadensis* showed a significantly higher potential for seed set compared to native *P. nigra*. The genotypic diversity of *P. nigra* was extremely low with only two (female) genets among 209 black poplars.

Conclusions – Natural hybridization with *P.* × *canadensis* occurs and may pose a significant threat to the conservation of *P. nigra* in West Flanders. To reduce the risk of introgression and genetic swamping of *P. nigra* by *P.* × *canadensis*, we strongly advise reforestation using multiple genotypes of *P. nigra* from neighbouring regions taking into account balanced sex-ratios.

Key words – *Populus nigra*, *Populus* × *canadensis*, hybridization, introgression, genetic diversity, potential seed set, reproductive potential.

INTRODUCTION

Natural hybridization of a rare species with a more common taxon may result in the extinction of the former (Ellstrand 2003). *Populus nigra* L., the European black poplar, is considered a locally endangered native tree species in Europe. Hybridization along with introgression and genetic swamping by commonly planted exotic poplars is assumed to threaten the genetic integrity of *P. nigra* (e.g. Lefèvre et al. 2001, Heinze 2008, Smulders et al. 2008a).

In Belgium, autochthonous *P. nigra* is only represented by individual relict trees occurring mainly in the valley of the river IJzer (West Flanders), the focus area of this study. Here, cultivated trees of *Populus* × *canadensis* Moench (*P.* ×

euramericana (Dode) Guinier cv., synonym.) frequently occur sympatrically with autochthonous *P. nigra*.

Trees of *P.* × *canadensis* are frequently planted for wood production. They are generally first-generation (F_1) hybrids resulting from a cross between a female tree of the North American *P. deltoides* Bartr. ex Marsh. and a male tree of the Eurasian *P. nigra*. Also, second-generation hybrids resulting from a backcross of a F_1 -hybrid to *P. nigra* are sometimes planted in commercial plantations. *P.* × *canadensis* generally appears in monoclonal plantations (Pinon 1992). Only 26 genets of *P.* × *canadensis* are listed in the Belgian List of Approved Basic Material for the Production of Forest Reproductive Material. From this list, only a few genets are frequently planted and many of them are highly related (full-sibs). For example, in Flanders and during the period 2005–

2007, afforestation with *P. × canadensis* supported by grants comprised only 13 different genets (Peter Simon, Flemish Government Department for Agriculture and Fisheries, personal communication). This resulted in very low genetic diversity within and among plantations of *P. × canadensis*.

The potential hazard from natural hybridization with cultivated poplars is the loss of *P. nigra* as a pure species. The loss of *P. nigra* may result from genetic assimilation by the more numerous cultivated poplars or from outbreeding depression. Outbreeding depression is the loss of specific, co-adapted gene complexes and occurs when hybrid offspring has lower fitness than offspring from intraspecific mating events. In particular, the potential hazard of interspecific hybridization is higher when *P. × canadensis* is more numerous and reproductively more effective by producing more pollen and / or seed compared to *P. nigra* (Ellstrand 2003). Although commercial hybrid poplar clones are assumed to be reproductively less effective than their related wild poplar species (Stettler et al. 1996, Strauss et al. 2001), information about the reproductive capacity of hybrid poplar plantations is scarce. Despite studied for many plant species (Burd et al. 2009), no report exists on the number of ovules per flower or on the potential for seed set of *Populus* species, to our knowledge.

In addition, natural hybridization with the cultivated clone *P. nigra* ‘Italica’, known as the Lombardy poplar, may

also pose a threat to autochthonous *P. nigra* resources. *P. nigra* ‘Italica’ is the most ancient poplar cultivar and the one with the widest distribution (Chenault et al. 2011). This male clone is frequently planted as a windbreak in rural and urban landscapes across the temperate zone and is supposed to be a major pollen producer (Chenault et al. 2011). Massive pollen flow from *P. nigra* ‘Italica’ may result in the loss of genetic diversity of the autochthonous *P. nigra* populations which can lead to their eventual extinction. Since the cultivar is part of the *P. nigra* species, barriers against introgression into autochthonous wild *P. nigra* populations could be assumed to be low (Chenault et al. 2011).

Vanden Broeck et al. (2004) presented evidence for natural hybridization between *Populus nigra* and *P. × canadensis*. They hypothesized that natural backcross events of *P. nigra* females with pollen of *P. × canadensis* are rare and seem to occur only occasionally in solitary *P. nigra* females when male hybrids are locally abundant. Nevertheless, this evidence for natural hybridization between *P. nigra* females and *P. × canadensis* males was based only on one case-study and one single tree-progeny. Furthermore, the results of the latter study contrast with results from other studies where no natural hybridization was detected in the offspring of open pollinated *P. nigra*, although males of *P. × canadensis* were present in the vicinity (Benetka et al. 1999, Fossati et al. 2003, Tabbener & Cottrell 2003, Heinze 2008). Former

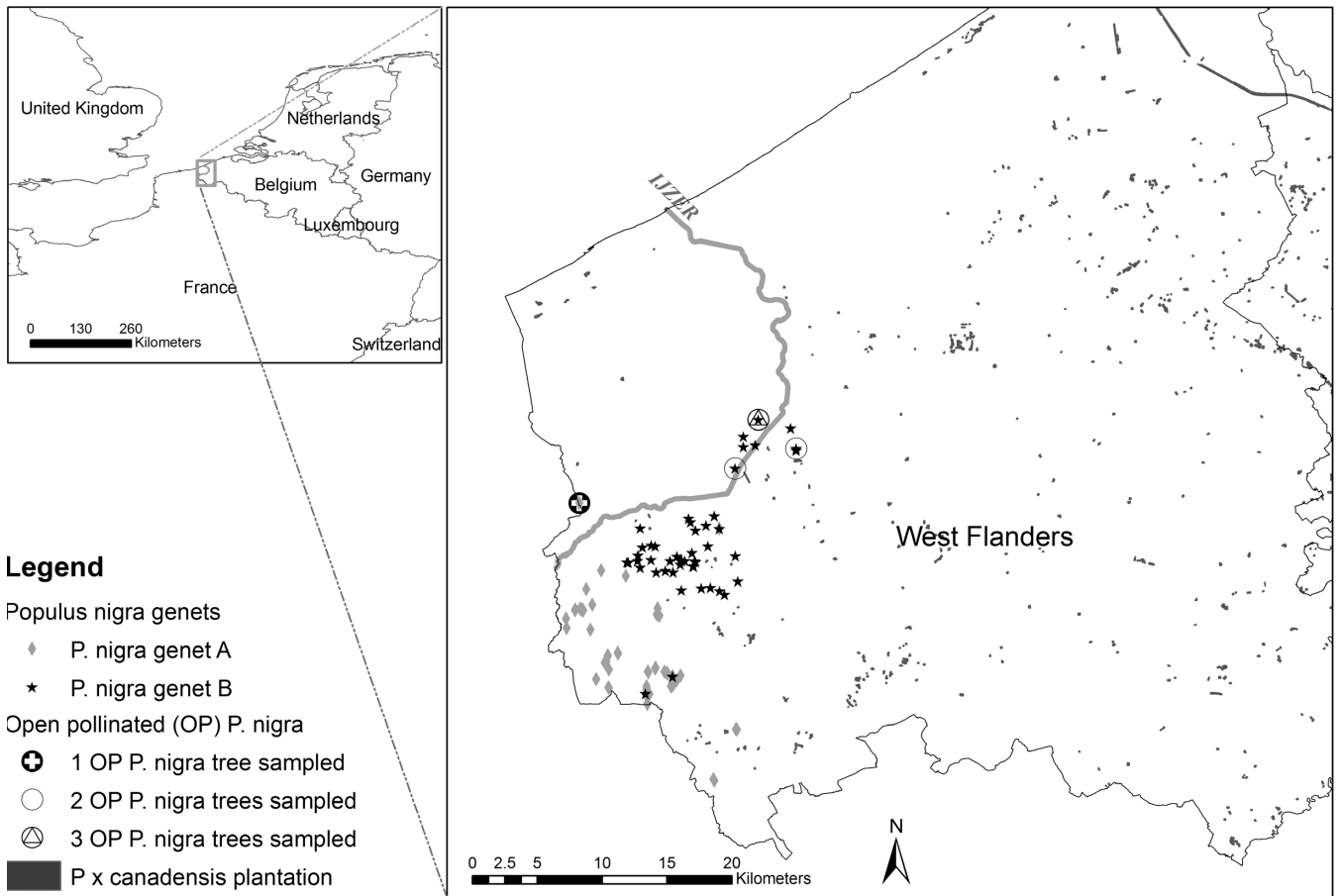


Figure 1 – Location of the sampled *Populus nigra* trees in the province West Flanders, Belgium. Seeds were harvested on eight open-pollinated black poplar trees located in the valley of the river IJzer.

Table 1 – Number of seedlings per progeny from open-pollinated *Populus nigra* carrying diagnostic microsatellite alleles of *P. deltoides* at loci Wpms09, Wpmgc14 and Wpms18.

Sampling Year	<i>P. nigra</i> mother tree	Genotype Code of mother tree	Number of seeds collected	Number of seeds germinated	Seedlings sampled for DNA-analysis	Number of seedlings with diagnostic microsatellite alleles of <i>P. deltoides</i> at loci Wpms09, Wpmgc14 and Wpms18			
						One diagnostic allele	Two diagnostic alleles	Three diagnostic alleles	Total
2006	Oosterzele	C	105	42 (40%)	42	7	10	5	22 (52%)
2007	Di_50C	A	200	46 (23%)	31	13	11	3	27 (87%)
2007	Di_50C2	A	13	9 (69%)	8	1	5	1	7 (77%)
2007	Di_50C3	A	6	4 (67%)	3	1	1	0	2 (67%)
2007	Di_33A	A	40	4 (10%)	4	0	0	0	0
2007	Di_33B	A	13	3 (23%)	3	1	1	0	2 (67%)
2007	Beveren1	B	27	10 (37%)	10	0	3	0	3 (30%)
2007	LR04/2	A	50	3 (6%)	3	0	0	0	0
2007	LR04/3	A	25	6 (24%)	6	0	0	1	1 (17%)
Total			479	127 (26%)	110	23	31	10	64 (58%)

studies indicate the local demographic context and pre-mating reproductive barriers as important factors determining hybridization frequencies (e.g. Vanden Broeck et al. 2004, 2006, Ziegenhagen et al. 2008, Field et al. 2011). However, as evidence for natural hybridization between *P. nigra* and *P. × canadensis* is scarce, the significance of hybridization in the context of the conservation of *P. nigra* is controversial (Ziegenhagen et al. 2008, Csencsics et al. 2009).

The goal of this study is to provide more insight into the significance of hybridization to the conservation of *P. nigra* along the river IJzer. More specifically, we determine whether native *P. nigra* females hybridize with cultivated *P. × canadensis* and *P. nigra* ‘Italica’. Also, we investigate the demographic and genotypic context of *P. nigra* in West Flanders. Finally, we compare the reproductive capacity in terms of the potential for seed set of the cultivated *P. × canadensis* with the native *P. nigra*. Based on the results we formulate practical guidelines for the restoration of black poplar along the IJzer in West Flanders.

MATERIAL AND METHODS

Study area and plant material

Putative autochthonous black poplar trees in the valley of the IJzer were selected from an earlier full inventory of autochthonous trees and shrubs in Flanders (Vander Mijnsbrugge et al. 2005). These trees were identified based on their morphological characters. The river IJzer is located in the province West Flanders, the westernmost province of Flanders (northern Belgium) that borders the North Sea and France. This river valley is characterized by a flat and open agricultural landscape. In spring 2007, we visited all 135 known locations with putative black poplars in the valley of the IJzer River. Young leaves were collected for DNA-extraction from generally one but up to 11 putative black poplar trees per location, because we expected a high level of clonality per location (Storme et al. 2004). This resulted in a total of 235

mature putative black poplar trees sampled to determine the genotypic diversity. When branches with flower buds were available, the sex of the tree was recorded. The locations of the sampled trees are given in fig. 1.

To assess hybridization between female trees of *P. nigra* and male trees of *P. × canadensis*, we harvested seeds (half-sibs) on eight open-pollinated black poplar trees located in the valley of the river IJzer (fig. 1). Furthermore, we harvested seed on one tree located in Oosterzele in the valley of river Scheldt in the province East Flanders. These mother trees were surrounded by adult commercial plantations of the hybrid *P. × canadensis* within a distance of 100 m. Seeds were sown in trays in the greenhouse within 24 h of collection. One month after sowing, seed germination percentages were recorded and leaf samples were collected from a total of 110 seedlings for DNA-extraction. The number of seeds genotyped per mother tree is listed in table 1. Young leaves were also sampled for DNA-extraction from the cultivated *P. nigra* ‘Italica’.

To investigate the potential for seed set of *P. nigra* compared to *P. × canadensis*, branches bearing pistillate inflorescences were collected before or shortly after bud burst in April 2010 from eight and nine female trees of *P. × canadensis* and *P. nigra*, respectively. They were selected from the poplar collection of the Research Institute for Nature and Forestry (INBO, Geraardsbergen, Belgium) based on the presence of flower buds. The trees of *P. × canadensis* originated from seeds obtained from controlled crosses and therefore represented different genets. The female trees of *P. nigra* represented different genets from the valleys of the rivers Dender, Meuse and Scheldt which were genotyped previously by Storme et al. (2004). The origin of the plant material used for studying the seed production potential is presented in table 2. At the time of collection, the inflorescences extended about 0.5 to 1 cm beyond the bud scales and thus were not yet fully expanded and unpollinated.

Table 2 – Characteristics of the *Populus × canadensis* and the *Populus nigra* trees analysed for seed set potential.
NA: not assessed due to dropped catkins.

Name	Species	Origin	# catkins sampled	# capsules per catkin (S.D.)	# ovaries sampled	# average number of ovules per ovary (S.D.)	Max. potential seed set / catkin
17.018/165	<i>P. × canad.</i>	Seedling of a controlled cross (S. 513-60 × S.157-3)	NA	NA	16	21.4 (2.2)	-
78.018/180	<i>P. × canad</i>	Seedling of a controlled cross (S. 513-60 × S.175-3)	2	33.5 (2.12)	15	20.7 (1.7)	693
78.018/192	<i>P. × canad</i>	Seedling of a controlled cross (S. 513-60 × S.175-3)	15	33.9 (3.6)	20	27.2 (2.8)	922
78.018/202	<i>P. × canad</i>	Seedling of a controlled cross (S. 513-60 × S.175-3)	7	27.8 (4.5)	15	20.8 (2.7)	578
78.018/203	<i>P. × canad</i>	Seedling of a controlled cross (S. 513-60 × S.175-3)	NA	NA	15	18.1 (3.3)	-
78.023/233	<i>P. × canad</i>	Seedling of a controlled cross (S. 513-60 × S.529-15)	NA	NA	15	18.1 (2.0)	-
Hees	<i>P. × canad</i>	Seedling of a controlled cross (Michigan × France)	16	32.5 (7.9)	30	15.3 (4.5)	497
Oudenberg	<i>P. × canad</i>	Seedling of a controlled cross (S. 513-60 × S.175-3)	11	27.6 (2.6)	15	19.4 (2.9)	535
		Mean	-	31.4 (4.2)	15.8	20.1 (2.8)	631
Hochter Bampt	<i>P. nigra</i>	natural setting	13	34.8 (5.5)	15	8 (1.6)	278
Hourraing	<i>P. nigra</i>	natural setting	15	50.1 (6.8)	15	9.7 (1.5)	486
Meers	<i>P. nigra</i>	natural setting	13	35.3 (9.4)	16	12 (2.5)	424
Oosterzele	<i>P. nigra</i>	natural setting	15	37.6 (4.5)	15	11 (1.3)	414
Papignies	<i>P. nigra</i>	natural setting	23	47.3 (12.2)	15	11.3 (1.1)	534
S.623-7	<i>P. nigra</i>	natural setting	20	20.3 (5.7)	15	15 (2.4)	305
Wannebecq-Ostiches	<i>P. nigra</i>	natural setting	20	45.6 (5.6)	15	10.8 (1.2)	492
Bilzen	<i>P. nigra</i>	natural setting	NA	NA	15	11.4 (1.9)	-
Wodecq	<i>P. nigra</i>	natural setting	NA	NA	17	9.6 (1.5)	-
		Mean	-	38.7 (7.1)	15.3	11.0 (1.6)	425

DNA-extraction and microsatellite genotyping

Young leaves were lyophilized prior to DNA-extraction. DNA was extracted using the Dneasy Plant Miniprep Kit (Qiagen, Helden, Germany). For the study of the genotypic diversity of *P. nigra* in the valley of the IJzer river, we analysed seven nuclear microsatellite loci: Pmgc14, Wpms05, Wpms09, Wpms14, Wpms16, Wpms19, Wpms22 (Smulders et al. 2001, van der Schoot et al. 2000). For the analyses of

the half-sib progenies collected in the field from the open pollinated *P. nigra* mother trees, we analysed four nuclear microsatellite loci: Pmgc14, Wpms09, Wpms18 and Wpms20. These loci have proven to be diagnostic for differentiating F₁ hybrids of *P. × canadensis* from non-hybrid *P. nigra* (e.g. Fossati et al. 2003, Vanden Broeck et al. 2004, Pospíšková & Šálková 2006). They display species-specific alleles or alleles with a significantly different frequency between *P. nigra*

Table 3 – Species-specific alleles (Pmgc14, Wpms09, Wpms18) or alleles with a significantly different frequency between *P. nigra* and *P. deltoides* (Wpms20) as found by Fossati et al. (2003).

Locus	Allele (bp)	Allele frequency		
		<i>P. nigra</i>	<i>P. deltoides</i>	<i>P. × canadensis</i>
Pmgc14	199	0	0.600	0.158
	193	0	0.400	0.408
Wpms09	234	0	1.00	0.583
Wpms18	220	0	1.00	0.566
Wpms20	224	0.154	0.800	0.625
	218	0	0.200	0.045

and *P. deltoides* (table 3). Locus Pmgc14 was selected from the IPGC (International Populus Genome Consortium) SSR database (<http://poplar2.cfr.washington.edu/pmgc>). Wpms09 was described by van der Schoot et al. (2000), the other loci were selected from the study of Smulders et al. (2001). PCR reactions were performed as described by van der Schoot et al. (2000) and Smulders et al. (2001). The markers Pmgc14, Wpms05, Wpms09, Wpms16 were mapped by Cervera et al. (2001) and are completely unlinked. PCR reactions were run on a Spectrumedix SCE9610 fluorescent capillary sequencer and analyzed using the BaseSpectrum (Version 2.0) (Spectrumedix LLC, State College, PA, USA).

In microsatellite genotyping, the observed size of a specific allele is generally an estimation of the absolute fragment size and may slightly vary between different studies. To enable the comparison of allele fragment sizes reported in former studies, we included the following control samples; five clones of *P. deltoides* (Harvard, S 9-2, S.4-89, S. 620-588 and S. 197B) and nine clones of *P. × canadensis* (Primo, Ghoy, Gaver, Gibecq, Ogy, Isières, Muur, Oudenberg, Vesten). Based on these control samples, we screened the genotypes of the half-sibs for alleles previously identified as diagnostic for *P. deltoides* by Fossati et al. (2003) (table 3). The widely planted male cultivar *P. nigra* 'Italica' was included to assess its role as a potential father for the open-pollinated progenies.

Assessment of the genotypic diversity of *P. nigra*

To detect if putative trees of *P. nigra* sampled in the field were in fact hybrids, we first screened the accessions for diagnostic alleles of *P. deltoides* at the loci Pmgc14 and Wpms09 (see also Fossati et al. 2003). Accessions showing a diagnostic allele of *P. deltoides* at one of these loci were considered hybrids and were eliminated from further analysis. Secondly, we defined the apparent number of different genets with the programme GENALEX 6.4 (Peakall & Smouse 2006) based on the multilocus microsatellite genotypes of the trees sampled. Samples were identified as replicates from the same genet when they shared identical multilocus genotypes for six nuclear microsatellites (Wpms22 was excluded because of scoring difficulties, see below).

Assessment of hybridization events with cultivated poplars

Hybridization events of *P. nigra* females with *P. × canadensis* males were assessed based on the multilocus genotype of the half-sibs from the open-pollinated *P. nigra* trees. The maternal allele at each locus was subtracted from the genotype of each sampled seedling. This process of maternal exclusion revealed the multilocus haplotype of the paternal contribution. When at least one diagnostic allele of *P. deltoides* was found in the paternal haplotype of the multilocus genotype of the seedling, the seedling was considered to have a clone of *P. × canadensis* as father. The genotype of *P. nigra* 'Italica' was considered the likely father of a seedling if its multilocus genotype matched the paternal haplotype of the seedling for the four microsatellite loci.

Assessment of seed set potential

The branches bearing pistillate inflorescences were placed in tap water in glass jars in the greenhouse (about 20°C) directly after collection and forced to bring the stigmas to receptivity. At the time the pistillate inflorescences were fully expanded and the ovaries were swollen, we collected one to three entire unpollinated inflorescences per tree from the branches in the greenhouse. We separated the flowers (capsules) from the catkins and dissected 15 to 30 (mean: 16) ovaries from 8 and 9 individual trees of *P. × canadensis* and *P. nigra*, respectively. The ovary was cut lengthwise with a scalpel and the number of unfertilized ovules per flower was counted using a stereomicroscope. Furthermore, we collected 2 to 23 (mean: 14) fully elongated catkins from the branches to count the number of flowers per catkin. The potential for seed set per catkin was calculated per genet and defined as the mean number of flowers per catkin multiplied by the mean number of ovules per flower. The number of collected catkins and the number of ovaries dissected per genet is given in table 2. Two-sided t-tests were used to test for significant differences between *P. nigra* and *P. × canadensis* for: (i) the mean number of ovules per flower, (ii) the mean number of flowers per catkin and (iii) the mean seed set potential per catkin. All these t-tests were performed using S-PLUS® 6.2 (Insightful).

RESULTS

Genotypic diversity of *P. nigra*

The tri-nucleotide microsatellite locus Wpms22 showed ladders of bands of equal intensity which resulted in scoring difficulties. Including this locus resulted in inconsistency within samples assigned to the same genet for one allele (in a heterozygous state). It is not clear if this is because of technical (PCR-) artefacts, scoring errors or a somatic mutation. We therefore did not include the microsatellite locus Wpms22 to detect samples sharing the same multilocus genotype.

Twenty-six [out of 235 (11%)] adult trees were identified as hybrids. They were likely F₁-hybrids because they were heterozygous at both loci Pmgc14 and Wpms09 showing one allele diagnostic for *P. deltoides* and another specific for *P. nigra*. These 26 trees represented four different hybrid

Table 4 – Multilocus microsatellite genotypes and number of replicates per genet of *Populus nigra* along the river IJzer.
For each locus, the mean fragment length (in base pairs) of the alleles is given.

Genet Code	# Replicates	Pmgc14	Wpms05	Wpms09	Wpms14	Wpms16	Wpms19
A	58	205 / 208	282 / 286	250 / 264	253 / 278	149 / 149	215 / 240
B	151	205 / 208	290 / 294	256 / 264	247 / 265	149 / 149	210 / 245

genets. After eliminating these hybrid genotypes only 2 different multilocus microsatellite genotypes remained within the 209 black poplar trees sampled. All the black poplar trees sampled were females. The multilocus microsatellite genotypes and the number of replicates per genotype are given in table 4.

Hybridization events with cultivated poplars

At least one diagnostic allele of *P. deltoides* was detected in 58% (64 / 110) of the seedlings from the seeds collected on the open pollinated black poplar trees. The probability of correctly identifying hybrid progeny from natural backcrosses of *P. nigra* with *P. × canadensis* combining the three diagnostic microsatellite markers (assumed to be unlinked) was 87.5%. The number of seedlings showing diagnostic alleles of *P. deltoides* per progeny collected from the open-pollinated *P. nigra* trees is given in table 1.

P. nigra ‘Italica’ was identified as a possible father of one seedling (from the tree DI50C).

For every seedling, the attribution of the maternal allele was feasible at each locus; there were no indications for null alleles. As expected, the diagnostic markers Wpms09, Pmgc14 and Wpms18 were heterozygous in the nine reference F₁-hybrids and homozygous in the five *P. deltoides* reference clones. Wpms09 showed a *P. deltoides*-specific allele of 234 bp, Pmgc14 revealed a *P. deltoides*-specific allele of 193 bp and Wpms18 of 220 bp. For locus Wpms20, Fossati et al. (2003) reported significantly different allele frequencies between *P. nigra* and *P. deltoides*. However, in this study

we did not find the diagnostic alleles for Wpms20 described by Fossati et al. (2003).

Seed set potential

The potential for seed set per catkin was significantly higher for *P. × canadensis* than for *P. nigra* ($t = 3.7818$, $df = 10$, p -value = 0.0036). The mean maximum potential seeds per catkin was 619 (S.D.: 175) for *P. × canadensis* and 425 (S.D.: 95) for *P. nigra* (fig. 2). *P. × canadensis* produced a significantly higher mean number of ovules per flower (19.9, S.D.: 4.7) compared to *P. nigra* (10.9, S.D.: 2.5; $t = 19.6074$, $df = 277$, p -value = 0; fig. 2).

In contrast, *P. × canadensis* contained a slightly lower mean number of flowers per catkin (30.9, S.D.: 5.8) compared to *P. nigra* (38.4, S.D.: 13; $t = -3.876$, $df = 157$, p -value = 0.0002; fig. 2).

DISCUSSION

The majority (if not all) of the relict black poplar trees in the valley of the IJzer are female trees. In early spring, conspecific pollen are scarce or even completely absent, except for pollen from the widely planted male cultivar *P. nigra* ‘Italica’. Unexpectedly, viable seeds could be collected in the field from all the nine open-pollinated black poplar trees investigated. The majority of the grown seedlings (58%) showed at least one allele specific for *P. deltoides*. As clones of *P. deltoides* are not planted in Belgium, alleles of *P. deltoides* in *P. nigra* offspring could only originate from natural hybridization with *P. × canadensis* males. In addition, it is likely that offspring without species-specific allele of *P. deltoides* at the three diagnostic microsatellite loci also originate from a backcross of *P. nigra* to *P. × canadensis*, as to our knowledge, no autochthonous *P. nigra* males were present in the vicinity. The expected proportion of hybrid seedlings is therefore likely to be 100%. The probability of correctly identifying hybrid progeny from natural backcrosses of *P. nigra* to *P. × canadensis* combining the three diagnostic microsatellite markers (assumed to be unlinked) was 87.5%. Combining data from more diagnostic molecular markers, for example additional microsatellites or Single Nucleotide Polymorphisms, would increase the chance of detecting backcross hybridization events (e.g. Meirmans et al. 2010). However, despite the low resolution, the results of this study provide solid evidence for natural hybridization between female *P. nigra* and male *P. × canadensis*, a process that may affect the evolution of the species-complex (Arnold 1997). It must be noted that, due to the low resolution of our genetic data and to our incomplete knowledge on the geographic location of the numerous cultivated poplar genotypes planted

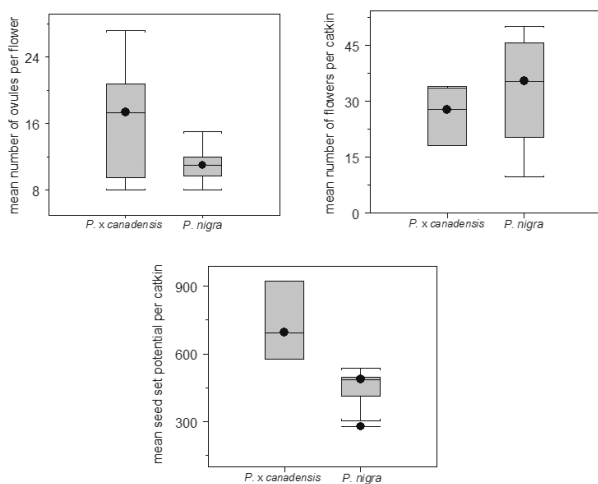


Figure 2 – Box plots of the mean number of ovules per flower, the mean number of flowers per catkin and the mean seed set potential per catkin for *Populus × canadensis* and *P. nigra*, respectively.

in the study area, a paternity analysis aiming to identify the location of individual siring trees was not possible.

Until now, evidence for natural hybridization between female *P. nigra* and male *P. × canadensis* was rarely found. Only one former study reports on evidence for introgression of genes of *P. deltoides* into the offspring of open pollinated *P. nigra* (Vanden Broeck et al. 2004). The latter study, also performed in Flanders, concerned only one single case and one single tree seed progeny of *P. nigra*. Also, the results of the latter contrast with other studies in which *P. × canadensis* growing in the vicinity failed to act as a father to any of the offspring of *P. nigra* females (Rajora 1986, Heinze 1997, Janssen 1998, Benetka et al. 1999, Fossati et al. 2003, Taberner & Cottrell 2003). Despite the rather small sample size, the findings of the present study provide further evidence for natural hybridization of solitary *P. nigra* females with hybrid *P. × canadensis*. The rather contrasting results between both studies performed in Flanders and similar other studies performed elsewhere in Europe investigating natural hybridization between female *P. nigra* and male *P. × canadensis*, may be explained by the local demographic mating environment. It should be noted that the situation of *P. nigra* along the river IJzer in West Flanders with extreme low densities of mainly or exclusively female trees of autochthonous *P. nigra* combined with a high abundance and proximity of *P. × canadensis*, is quite exceptional for Europe. The strong limitation of conspecific pollen results in *P. nigra* receiving mainly foreign, heterospecific pollen which may enhance the rate of hybridization. Vanden-Broeck et al. (2012) investigated the potential for hybridization between *P. × canadensis* and *P. nigra* by a series of pollination experiments in the greenhouse. Using pollen lots of *P. nigra* pollen mixed with pollen of *P. × canadensis*, *P. nigra* sired a significantly greater number of seeds on its own flowers than did *P. × canadensis*, even when higher proportion of pollen from *P. × canadensis* was present in the pollen mix (Vanden-Broeck et al. 2012). This suggests that the risk of hybridization between female *P. nigra* and male *P. × canadensis* may be reduced when conspecific *P. nigra* pollen are present. In the absence of *P. nigra* pollen, the risk for hybridization with male *P. × canadensis* may be higher (Vanden-Broeck et al. 2012).

Only one seedling (0.9%) may have been fathered by *P. nigra* 'Italica' as this male cultivar could not be excluded as a potential male parent for one seedling. Former studies report on natural hybridization levels between female autochthonous *P. nigra* with *P. nigra* 'Italica' (F_1 -hybrids) of 0% in the UK (Taberner & Cottrell 2003), 1.6% on the Dutch-Belgian border (Vanden Broeck et al. 2004) to 2.6% along the Loire River in France (Chenault et al. 2011). As *P. nigra* 'Italica' belongs to the species *P. nigra*, flowering synchrony, rather than genetic incompatibility, is likely an important factor in determining natural hybridization. In Belgium *P. nigra* 'Italica' generally starts flowering before autochthonous *P. nigra* although an overlap in flowering can occur (Vanden Broeck et al. 2003). Flowering synchrony might be more significant in Southern regions, which, at least partly, may explain the higher levels of hybridization observed in France along the Loire River by Chenault et al. (2011).

The extremely low genotypic diversity of *P. nigra* in the IJzer valley may increase the potential risk of genetic assimilation

through hybridization by the more numerous cultivated *P. × canadensis*. The fact that hybridization frequencies can be highly variable between populations and strongly depend on the local mating environment was also discussed by Field et al. (2011) in a study of mating patterns between eucalyptus species. Only two different multilocus genotypes or genets, both replicated dozens of times, were detected within the samples of *P. nigra* collected in the IJzer river valley. A study of the genetic diversity in nine gene bank collections of *P. nigra*, including the Belgian *P. nigra* gene bank, indicated that the combined data of five specific microsatellite loci (Pmgc14, Wpms09, Wpms14, Wpms16 and Wpms20), of which four are used in this study, revealed sufficient statistical power for the identification of duplicates of the same genet (Storme et al. 2004). Because we used even more microsatellite loci, we are confident that the microsatellite data used in this study were sufficient to reliably assign samples with the same genotype to the same genet.

The high number of genetic replicates is probably due to the human influence on the species. Nowadays, black poplars growing in the valley of the IJzer River generally grow in the neighbourhood of farms. It is very likely that, several decades ago, autochthonous poplar trees growing near the river have been propagated by farmers and planted near their farms as multipurpose trees (for fuel wood, fodder, timber, shading or as a windbreak). Poplars can be easily propagated through striking of cuttings. The collection of plant material from a few trees may have led unconsciously to multiple replicates of a few genets. Additionally, these might have been propagated and exchanged between neighbouring farmers resulting in the high number of duplicates of only two genets of *P. nigra* in the valley of the IJzer. A similar situation was reported for *P. nigra* in the region of the Usk River, on the border between England and Wales (UK) (Cottrell et al. 2002, Smulders et al. 2008b). Only two different genets were identified within 72 black poplar trees sampled (all of them males) along the Usk based on seven microsatellite markers. Likewise, the area around Aylesbury (northwest of London), which has a large number of black poplars, contains a very low number of genets (Cottrell et al. 2002). A high number of ramets from a single genet where also found along the Rhine in the Netherlands (Smulders et al. 2008b). In contrary, no duplicates of genets were found in samples collected from black poplar populations along more dynamic southern and eastern European river systems like the French river Drôme, the Spanish river Ebro, the Czech part of the river Labe and in Ukraine along the rivers Tysa and Prut (Smulders et al. 2008b). The results of this study support the findings of Smulders et al. (2008b) indicating that the genetic diversity of black poplar is related with the river system dynamics. In Flanders, the UK and some regions of the Netherlands, natural patterns of river flow and water table levels are altered. This limits the opportunities for sexual reproduction and explains the current lack of natural populations of black poplar in Belgium and in the UK (Cottrell et al. 2002). In these countries, man unconsciously prevented the extinction of black poplar by vegetative propagation. However, this has resulted in an extreme low genetic diversity, limiting the evolutionary potential of the species and increasing the susceptibility to diseases.

In addition to the ‘pollination threat’ discussed above, *P. × canadensis* may also outcompete *P. nigra* in terms of numbers of seeds produced. To our knowledge, this is the first study comparing the potential for seed set of *P. × canadensis* and *P. nigra*. Despite the small sample size, our results suggest that the potential for seed set per catkin is higher for *P. × canadensis* compared to *P. nigra*. In addition, the number of ovules per ovary was significantly higher for *P. × canadensis* compared to *P. nigra*. This indicates that the cultivated *P. × canadensis* may have a higher reproductive seed set potential per catkin compared to the native *P. nigra*. However, there are numerous other factors that influence reproductive potential like the number of catkins per tree, pollen production, pollen availability, pollen viability, pollen interaction, seed production and seed viability. Vanden-Broeck et al. (2012) investigated the impact of pre-zygotic mating barriers like pollen viability and heterospecific mixed pollen loads on the reproductive potential of *P. × canadensis* and *P. nigra*. They found that, in greenhouse experiments sterility of *P. × canadensis* can be overcome by the interaction with untreated pollen of the related *P. nigra*. Insight in the different aspects determining the reproduction potential of the cultivated poplars will help to explain the observations of this and former field studies.

There is currently some interest in Flanders in the restoration of river dynamics and floodplain ecosystems due to several recent floods that have caused high damage in densely populated regions. Riparian forests with *P. nigra* as a key species can help in flood control, for example by stabilizing stream banks and by reducing erosion. It is clear from this study that in the valley of the IJzer, hybridization between native and cultivated poplars occurs and that there is a lack of adult genetic resources to establish new populations of *P. nigra* on sites where river dynamics are restored. To reduce the risks for introgression and genetic swamping of *P. nigra* by *P. × canadensis*, we strongly advise reforestation with plant material including other genotypes of *P. nigra* from neighbouring regions taking into account balanced sex-ratios. Plant material for reforestation may originate for example from the Netherlands or from populations along the French rivers Oise and Seine. Forest reproductive material of known provenance is available on the market in the Netherlands and will soon be on the market from the French rivers Oise and Seine. Enlarging the genetic diversity will promote the survival of the population on the long term and will likely limit the potential for introgression of cultivated genes into the offspring of *P. nigra* along the IJzer river. Regular genetic monitoring is strongly advised to enable the evaluation of restoration actions and provide good opportunities to track hybridization events over time (Schwartz et al. 2007).

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