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Complex patterns of hybridization between exotic and native North American poplar species[1]

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- *Premise of the study:* Poplars and their hybrids are seen as important candidates for bioenergy initiatives. However, many concerns have been raised about large-scale plantations of new poplar cultivars. The deployment of such plants with novel traits brings the risk of potential spread of novel genome regions (including exotic genes, transgenes, or other heritable modifications) into natural populations of related species. The possibility of introgression is especially high in poplars because reproductive barriers between species are weak. Knowledge of the frequency of hybridization between cultivated trees and natural populations is one important step in the risk-assessment process.
- *Methods:* We studied the rate of spontaneous hybridization from two sexually mature poplar plantations into adjacent natural populations of *Populus deltoides* and *P. balsamifera*. The two plantations, both in eastern Canada, contain many different complex hybrid clones with components from exotic species, mostly *P. nigra*, *P. trichocarpa*, and *P. maximowiczii*. We analyzed 12 species-specific single nucleotide polymorphisms from six different genes in 5373 offspring sampled from the natural populations.
- *Results:* Contributions from all three exotics were found in the offspring, confirming low reproductive barriers among poplar species in these sections. The frequency of hybrid offspring varied among pollen donors, recipient populations, and years.
- *Conclusions:* The remarkably high rate of hybridization that was found in the smallest natural population sampled suggests that small peripheral populations carry a higher risk of introgression. These results could be used as a starting point for developing regulatory guidelines for the introduction of plants with novel traits.

Key words: exotic species; gene flow; introgression; *Populus balsamifera*; *P. deltoides*; single nucleotide polymorphism.

In light of the decline of the global forestry industry and growing energy requirements, some countries are showing an increasing interest in the cultivation of fast-growing trees. Poplars and their hybrids are seen as good candidates to play an important role in bioenergy initiatives because of the variety of strategies that can be used to increase their productivity and adaptability (Hinchee et al., 2009). These strategies include conventional breeding with exotic species, genetic engineering, and genomics-guided transgenics (Strauss, 2003). However, many concerns have been raised about large-scale plantations of new poplar cultivars because these trees are not domesticated and have a propensity for long-distance movement of pollen and seeds (Strauss et al., 2004). In Canada and many other countries, regulatory guidelines have therefore been created regarding the introduction of such plants with novel traits (which in Canadian regulation includes exotics as well as transgenics; Bonfils, 2006). One of the key issues concerning such introductions is the introgression of novel genome regions (including exotic genes, transgenes, or any type of heritable genomics-derived modification) into natural populations of wild species (Ellstrand 2003). Monitoring the rate of introgression between native and exotic species has therefore recently been the focus of a large research effort (e.g., Fossati et al., 2003; Vanden Broeck et al., 2004; Hooftman et al., 2007; Smulders et al., 2008; Ziegenhagen et al., 2008). However, the occurrence of introgression depends on many factors, including the interfertility of the species, the actual occurrence of hybridization, the fitness and fertility of the hybrids produced, and the degree of backcrossing.

The possibility of introgression is especially high among species of poplar (Salicaceae: *Populus*) because for centuries many poplar species have been planted around the northern hemisphere because of their ornamental and commercial value (Dickmann, 2001). Furthermore, several of the above-mentioned processes are known to take place in poplar. However, most available knowledge of these processes comes from crossing experiments for breeding purposes, and there is a lack of information regarding their occurrence in natural populations. For example, crosses have shown that the reproductive barriers can

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be weak between many combinations of poplar species (Eckenwalder, 1996), which makes artificial hybridization between native and exotic species relatively easy (though this depends on their relatedness). Furthermore, we know that at least some of the hybrids are able to survive to sexual maturity and successfully backcross with the parental species or with other poplar species (Thompson et al., 2010). In fact, hybrid poplars are widely used because they display many commercially desirable properties, such as fast growth, disease resistance, and tolerance to abiotic stress. However, despite their widespread use, there is very little information regarding the rate at which these hybrids cross with native species when there is open pollination. The rate of spontaneous hybridization in adjacent natural populations may well differ from expectations based solely on crossability, because of phenological differences or competition effects between the pollen from the native and the exotic species (Rajora, 1989; Tabbener and Cottrell, 2003; Vanden Broeck et al., 2003).

Several Europe-based studies have addressed the presence and extent of spontaneous hybridization from plantations containing exotics to neighboring populations of native species (Heinze, 1997; Fossati et al., 2003; Tabbener and Cottrell, 2003; Vanden Broeck et al., 2004; Pospíšková and Sálková, 2006). All of these studies have used genetic markers to assess whether offspring of native open-pollinated *Populus nigra* L. contained genetic material from plantations of *P.* × *canadensis* Moench (*P. deltoides* Marsh. × *P. nigra*). Most of these studies found at most a low rate of spontaneous hybridization of only a few percent, in line with the results of crossing experiments that have shown that backcrosses of *P.* × *canadensis* with *P. nigra* can be difficult (Zsuffa, 1974). The situation is likely to be more complex in North America than in Europe. Not only is a more diverse array of clones used in plantations, but there are also multiple native species that may hybridize with these clones. Many of these clones are of a complex nature, consisting of second- or third-generation hybrids of up to four different native and exotic species. Most of these clones are thought to be interfertile with at least one or two native poplar species of eastern North America, most importantly with *P. deltoides* and *P. balsamifera* L. However, given the large number of clones, experimental data on their intercompatibility is scarce. Furthermore, because of the long generation cycle of poplars, most experiments have focused on first- and second-generation hybrids (for an overview, see Vanden Broeck et al., 2005). Clearly, there is a need for data about the rate of spontaneous hybridization taking place between poplar species that are native to North America and complex hybrid clones that contain exotic components.

We measured the rate of spontaneous hybridization from two experimental plantations of sexually mature poplar clones that contain components of the exotics *P. trichocarpa* Torr. & Gray, *P. maximowiczii* Henry, and *P. nigra* into neighboring populations of two native species: *P. deltoides* and *P. balsamifera*. Thousands of seeds produced by trees from the natural populations were sampled over 3 yr and analyzed using an array of 12 species-specific SNPs located on several different gene loci. We found that the rate of hybridization differed markedly among pollen donors, years, and recipient populations.

MATERIALS AND METHODS

Sampling—Seed capsules were sampled from naturally occurring sexually mature female *P. deltoides* and *P. balsamifera* surrounding experimental plantations of sexually mature poplar clones containing exotic components in two different locations. At the time this study was conducted, these plantations were the only sexually mature ones that were available in eastern Canada. The first sampling site was located near Sorel in southern Quebec (Table 1) and consisted of a ruderal site that contains a large natural population of 15- to 30-year-old *P. deltoides* and a small population of 15-year-old *P. balsamifera* at the southern limit of the species' distribution. We sampled seeds from female trees of both species located on the northeastern side of the plantation, since the plantation was surrounded by agricultural fields on most of the other sides (Fig. 1). The region surrounding the plantation mainly consists of agricultural land, interspersed with 24% small wooded areas. Poplars are relatively scarce in this region, with the highest density located to the north of the plantation (Appendix S1; see Supplemental Data at http://www.amjbot.org/cgi/content/full/ajb.0900271/DC1). During the flowering period, the dominant wind direction shifts from northeast to southwest. In addition, seeds were sampled from planted female *P. deltoides* located inside the northwestern part of the plantation, in an area where only pure *P. deltoides* has been planted. The second site was located in a wildlife reserve near Matane in eastern Quebec (Table 1) and contained a natural population of ~50-year-old *P. balsamifera*, but no *P. deltoides* because the site is located a few hundred kilometers north of the species' natural range. In the Matane site, we sampled female trees from two plots that were located directly to the west and to the east of the main plantation, though the eastern plot was also in the vicinity of several smaller plantations (Fig. 1). The region surrounding the plantation is dominated by natural forests (94%) and plantations (Appendix S1). However, the plantations immediately adjacent to the sampling sites are the only poplar plantations in the region; all the others contain conifers. Natural poplars (*P. balsamifera*) are more common here, especially to the west and southwest of the sampling site, and the dominant wind direction during the flowering period is southwest.

The plantations at both sites were established by the Ministère des Ressources naturelles et de la Faune du Québec. In the Matane plantation, the first cohort of hybrid clones with exotic components was planted from 1969 to 1977, followed by a second cohort in 1985. In Sorel, the first cohort was planted in 1980, followed by a second cohort in 1991 and 1993. Plantations on both sites cover several hectares and contain hybrid clones, mostly involving the species *P. deltoides*, *P. balsamifera*, *P. nigra*, *P. trichocarpa*, and *P. maximowiczii* (Table 1). The plantations in Matane and Sorel contained 325 and 1563 different clones, respectively. Most clones were represented by more than a single individual, giving a total of 2852 planted trees in Matane and 7423 planted trees in Sorel. Because the great majority of clones were hybrids and many types of hybrids were present, Table 1 lists the genomic contributions of these five species only rather than distinct species and hybrid types. Male and female clones (poplars are dioecious) were present in approximately equal frequencies and had all reached sexual maturity by the first year of sampling (2003).

TABLE 1. Location and climatic parameters (Environment Canada, 2002) of the two sampling sites (natural populations) and species composition of the adjacent poplar plantations. Because the great majority of clones were of hybrid origin, the percentages given for each species represent the extent to which each species contributed to clones in that plantation.

Parameter/Species	Matane	Sorel
Latitude (N)	48°53′	45°80′
Longitude (W)	67°13′	73°10′
Mean annual temperature (°C)	3.1	5.9
Total annual precipitation (mm)	929	906
Number of days >0°C	257	282
Dominant wind direction (April–May)	NE	NE/SW
Wind speed (km/h; April–May)	17.5	12.4
Number of planted clones	325	1563
Number of planted trees	2852	7423
Species composition of clones (%)		
Populus balsamifera	3	6
P. deltoides	26	36
P. maximowiczii	2	20
P. nigra	27	28
P. trichocarpa	26	5
Other species	9	1
Unknown	7	3

Fig. 1. Maps of the two sampling sites in Sorel (top) and Matane (bottom). Dark lines delineate the borders of the poplar plantations. Filled circles indicate the locations of sampled female *P. balsamifera*, and white circles indicate the locations of sampled female *P. deltoides*. In both maps, north is at the top.

To detect year-to-year variation in the hybridization rate, the natural poplar stands on both sites were sampled in 2003, 2004, and 2005. The numbers of trees from which seeds were sampled depended on the available numbers of female trees with catkins and therefore differed between sites, years, and species (Table 2). In Matane in 2003, seeds were not directly collected from tree crowns but were taken from female catkins that were clustered on the forest floor directly under each female tree; care was taken to sample only catkins that were lying close to the trunk of each sampled tree and when there were no other female poplar trees flanking the sampled tree. In Matane in 2004, all collected seeds were found to be sterile, most likely because of a late frost that killed the embryos. In 2003 and 2005, seed collection was more successful on that site, with 23 and 9 females sampled, respectively. The spatial coordinates of sampled females were recorded, and leaves were collected also to determine their genotypes. The distance between the plantations and the sampled females ranged from 18 to 282 m in Matane (average: 124 m) and from 17 to 531 m in Sorel (average: 190 m).

DNA extraction—To get enough plant material for DNA extraction, the seeds collected in 2003 and 2004 were germinated in the greenhouse. The collected seed capsules were stored in a dryer until the seeds shed. The seeds were directly sowed onto plastic containers filled with mixed substrate and covered with a thin layer of sand. The containers were placed under a shade-cloth in a greenhouse at 22°C and were watered twice a day to maintain a moist surface. After 3 weeks, the shade-cloth was removed, the nighttime greenhouse temperature was reduced to 18°C, and 20/20/20 fertilizer was applied once a week at 1 g/L. A few days after the germination of a seed, the cotyledons were removed and lyophilized for subsequent DNA extraction. Later, we discovered

TABLE 2. The number of females sampled in each natural population of *Populus balsamifera* and *P. deltoides* and the average number of offspring per female with successful SNPstream genotypes included in the analysis.

		2003		2004		2005		Total	
Site	Species	No. of females sampled	No. of offspring per female	No. of females sampled	No. of offspring per female	No. of females sampled	No. of offspring per female	No. of females sampled	No. of offspring per female
Matane	*P. balsamifera*	23	22.4	–	–	9	38.9	26	27.1
Sorel	*P. balsamifera*	2	37.0	4	87.5	2	55.0	4	66.8
	P. deltoides	22	13.0	32	68.3	21	43.0	36	45.0

that sufficient quantities of DNA could be recovered by performing the DNA extractions directly on the tiny poplar seeds using a different procedure (see below). This allowed us to genotype ungerminated seeds and, thus, to test whether there were any differences in germination frequency between hybrid seeds and nonhybrid seeds. Therefore, for the seeds in the collection year 2004, we extracted DNA both from cotyledons and from ungerminated seeds. In 2005, DNA was obtained directly from the seeds.

Genomic DNA was extracted with the DNeasy 96 Plant kit (Qiagen, Mississauga, Canada) for samples of 2003 and with the MagAttract 96 DNA Plant Core Kit (Qiagen) for samples collected in 2004 and 2005. Between 1 and 121 offspring were analyzed per collection per female, with an average of 41.6 offspring.

Genotyping—It is widely appreciated that species-specific markers represent one the best tools for detecting hybrid offspring (Holderegger et al., 2005; Hamzeh et al., 2007; Ziegenhagen et al., 2008), because they allow for unambiguous parental assessments of first-generation hybrids and easy scoring and analysis. If available, species-specific single nucleotide polymorphisms (SNPs) are preferable to other markers, such as amplified fragment length polymorphisms or microsatellites, because their use in genotyping arrays allows for affordable and fast genotyping of large numbers of offspring. We therefore used the species-specific SNPs developed by Meirmans et al. (2007) to identify the contribution of each of the five target species to the sampled offspring. An SNPstream (Bell et al., 2002) array with 12 species-specific SNP loci (the standard number for an SNPstream, which was the most affordable SNP genotyping system at the time), located on six different genes, was used to genotype the sampled females and progeny (Appendix S2). Three of these loci distinguished *P. deltoides* from the four other species, three distinguished *P. maximowiczii*, three distinguished *P. nigra*, one distinguished *P. trichocarpa*, and two distinguished *P. trichocarpa* and *P. balsamifera* together from the three other species. The SNP loci were determined as species specific by sequencing the genes for 25–60 individuals per species (Meirmans et al., 2007). The SNP markers were further assessed on hundreds of individual *P. balsamifera*, *P. trichocarpa*, and *P. deltoides* (S. Thompson et al., unpublished data). Although these sample sizes do not give any absolute guarantee that the markers are indeed fully species specific, they should be suitable for our purposes. The usefulness of these markers for detecting hybrid poplars was assessed using a large number of hybrid trees of known denomination (Meirmans et al., 2007). Furthermore, the markers have already been used successfully to detect hybrids in natural populations by Thompson et al. (2010), whose results were verified using a Bayesian admixture analysis.

Data analysis—Normally, the rate of hybridization is calculated by simply taking the number of hybrid offspring found and dividing this by the total number of offspring. This approach, though simple and intuitive, has multiple problems when some of the fathers themselves are hybrids and only a limited number of markers is used. In such cases, because of the stochasticity of Mendelian inheritance, not all real hybrid offspring may be recognized as such, leading to an underestimation of the hybridization rate. Another problem occurs when the offspring are backcrosses, when the father is a hybrid that shares part of its genome with the mother species. In that case, the hybridization rate is not representative of the rate of gene flow into the mother species but is instead an overestimate. For example, when a female *P. balsamifera* is pollinated by a male *P. balsamifera* × *P. deltoides*, the frequency of hybrids among the offspring will be 100%. However, 50% of the paternal alleles will originate from *P. balsamifera* and, therefore, not constitute any introgression. Therefore, a better estimate for the rate of gene flow between the species in this case is 50%.

To circumvent these problems, we used a different method to estimate the rate of gene flow between species (though we also present the results of the "classical" method of calculating the frequency of hybrids). This method has the advantage that it is also more suitable for comparing rates among donor species. This per-species rate of gene flow equals the Mendelian probability that a single biallelic locus from the paternal contribution to the offspring contains an allele specific to that species. This rate can be estimated by calculating per species the average frequency of the alleles specific for that species in the genotyped offspring:

$$H_s = 2 \cdot \frac{\sum_{l}^{L_s} p_l}{L_s}$$

Here, L_s is the number of loci carrying alleles that are diagnostic for the species s, and p_l is the observed frequency of the diagnostic allele at locus l. Because we consider only the paternal contribution to the offspring and do not take the maternal alleles into account, this statistic would have a maximum of 0.5. Therefore, the estimates were doubled to range from 0 to 1 instead of 0 to 0.5. The idea behind this estimate is that when the father of a seed is a complex artificial hybrid, there is the potential for hybridization from its multiple constituent species, though for each species at a lower rate than if the father were a pure individual from a species. For example, when a female *P. balsamifera* is pollinated by a male *P. deltoides* × *P. nigra*, the average male contribution to the offspring will be 0.25 for each of the genomes of *P. deltoides* and *P. nigra*. The estimated per-species hybridization rate in that case will be 0.5 for both *P. deltoides* and *P. nigra*.

A standard chi-square test was used to test for an association between the per-species gene flow rates and the expected values based on the species composition of the plantation (Table 1). We used permutations to test for significant year-to-year variation. For this, we calculated the difference in the average frequency of hybrids between the years, weighted for the number of offspring sampled per female. We then randomized females over years and recalculated the difference for the randomized data set, with 9999 permutations. In addition, we tested whether there was any difference in germination between hybrid seeds and nonhybrid seeds in the 2004 sample from Sorel. For this, we calculated the difference in the weighted average frequency of hybrids in the seeds and in the seedlings. The significance was tested by randomizing per female the two weighted frequencies and then recalculating the overall difference, with 9999 permutations. All permutation tests were performed using custom scripts in R, version 2.6.1 (R Development Core Team, Vienna, Austria).

RESULTS

Genotyping success—Of the 64476 SNP-genotype calls obtained for 5373 individuals, only 6800 calls failed, giving a success rate of 89.5%. Out of those 5373 individuals, there were 580 for which SNP-genotype calls were unsuccessful at more than two loci. In line with earlier work using this SNPstream assay (Meirmans et al., 2007), we removed these individuals from the data set. We also removed 19 individuals (0.4%) for which the resulting genotypes were, for unknown reasons, incompatible with those of their apparent mothers, indicating that the rate of technical errors was low. In total, 4774 offspring were used for the estimates of the rate of spontaneous hybridization, stemming from the 66 female trees sampled (Table 2). The

two methods of DNA isolation that we used had an effect on the success rate of the genotyping when compared for the 2004 sample from Sorel, for which both methods were used: the success rate for DNA extracted from cotyledons was 99%, compared with 95% for DNA extracted directly from tiny seeds.

Observed rates of spontaneous hybridization—The observed frequency of hybrid offspring varied strongly among the three samples (Table 3A). A relatively low frequency was found for the *P. deltoides* population from Sorel; averaged over the 3 years, only 3% of the offspring contained nonconspecific alleles. The frequency of hybrids in the *P. balsamifera* population from Matane was intermediate, at 20%, averaged over the 2 yr for which seeds were available. A much higher percentage of hybrid offspring was found among the seeds produced by the small *P. balsamifera* population from Sorel; averaged over the 3 yr, 72% of the offspring were found to contain alleles that were nonspecific to *P. balsamifera*. However, these observed percentages of hybrid offspring do not necessarily mean that there was an equally high rate of gene flow into these populations. Because many of the fathers were hybrids themselves (see below), a part of the offspring resulted from backcrosses, lowering the actual rate of gene flow. This effect can be observed in Table 3B, which contains estimates of the rate of gene flow (H_s) taking into account that some of the offspring are backcrosses. The estimates of the rate of gene flow from Table 3B are, on average, about one-third lower than the estimates of the frequency of hybrids in Table 3A. This means that for the *P. balsamifera* population from Matane, the overall rate of gene flow is 9%. In Sorel, the rate of gene flow for the *P. deltoides* population is 2% and the 72% of hybrid offspring for *P. balsamifera* represents a rate of gene flow of 49%.

The differences among years were less pronounced than the differences among the three populations. Because of the different DNA-extraction methods used, the peripheral nature of *P. balsamifera* in Sorel, and the lack of seeds from 2004 in Matane, we could perform only two tests for differences between years in spontaneous hybridization rate, both for the *P. deltoides* population in Sorel. The difference in hybridization rate between the years 2003 and 2004 (DNA extracted from cotyledons) was strongly significant ($P = 0.007$; 9999 permutations). The difference in hybridization rate between the years 2004 and 2005 (DNA extracted from seeds) was nonsignificant ($P = 0.50$; 9999 permutations).

TABLE 3. (A) The percentage of hybrids found in the offspring in three natural population of *Populus* spp. (B) The resulting rates of gene flow from nonconspecifics, taking into account that some of the offspring are backcrosses. Values are combined for all sampled female trees per population and per year. The values within brackets give the standard error, calculated over females weighted by the number of offspring.

	Matane	Sorel	
A. Year	*P. balsamifera*	*P. balsamifera*	*P. deltoides*
2003	18.2 (3.3)	49.3 (26.1)	9.1 (2.5)
2004	–	76.6 (5.8)	2.3 (0.3)
2005	21.6 (3.9)	74.5 (2.1)	2.2 (0.6)
Overall	19.6 (2.5)	72.4 (4.9)	2.9 (0.5)
	Matane	Sorel	
B. Year	*P. balsamifera*	*P. balsamifera*	*P. deltoides*
2003	11.7 (3.1)	41.4 (30.1)	7.3 (2.0)
2004	–	50.5 (2.6)	1.8 (0.3)
2005	4.8 (1.7)	49.1 (5.6)	1.5 (0.5)
Overall	8.9 (2.1)	49.0 (2.5)	2.2 (0.4)

Per-species rates of gene flow—Because the genotypes of all female trees sampled were known, it was possible to infer the alleles that were contributed by the fathers. We used this information to calculate a per-species rate of gene flow by estimating each species' average contribution to the paternal alleles of the offspring. This approach has the advantage that the rates can be directly compared among species, rather than among the many different male types. Table 4 shows that the per-species rate of gene flow differed both among pollen-recipient species and among pollen-donor species. There were also marked differences between populations, whereas the differences among years were relatively slight (Table 4). In the natural *P. balsamifera* population in Matane, the largest interspecific contributions were from *P. deltoides* (5.4% over all years) and *P. trichocarpa* (3.2%), whereas the contributions of *P. nigra* (0.3%) and *P. maximowiczii* (0.1%) were negligible. For the natural *P. deltoides* population in Sorel, the differences between native and exotic species were not compelling, the values for *P. balsamifera* (0.8% over all years) and *P. trichocarpa* (0.9%) being in the same order of magnitude as the values for *P. nigra* (0.3%) and *P. maximowiczii* (0.2%). The small peripheral *P. balsamifera* population in Sorel stood out not only because of its high rate of spontaneous hybridization but also because of the large contribution of exotic species. The highest per-species contribution of nonconspecific alleles was from *P. maximowiczii* (22.2% over all years), with approximately equal contributions from *P. trichocarpa* (10.0%), *P. nigra* (7.8%), and the native species *P. deltoides* (9.0%). Comparison of the overall per-species gene-flow rates (Table 4) with the species composition of the plantations (Table 1) revealed strongly significant differences (Matane, *P. balsamifera*, $\chi^2 = 33.2$, df = 3, $P < 0.001$; Sorel, *P. balsamifera*, $\chi^2 = 108.0$, df = 3, $P < 0.001$; Sorel, *P. deltoides*, $\chi^2 = 87.0$, df = 3, $P < 0.001$). This means that some species were much more frequent in the hybrid offspring than in the plantation (most notably *P. maximowiczii* and *P. trichocarpa*), whereas others were much more frequent in the plantation than in the hybrid offspring (most notably *P. nigra*).

Inference of father types—When the data were combined over the 12 SNP loci, 210 different genotypes were observed among the offspring of both sites. On the basis of these genotypes, we determined, by hand, for every individual offspring a putative father species or hybrid (Table 5). Of course, the stochastic nature of Mendelian inheritance means that such an inference is necessarily rough when the number of loci is limited. Combined over the two recipient species, a total of 23 different father types were found (Table 5). Of these, 18 different father types were hybrids, of which three were trihybrids. However, it is important to note that because of the limited number of markers, the frequency of hybrid fathers represents an underestimate, given that some of the offspring that have genotypes that resemble an F1 may in fact be later-generation hybrids. This also holds for the distinction between simple and more complex hybrids. For example, it would have been difficult to observe any tetrahybrid fathers, even though some were present in the plantation. Overall, among all the hybrid offspring that we found, at least 81% had a father that was a hybrid itself, meaning that at most 19% of the observed hybrid offspring were first-generation hybrids.

TABLE 4. Spontaneous gene-flow rates per species: the average percentage of alleles specific to each of the five *Populus* species, found among the paternal alleles of the offspring in the three natural populations.

		Matane	Sorel	
Year	Species	*P. balsamifera*	*P. balsamifera*	*P. deltoides*
2003	*P. balsamifera*	82.3	57.1	3.1
	P. deltoides	7.2	14.9	91.4
	P. maximowiczii	0.0	5.0	0.3
	P. nigra	0.1	2.7	0.7
	P. trichocarpa	4.3	18.9	3.1
2004	*P. balsamifera*	–	38.2	0.8
	P. deltoides	–	6.2	97.8
	P. maximowiczii	–	27.7	0.3
	P. nigra	–	9.1	0.3
	P. trichocarpa	–	7.5	0.5
2005	*P. balsamifera*	87.8	42.2	0.3
	P. deltoides	2.7	13.9	98.8
	P. maximowiczii	0.2	16.4	0.0
	P. nigra	0.5	7.0	0.1
	P. trichocarpa	1.4	11.8	1.1
Overall	*P. balsamifera*	84.5	41.7	0.8
	P. deltoides	5.4	9.0	97.5
	P. maximowiczii	0.1	22.2	0.2
	P. nigra	0.3	7.8	0.3
	P. trichocarpa	3.2	10.0	0.9

Given that the two native species are known to spontaneously hybridize in nature (Thompson et al., 2010), there is the possibility that some of the hybrid offspring stem from the natural populations and not from the plantations. By considering only fathers with exotic components, we can be sure that these represent gene flow from the plantations. In Matane, only 34% of the observed hybrid offspring had fathers with exotic components and 66% had fathers that were F1-or-higher combinations of only *P. balsamifera* and *P. deltoides*, the two species native to eastern Canada (though the sampling site lies outside the range of *P. deltoides*). In Sorel, the site where both species occur naturally, only 30% of the hybrid offspring were native, and 70% had fathers with exotic components. Including only fathers with exotic components gives estimates of the minimum frequency of hybrids originating from the plantation of 3.6% (31 offspring) for *P. balsamifera* in Matane, 53.6% (288 offspring) for *P. balsamifera* in Sorel, and 1.5% (49 offspring) for *P. deltoides* in Sorel. Overall, 368 offspring were found with alleles from exotic species, giving a minimum frequency of hybridization with fathers from the plantation of 7.7%. When it is assumed that hybrids between the native species also originate from the plantation, this gives a maximum frequency of 13.6%. To produce conservative estimates, hybrids with unknown components, such as the putative "Bx" fathers (Table 5), were counted as native species. These fathers may themselves be the results of backcrosses from unknown hybrids into *P. balsamifera*. Most likely, those unknown hybrids are *P. balsamifera* × *P. deltoides*, as this type of hybrid is known to preferentially backcross to *P. balsamifera* (Thompson et al., 2010). They are less likely to be attributable to artifacts in that they are not restricted to a specific mother, population, year, locus, or reaction plate.

TABLE 5. The putative father types inferred from the genotyped offspring, combined over all years (B = *Populus balsamifera*, D = *P. deltoides*, M = *P. maximowiczii*, N = *P. nigra*, and T = *P. trichocarpa*; an "x" indicates that a father was inferred not be of a pure species, but not all components could be identified).

	Matane	Sorel	
Putative father	*P. balsamifera*	*P. balsamifera*	*P. deltoides*
B	698	147	7
BD	54	36	13
BDM	–	–	2
BDN	–	–	1
BM	2	154	1
BN	1	9	–
Bx	79	51	–
D	6	15	3277
DM	–	4	4
DMT	–	1	–
DN	1	1	5
DT	17	23	16
Dx	–	–	22
M	–	27	2
MN	–	3	–
MT	–	16	2
Mx	–	4	2
N	–	32	4
NT	1		–
Nx	–	2	–
T	9	10	10
Tx	–	2	–
x	–	–	1

The genotypes of the sampled mothers were always in complete agreement with their species designations based on morphology, and no alleles were found that were diagnostic for any other species. Though there was some variation among female trees in the frequency of hybrid offspring, there were no clear patterns: the great majority of females had some hybrid offspring in at least one of the years. Some females produced a high number of hybrids in one year but none in another, whereas other females produced approximately the same proportion of hybrids over multiple years (Appendix S3). Our analysis of multiple paternity showed that a large fraction of the sampled females were pollinated by multiple males. We analyzed this by manually inferring the minimum number of male types needed to produce the hybrids produced by each female. It should be noted that because the markers are species specific, we can distinguish the ancestry of the males only with respect to their hybrid conformation, and not the individual males within a given type of hybrid. For example, this means that we could distinguish the offspring of a male *P. deltoides* × *P. nigra* from that of a male *P. balsamifera* × *P. maximowiczii* but could not distinguish the offspring of two male *P. deltoides* × *P. nigra*. Of the 104 female–year combinations with at least five offspring genotyped, 31 showed the presence of only nonhybrid conspecific offspring, 53 had both nonhybrid offspring and hybrid offspring of a single type, and 20 had both nonhybrid offspring and hybrid offspring of more than one type. Therefore, we could infer that the great majority of female trees were pollinated by multiple different males and that many females were pollinated by males with at least three different genomic compositions.

When DNA extracted from cotyledons of germinated seeds was used for genotyping, a higher fraction of hybrids was found than when DNA extracted from seeds was used. We could perform this test only for the 2004 sample of the *P. deltoides* population in Sorel, because this was the only population with enough females for which we had both seeds and cotyledons. The weighted average percentage of hybrids in the females was 3.5 for the cotyledons and only 1.2 for the seeds. Note that this number is different from the overall fraction of 9% hybrids

(Table 3) because only a subset of females was available for which we had both seeds and cotyledons. The difference between the fraction of hybrids in the seeds and in the cotyledons was found to be significant in the permutation test ($P = 0.022$; 9999 permutations).

DISCUSSION

One of the first steps of the process of introgression of novel genome regions (including exotic genes, transgenes, or other heritable genomics-derived modifications) into natural populations of native species consists of hybridization between the native and cultivated species. Here, we studied the offspring of two poplar species at two sites where the natural populations were adjacent to plantations containing hybrid clones with exotic components. We found that the frequency of hybrid offspring in the two species ranged from 2% to 72%. The observed patterns of interspecific hybridization were remarkably complex; not only did we find evidence of hybridization involving all five included species, we also found evidence that the siring fathers had a wide range of genomic compositions, including pure species, first-generation hybrids, backcrosses, and trihybrids. The large range in interspecific hybridization rates that we observed was due to the fact that there were large differences between the two recipient species and between sites: *P. balsamifera* displayed a higher hybridization rate than *P. deltoides*, and *P. balsamifera* exhibited a much higher rate at the southern Sorel site than at the more northern Matane site. The northern site of Matane was outside the natural range of *P. deltoides*, so a direct comparison between the two native species was not possible for this site. Nevertheless, the observed hybridization rate of *P. balsamifera* in Matane was one order of magnitude higher than that of *P. deltoides* in the southern site of Sorel.

The unbiased, allele-based breakdown of hybridization (the per-species rate of gene flow) showed distinct differences in the rate of hybridization among pollen donors. Comparison of these rates with the species composition of the plantations revealed that there was no clear-cut correspondence between them. Most of the nonconspecific contributions found in *P. deltoides* offspring came from the North American species, *P. balsamifera* and *P. trichocarpa*, whereas very little hybridization was seen from the Eurasian species *P. nigra* and *P. maximowiczii*. The per-species rates of hybridization with *P. balsamifera* varied between populations and years. In the northern population in Matane, most nonconspecific contributions were from North American species, with a much smaller contribution from the Eurasian species. On the other hand, in the small *P. balsamifera* population in Sorel, there were substantial contributions from both native and exotic species, though there were strong differences between years. This suggests that for *P. balsamifera*, populations in the periphery of its natural range may be more affected by the hybridization than more central populations.

We found a remarkably high rate of gene flow in the *P. balsamifera* population in Sorel. Though multiple explanations are possible, we think that it is most likely that the high rate of gene flow is due to the very small size of the local population. The overall population density of *P. balsamifera* was very low in the local agroforestry landscape dominated by fields and maple stands. Next to the four sexually mature females we sampled, we detected only three sexually mature *P. balsamifera* males in the surroundings of the sampling site. This low population density may therefore lead to a lack of suitable partners, which may in turn decrease the competition that nonconspecific pollen experiences from *P. balsamifera* pollen. Such a relationship between population size and hybridization rate has been observed repeatedly, for example in the tree genera *Eucalyptus* (Field et al., 2008) and *Nothofagus* (Marchelli and Gallo, 2001). An extreme form was studied by Vanden Broeck et al. (2004), who analyzed the offspring from a single isolated female of *P. nigra* and found that it consisted completely of hybrids. Small populations are therefore at a higher risk of extinction by hybridization, a phenomenon that is known as "demographic swamping" (Levin et al., 1996).

Introgression vs. hybridization—We found clear differences in hybridization rate between the two studied recipient species. For *P. deltoides*, most detected hybrids involved contributions from the native *P. balsamifera*. For *P. balsamifera*, most hybrids involved contributions from the native *P. deltoides* or *P. trichocarpa*, a native of western North America that is phylogenetically closely related to *P. balsamifera*. *Populus deltoides* and *P. balsamifera* are known to spontaneously hybridize when growing together in their zone of contact (Hamzeh et al., 2007; Thompson et al., 2010), yet they are still identifiable species that are easily distinguishable using genetic markers (Meirmans et al., 2007; Thompson et al., 2010) or morphology (Eckenwalder, 1996). It is therefore likely that for the population from Sorel, where the two species co-occur, a part of the observed hybrids were fathered by trees from the natural population. However, only 30% of the hybrid offspring in this population consisted of a combination of the two native species, which means that 70% of the hybrid offspring had exotic components.

It is possible that there is strong selection against the hybrids, which prevents introgression from occurring. Hybridization will only lead to realized introgression if the produced hybrids are able to establish themselves long enough to reach sexual maturity and backcross with the parental species, processes about which we have only limited data for poplars. Given the number of different clones used in populiculture and the complexity of their genetic make-up, gathering such data is difficult and costly. However, the better performance of certain hybrid clones has often been observed in poplars and is one of the main reasons for their widespread use. An experimental study showed that, for most traits analyzed, F1 hybrids of *P. fremontii* and *P. angustifolia* outperformed the least-fit parent species, and, for one trait (asexual reproduction), performed two to four times better than the most fit parent (Schweitzer et al., 2002). Therefore, there are good reasons to believe that at least some introgression may be occurring. One of the most limiting steps in the process of introgression is backcrossing the F1 hybrids to the parental species, a step that is often more difficult than subsequent backcrosses or the first-generation hybridization itself (Rieseberg and Carney, 1998; Arnold et al., 1999). In our study system, this difficult step is bypassed because the great majority of the clones in the plantations we studied are not pure species but hybrids, some of which are in fact already backcrosses with *P. deltoides* or *P. balsamifera*. The presence of F1 and BC1 hybrids growing under controlled conditions in the plantations alleviates the selection acting against these hybrids that would otherwise result from competition with the parental species in their parental species' native habitat. BC2 offspring are less likely to suffer from such competition than F1 or BC1 because the former share a greater part of their genome with the native species. We indeed found that a minimum of 81% of the hybrid

offspring had fathers that were hybrids themselves. As a result, some of the hybrid offspring we found actually consisted of second-generation backcrosses (BC2).

From our germination experiments, it is clear that the hybrid seeds produced in these sites are viable. In fact, we found a significantly higher proportion of hybrids after seed germination, compared with direct analysis of the seeds. One explanation for this result is heterosis, a higher fitness of the hybrid offspring, which has been shown to occur in many plant species (Rieseberg and Carney, 1998). Unfortunately, we did not measure the germination rate for the sampled mothers, so we cannot compare this to the frequency of hybrids in the offspring. Another explanation for the higher proportion of hybrids after germination may be that SNPstream analysis performed on DNA extracted from empty seeds returned the parental genotype. On the other hand, the lower genotyping success for DNA obtained from seeds suggests that empty seeds likely lead to failed SNPstream reactions.

Pollen source—Even though the main goal of our study was to determine the effect of pollen flow from plantations into natural populations, we cannot be fully assured that the fathers of all observed hybrid offspring actually came from the adjacent plantations. There is the possibility that the natural populations to which the sampled female trees belong retain a low level of variation at the loci that we assumed to be diagnostic. Such a low level of variation may, for example, result from historical introgression or incomplete lineage sorting. In our study, it is likely that historical introgression between *P. deltoides* and *P. balsamifera* has influenced the results. We therefore also presented a minimum estimate of gene flow from the plantation, for which we included only offspring that could unambiguously be assigned to the plantation on the basis of the presence of markers specific for any of the exotic species. This showed that over all years and populations, at least 7.7% of the offspring were derived from the plantation.

However, we think that the influence of historical introgression would mainly be present at the site in Sorel and would be very minimal in Matane. In Sorel, both native species are present, and, given that they are known to spontaneously form hybrids (Hamzeh et al., 2007; Thompson et al., 2010), it is likely that we picked up hybrids that were pollinated by fathers from other natural populations rather than from the plantations. Therefore, the influence of the plantations on the natural populations may be smaller than it would seem on the basis of the rates displayed in Table 3. In Matane, the situation is different in that only *P. balsamifera* is present and the site is hundreds of kilometers removed from the hybrid zone with *P. deltoides*. Even if some introgressed alleles may have spread over such a distance (e.g., as a result of selection; Martinsen et al., 2001), it is unlikely that this happened at all three SNP loci we used to detect *P. deltoides*. Moreover, when we genotyped the 26 female trees sampled at this site, we found no alleles diagnostic for *P. deltoides*, which means that if there is residual variation, it is present at frequencies that are much lower than the frequency that was observed in the offspring. Given the presence of a large, sexually mature plantation next to the *P. balsamifera* population in Matane, we think that gene flow from the plantation is a more parsimonious explanation than gene flow or introgression from natural populations over hundreds of kilometers.

Comparison with other studies—This study is one of the first to estimate spontaneous hybridization in poplars in North America. Most similar studies have focused on the case of *P. deltoides* and *P. nigra* in Europe (Heinze, 1997; Fossati et al., 2003; Tabbener and Cottrell, 2003; Vanden Broeck et al., 2004; Pospíšková and Sálková, 2006; Smulders et al., 2008; Ziegenhagen et al., 2008). Their hybrid *P.* × *canadensis* has been widely introduced for commercial purposes, and this has triggered concern that the rare Eurasian native *P. nigra* may be negatively affected by backcrosses with *P.* × *canadensis*. The results are mixed, with some studies finding no evidence of hybridization and others finding remarkably high levels of hybrids. Fossati et al. (2003) studied the offspring of a large population of *P. nigra* that was close to a plantation of *P.* × *canadensis* and found no offspring that contained markers specific to *P. deltoides*. Likewise, Tabbener and Cottrell (2003) found no hybrids in offspring of *P. nigra* growing in a park intermingled with *P. deltoides* and *P.* × *canadensis*. By contrast, Ziegenhagen et al. (2008) found that among young poplars along a riverbank in Germany, almost 20% were hybrids. Smulders et al. (2008) found that among young trees sampled along the Rhine, almost 50% were hybrids. These results show that, as in our case, the observed hybridization rates can differ dramatically among populations. The hybridization rates we found between *P. deltoides* and *P. nigra* were generally low. More hybridization was seen from *P. maximowiczii* into *P. balsamifera*, and it likely reflects the fact that they are phylogenetically closely related (Eckenwalder, 1996). We found the highest levels of spontaneous hybridization and backcrossing between the native species *P. deltoides* and *P. balsamifera*, whether these were considered as male donor or female recipient, echoing reports on natural hybridization in their zone of contact (Hamzeh et al., 2007; Thompson et al., 2010).

Our results stand out in comparison with those of the other studies on natural hybridization in poplars (Fossati et al., 2003; Tabbener and Cottrell, 2003) in that we found a relatively high rate of spontaneous hybridization in certain situations. Both previous studies sampled only a single native species in a single year, whereas we investigated two native species with samples taken in multiple years. Furthermore, our study is the first to analyze hybridization from multiple poplar species simultaneously. Given that many poplar clones used in North America are hybrids, such a multispecies approach is strictly necessary. However, our study lacks a replication of populations because *P. deltoides* was present at only one of the two sites and the population of *P. balsamifera* in Sorel was too small to allow any meaningful comparisons with the population in Matane. Therefore, even larger studies are needed to get a view of how the rates of spontaneous interspecific hybridization differ among populations. A fully comprehensive research project should ideally also include natural populations more remote from the plantations and different levels of forest landscape fragmentation.

Genotyping strategy—The success rate of 89.5% of the SNPstream (Bell et al., 2002) genotyping method compares favorably with that of other high-throughput studies using Sequenom (e.g., 84.4%; Thompson et al., 2010) or Illumina (e.g., 78.5%–82%; Pavy et al., 2008). The SNPstream assay with 12 SNPs representative of six genes that we used permitted us to distinguish the five different poplar species (Meirmans et al., 2007) with sample sizes that are hard to obtain with other markers, such as microsatellites. The different species were represented by different numbers of markers on the assay. This should not bias the allele-based estimates of the per-species

hybridization rate, though it affects the accuracy of the estimates. Therefore, we believe that such an allele-based approach should be used for any study that estimates the rate of spontaneous hybridization from complex hybrids.

The number of available markers was the lowest for *P. trichocarpa*, for which only a single species-specific marker was included. Therefore, we expect that the error in the estimates of gene flow from *P. trichocarpa* will be higher than in the estimates for the other species. Estimating gene flow from *P. trichocarpa* is further complicated by its close relationship to *P. balsamifera*, with which it shares a lot of its genetic variation (Meirmans et al., 2007). These factors make inferences of pure *P. trichocarpa* fathers especially suspect in that these could be attributable to earlier introgression of the marker from *P. trichocarpa* into the local *P. balsamifera* population or to the retention of an ancestral polymorphism at the marker locus. However, inferences of fathers that are hybrids between *P. trichocarpa* and another species (such as the 18 offspring with putative *P. trichocarpa* × *P. maximowiczii* fathers) are not affected by these processes.

CONCLUSIONS

Populus species and their hybrids are seen as good candidates for short-rotation intensive plantations to increase productivity from less land and for applications in biofuels (e.g., Hinchee et al., 2009). This includes new varieties of poplars derived from conventional breeding with exotic species, from genetic engineering, or from genomics-guided transgenics. However, the large-scale deployment of these new poplar cultivars across the landscape raises concerns about the consequences of gene flow of novel gene regions into wild populations. Governmental regulations prohibit the direct study of gene flow from research trials of such plants with novel traits (Bonfils, 2006). Although not perfect, we think that studying gene flow from existing plantations of exotic poplar can help address some of the concerns. Accordingly, estimation of effective gene-flow levels from cultivated exotic poplars toward native species represents the first step in the assessment of ecological risks linked to the introduction of trees with novel traits in Canada.

We found a complex pattern of spontaneous hybridization from exotics in poplar plantations into adjacent natural populations of North American poplars. Overall, our study confirms earlier evidence that reproductive barriers are weak among the studied poplar species (Eckenwalder, 1996). Because we did not directly study the establishment and fitness of the hybrid offspring, we cannot make conclusive remarks about the fate of the hybrids produced. Nevertheless, for reasons outlined above, we think that there is a good possibility that the ongoing spontaneous hybridization will result in introgression. These processes will have the strongest effect on the *P. balsamifera* populations in the disturbed agroforestry landscape of southern Quebec, where the species is rare. Our results on *P. balsamifera* indicate that small peripheral populations are more likely to be subject to genetic swamping (Levin et al., 1996), either through gene flow from plantations or from naturally occurring *P. deltoides* in the agroforestry landscape. However, because *P. balsamifera* has a distribution that spans from eastern to western Canada, the effect of these processes on the whole species may be limited.

Our results can therefore be used as a first approximation for the rate of spontaneous hybridization between exotic and native poplars in eastern Canada. These rates, together with other factors such as the selection acting on the genes and population structure, are important factors in determining the actual rate of introgression into wild populations (e.g., Meirmans et al., 2009). In particular, the observed differences in the rate of spontaneous hybridization between central and peripheral populations have important implications for modeling introgression and for the development of regulatory guidelines for the commercial release of plants with novel traits, and for other cases where the rate of gene flow from plantations into natural populations should be kept to a minimum (Forest Stewardship Council certification; see http://www.fsccanada.org/).

Research on the strategies and risks of introducing plants with novel traits into natural populations is still in its infancy (e.g., DiFazio et al., 2004; Meirmans et al., 2009). Trees are much longer lived and have much longer generation times than annual crops, which makes research a more lengthy and difficult process. Given the high and idiosyncratic rates of gene flow we found between the poplar plantations and the natural populations, tools for mitigating gene flow should be developed if the introduction of the novel trait is deemed to pose a risk. Reproductively sterile genotypes might alleviate these concerns and facilitate regulatory approval for long-term field studies (Strauss et al., 2004). Plants with novel traits, which include both exotics and genetically engineered plants in Canadian regulation, could make an important contribution to tree breeding, to conservation of natural forests, and to conservation of endangered tree species (Hoenicka and Fladung, 2006). However, social acceptance of forest trees with novel traits will be highly dependent on our capacity to show that environmental issues (especially long-term effects) have been properly addressed.

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