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ESTIMATING GENETIC FLOW BETWEEN EXOTIC AND NATIVE POPLAR SPECIES IN QUÉBEC

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RÉSUMÉ

Notre étude a consisté à détecter et à quantifier, le cas échéant, la présence de flux génique entre des peupliers à composantes exotiques et leurs cousines indigènes. Nous avons récolté sur deux sites différents (Matane et Sorel) et sur deux à trois années consécutives, des graines provenant d'arbres-mères des espèces indigènes, *Populus balsamifera* et *P. deltoides*, situés autour de plantations de peupliers à composantes exotiques. Nous avons génotypé les graines à l'aide de marqueurs génétiques spécifiques de ces espèces. Ceci nous a permis de détecter la présence d'allèles provenant des arbres mâles à composantes exotiques de la plantation au sein des graines récoltées sur les arbres-mères indigènes présents autour de ces plantations. Nos résultats ont ainsi révélé la présence d'hybridation entre les arbres indigènes et les arbres à composantes exotiques des plantations. Cette hybridation spontanée pourrait affecter à plus long terme la constitution génétique des populations locales des espèces indigènes.

ABSTRACT

The goal of this study was to detect and quantify, when necessary, the presence of gene flow between poplars with exotic components and their native relatives. Seeds were collected from native mother trees, *Populus balsamifera* and *P. deltoides*, located in the surroundings of plantations of poplars with exotic components, in two different sites (Matane and Sorel) and during two to three consecutive years. We genotyped these seeds using genetic markers specific for these species. This method allowed us to detect, in the seeds collected from native mother trees present around these plantations, the presence of alleles brought about from male trees with exotic components located within the plantations. Our results revealed the presence of hybridization between native trees and trees with exotic components from the plantations. This spontaneous hybridization could affect in the long term the genetic constitution of local populations of native species.

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CHAPTER 1

INTRODUCTION

Poplars grow naturally in many parts of the world. They grow fast, are easy to propagate and provide an excellent source of different wood products (Eckenwalder 1996). These characteristics have made poplars an excellent candidate for short-rotation plantations. In different parts of the world populiculture has seen a major increase in the past decades. In an effort to provide the forest industry with resources that will increase wood production, poplar breeders have set up improvement programs to select superior and well adapted genotypes. Clones that are resistant to common diseases or drought will allow a gain in wood production without the need to increase the area of land used for plantation or the harvesting of natural forest (DiFazio *et al*. 1999).

The integration of biotechnology into tree improvement programs can accelerate the introduction of desired traits in existing elite clones. However, the development and use of genetically modified trees is presently restricted due to a lack of information regarding their potential environmental impacts. One of the biggest concerns is the possibility of genetic contamination of natural populations by gene flow from transgenic trees. Pollen is capable of traveling long distances and hybrids between natural and genetically modified trees could be created, with potential short- and long-term impacts.

Because plantations of mature genetically modified trees are prohibited in Canada, an alternative approach is used to estimate the potential gene flow between genetically modified trees and native species. This approach consists of quantifying gene flow between plantations of hybrids with exotic components and native species in the natural forest. In this study, we use poplar as a model to study gene flow between native and exotic species in Québec. The high genetic variability among poplar species and their ability to produce viable interspecific hybrids are among others, desirable characteristics that make poplar the ideal candidate for this work.

In the following introduction, I discuss the various aspects of poplar biology and the attributes that have helped establish poplar as a model for tree molecular biology. I then go on to review hybridization among poplar species and the risk it poses to the conservation

of their genetic variation. Finally, I describe different aspects of populiculture, making an emphasis on the Canadian situation.

1.1 Poplar biology

1.1.1 Taxonomy / Phylogeny

The genus *Populus* L. belongs to the family Salicaceae (order Malpighiales), and includes cottonwoods, poplars and aspens, all of which are sometimes termed poplars. They are widely distributed in the northern hemisphere and can be found from subtropical to boreal forests (Braatne *et al.* 1992). Although the actual number of poplar species is low, their distinctive morphology and ecology allows classifying them into 6 sections: *Abaso*, *Turanga*, *Leucoides*, *Aigeiros*, *Tacamahaca* and *Populus* (Cagelli and Lefèvre 1995; Eckenwalder 1977; 1996). There is a large consensus in the literature on the characteristics and species composition of the sections, and the major barriers to hybridization in the genus have been observed to mostly lie between sections (Zsuffa 1975). Nevertheless, some species have created controversy. One example is *P. mexicana* which was initially placed in the *Aigeiros* section but several years later a new section, *Abaso*, has been created to contain it (Eckenwalder 1996). Another example of questioned sectional affiliation is the relationship between section *Aigeiros* (cottonwoods) and *Tacamahaca* (balsam poplars), since these two sections are the only ones known to intercross freely (Zsuffa 1975; Eckenwalder 1984). Cottonwoods and balsam poplars present no differences in the morphology of flowers and inflorescences so they can be accommodated in a single section. Nevertheless, present evidence, including phylogenetic analyses, seems to favor keeping them apart (Eckenwalder 1996). Another difficulty involves the placement of *P. nigra* that, although belonging to section *Aigeiros*, is very similar to some species of the balsam poplars, section *Tacamahaca.* Moreover, in a phylogenetic analysis based on chloroplast DNA sequences, *P. nigra* actually fell in a clade containing only species from section *Populus* (Hamzeh and Dayanadan 2004). Ultimately, the North American cottonwoods may need to be removed from section *Aigeiros* into their own section (Eckenwalder 1996), but more research is needed to resolve this and other affiliation dilemmas.

Although there is a major agreement among taxonomists about the sectional classification, the number of existing poplar species is largely debated. Biologists from China and Russia are more inclined to emphasize variation and acknowledge any entity that can be recognized in the field or herbaria. Whether this process is the cause or the effect of the high poplar diversity that is observed in these regions is open to debate. On the other hand, North American and Western European taxonomists are more conservative and are willing to accept a broader range of variation within species (Eckenwalder 1996). Discrepancies in the number of species may in a large part be attributable to such differences in the interpretation of species boundaries, but also to the misinterpretation of some hybrids.

The above difficulties in the classification of poplar species can largely be attributed to the ambiguities that are introduced by using only morphological characters. The development of biochemical and molecular markers has provided biologists with important tools to help reach a conclusion. Hamzeh and Dayanadan (2004) used sequences of chloroplast and nuclear DNA to reconstruct a phylogeny of the genus *Populus* (Fig 2.). The authors concluded that although *Populus* consists of well-marked sections, at least *Tacamahaca* and *Aigeiros* are not monophyletic in origin and the lineage comprising species of section *Populus* is distinct from *Tacamahaca* and *Aigeiros*. In addition, the phylogenetic trees obtained from the analysis of nuclear and chloroplast DNA data were not fully congruent suggesting a reticulate evolution of the genus *Populus*.

Table 1-1 Species of the sections within the genus *Populus* with their generalized distribution (Eckenwalder 1996; Taylor 2002)

Section	Leaf characteristics	Major species	Distribution
Abaso		*P. mexicana* Wesmael	North America
Turanga	Lanceolate-linear	*P. euphratica* Olivier	E. Eurasia + Nothern Africa
		P. pruinosa Schrenk	E. Eurasia
		P. ilicifolia (Engler) Rouleau	E. Africa
Leucoides	Very large, cordate, underside violet when young	*P. heterophylla*	Eastern USA
		P. lasiocarpa Oliver	Central and Western China
		P. wilsonii Schneider	
		P. violascens	China
Aigeiros	Large, deltoid, cordate	***P. deltoides*** Marshall	**Eastern North America**
		P. fremontii S. Watson	Western USA
Black poplars and cottonwoods		***P. nigra***	**Europe to central Asia**
		P. sargentii Dode	North America (east of Rocky Mountains from Saskatchewan to New Mexico; west to Texas)
		P. wisilizeni Dode	USA (west Texas, New Mexico)
Tacamahaca	Small-large ovate-lanceolate.	*P. acuminate*	USA (Montana and South Dakota to New Mexico and Arizona)
Balsam poplars	Underside silvery-brownish.	*P. angustfiolia* James	Western USA (east of Rocky Mountains)
		P. balsamifera	**North-eastern USA**
	Without translucent margins	*P. cathayana* Rehder	North-western Chile to Manchuria and Korea
		P. ciliate Royle	Himalayas
		P. koreana Rehder	Korea
		P. laurifolia Ledebour	Siberia
		P. maximowiczii A. Henry	**North-eastern Asia-Japan**
		P. purdomii	North-western China
		P. simonii Carrière	North and west central China
		P. suaveolens Fischer	East Turkey, Siberia, Far East
		P. szechuanica Schneider	Western China
		P. trichocarpa T. & G.	**Alaska and British Columbia to California Rocky Mountains plains (Idaho to Montana)**
		P. tristis	Central Asia
		P. yunnanensis Dode	South-west China
Populus	Large three lobed or palmate leaves.	*P. davidiana* (Dode) Schneider	Northeast Asia
		P. alba	North and Central Europe
White and grey poplars and aspens	Dense white hairs.	*P. tremula*	Europe, Western Asia, North Africa
		P. tremuloides Michaux	North America

Note: Names in bold characters represent the species used in this study.

The extensive interspecific hybridization and the high levels of morphological variation among poplars have presented great difficulties in species delimitation for systematic and comparative evolutionary studies (Hamzeh and Dayanadan 2004). Depending on which taxonomic classification is considered, the genus contains between 22 and 85 species, although it is reasonable to assume approximately 29 species (Eckenwalder 1996), with some broad agreement that clear morphological characteristics are present. Among these 29 species of *Populus*, only 5 are indigenous to Canada: *P. trichocarpa* (black cottonwood), *P. balsamifera* (balsam poplar), *P. angustifolia* (narrowleaf cottonwood), *P. deltoides* (eastern cottonwood and plains cottonwood varieties) and *P. tremuloides* (trembling aspen) (Farrar 1995).

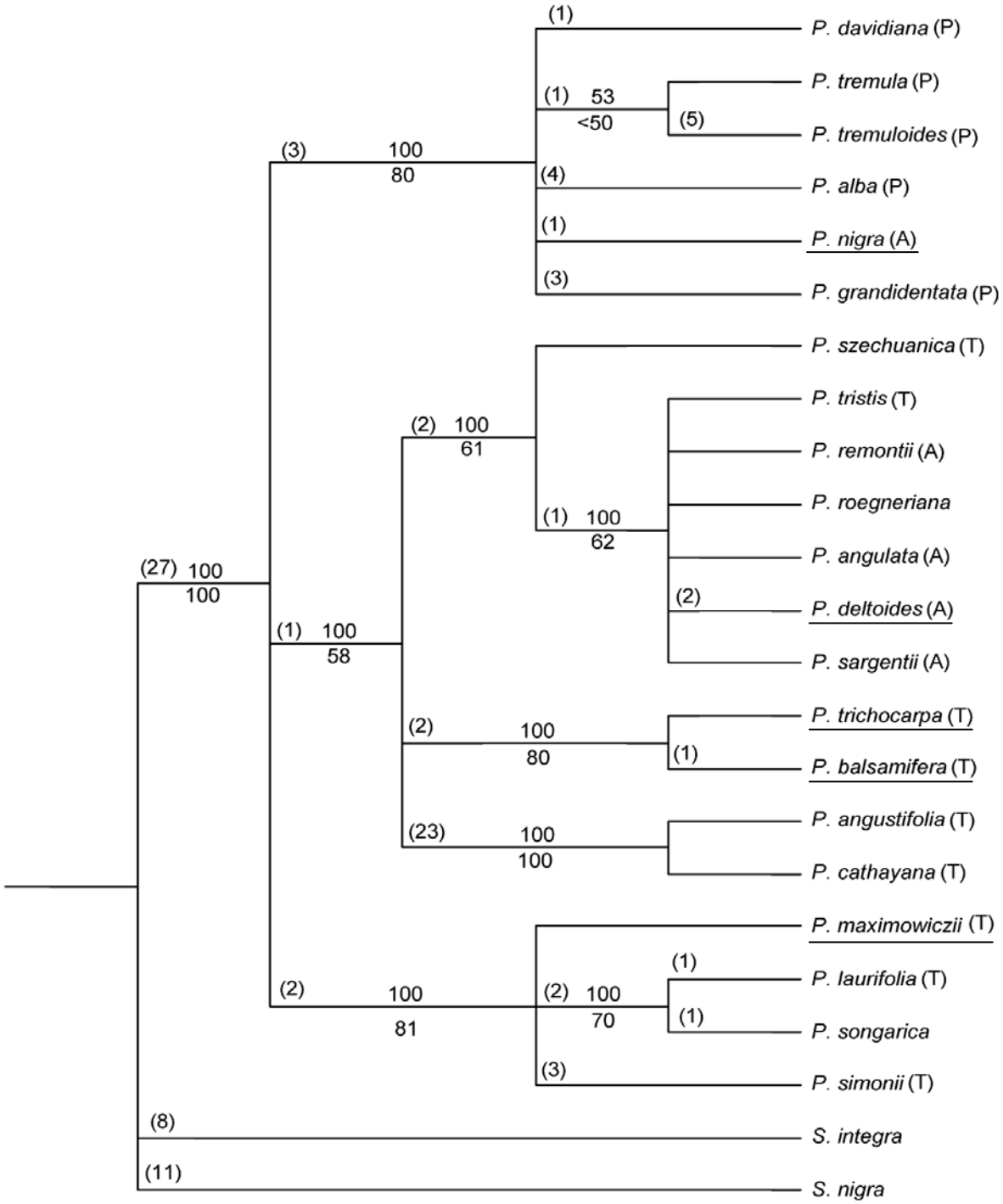


Figure 1-1 Majority rule consensus tree based on three noncoding regions of trnT-trnF of cpDNA sequences from *Populus* species. Numbers above branches show the frequency of occurrence in 50% majority rule consensus tree. Numbers below branches indicate boostrap percentage values. Numbers in brackets show the number of nucleotide substitution (Hamzeh and Dayanadan 2004).

Note: Names underlined represent the species used in this study. A, *Aigeiros*; P, *Populus*; T, *Tacamahaca*.

1.1.2 Morphology

Species of the genus *Populus* are all single-trunked, deciduous trees that are able to reach large sizes (Farmer 1996). Their fast growth is tied ecologically to their role as pioneer species as well as functionally to their heterophyllous growth habit. All poplars have resinous buds and alternate leaves with long stalks; in many species, these stalks are flattened laterally. In autumn, the leaves turn bright gold to yellow before falling. Poplar shoots continue to grow after bud burst by initiating, expanding and maturing leaves throughout the growing season (so-called neoformed or late leaves) (Critchfield 1960). In many poplar species, photoperiod induces the cessation of growth and bud formation (Pauley and Perry 1954). In a recent study, Böhlenius *et al.* (2006) used *P. tremula*, *P. trichocarpa* and *P. tremuloides* to demonstrate how the *CO*/*FT* (*Constans* and *Flowering locus T*) regulatory gene controls the variation in the critical daylength that induces growth cessation and bud formation in trees.

Poplars are dioecious, meaning that they bear the two sexes on separate trees. Both male and female flowers are placed on pendulous catkins that open before leaf emergence. In most places there is at least partial separation of flowering times in co-occurring species of poplar. Details of flower and inflorescence morphology of both males and females are used to define the sections of the genus (Eckenwalder 1996).

1.1.3 Poplar reproduction

Poplars are able to reproduce both sexually and asexually. Asexual reproduction takes place by means of root-borne sucker shoots, a trait uncommon among trees and is an effective mechanism for maintaining populations during long periods of relatively stable environmental conditions. Both asexual and sexual reproductions play a role in the capacity of populations to adapt to changing environmental conditions and to occupy new suitable habitat (Eriksson 1992; 1993). On one hand, asexual reproduction is crucial to re-

establish a population after major disturbances such as forest fires. On the other hand sexual reproduction and seed dispersal is necessary to maintain genetic variability.

1.1.3.1 Sexual reproduction

The age of reproductive maturity varies among poplar species and species from section *Aigeiros* typically reach sexual maturity earlier than species from section *Tacamahaca* (Reichenbacher 1984; DeBell 1990; Cooper and van Haverbeke 1990). In contrast with other tree genera most poplars reach maturity very early, at 5-10 years of age. This characteristic is useful in breeding as it allows a rapid progression into advanced generations (Stettler and Bradshaw 1996). In most poplar species, males initiate flowering before females but there is significant variation in the timing and duration of flowering (Braatne *et al*. 1996). Significant variation in flowering period has also been reported within populations (Dunlap 1991; Farmer 1993).

Early studies of gene flow in forest trees focused on the physical dispersal potential of pollen and seeds. The first measurements were taken as early as 1919 in *Pinus sylvestris* (Lindgren *et al.* 1995). Later studies confirmed long-distance movements for both pollen and seeds (Levin and Kerster 1974) and provided essential insights in the potential rate of gene flow. However, the effective rate of gene flow not only depends on the actual dispersal distances, but also fertilization and establishment success. Factors such as pollen competition, phenological synchrony, and seed predation can result in levels of gene flow far below those predicted by propagule movement alone (Adams *et al*. 1992). Nonetheless, it has become clear that pollen and, to a lesser extent, light seeds have an astonishing mobility (Di-Giovanni *et al*. 1996).

In poplar, pollen is wind-dispersed and seeds are formed and dispersed within 3-6 weeks after fertilization of the ovule. Females produce a very large number of small seeds that are surrounded at the base by long silky hair which aid dispersal by wind and water. In most

riparian species of poplars (e.g. *P. balsamifera*, *P. trichocarpa*, *P. angustifolia*, *P. deltoides*) seed dispersal combines an initial wind-mediated phase, i.e. transport of seeds from the maternal plant to the ground or water, with a secondary hydrochorous phase (Barat-Segretain 1996). Seeds and pollen are thought to be able to disperse over several kilometres (Ridley 1930; Nilsson *et al*. 1991) and gene flow is therefore supposed to be continuous, giving rise to large population sizes (Imbert and Lefèvre 2003). The seeds mature at a time where there is still high soil moisture content (from spring floods in riparian species), which is needed for immediate germination and establishment (Woolward 1907; Faust 1936). In nature, seed viability is a limiting factor in the life cycle of poplars because it generally lasts only 1-2 weeks (Cooper 1990; DeBell 1990; Zasada and Phipps 1990). Once a seed is wet, viability is generally lost within 2-3 days if it does not find a favourable microsite (Braatne *et al*. 1996).

1.1.3.2 Asexual reproduction (vegetative reproduction)

Asexual reproduction leads to clonal structure, in which one clone (genet) may consist of several trees (ramets). Members of the same genet are identical in their genotype and often also in their phenotype.

Poplars are very easy to propagate vegetatively. Clonal selection and propagation of commercial cultivars allow reaching economic goals in a short period of time. The power of clonal selection has been demonstrated by the impressive number of clonal cultivars that have been produced, as well as by the large number of ramets that exist of some of these clones (e.g. Walker). The latest but still incomplete version of the Catalogue of Poplar Cultivars (Viart 1992) includes 280 cultivars that are or have been cultivated throughout the world, mostly represented by clones (Bissoffi and Gullberg 1996).

1.1.3.3 Clonal identification

For the purpose of clonal selection, a proper identification of clones is essential. In the past this was accomplished mainly using morphological characters such as stem form, branch type, spring and autumn foliage (Barnes 1969). An often used list of characters to distinguish the commercially most important clones contains no less than 64 morphological traits. Such a long list makes the identification of clones difficult and unreliable since the list includes traits from several life stages and observable traits are often affected by year to year variation (Cheliak and Pitel 1984; Rogstadt *et al*. 1991).

Genetic markers provide a more reliable method for the identification of clones than morphological characters. Allozymes (Rajora and Dancik 1992; Culot *et al*. 1995) and RAPDs (random amplified polymorphic DNAs, Castiglione *et al*. 1993) were the first to be used for the identification of *Populus* clones. However, both allozymes and RAPDs have some substantial drawbacks. Allozyme loci are often not variable enough for clone identification (Lin *et al*. 1994), while RAPDs are dominant markers, and a homocygote and a heterozygote with the same allele cannot be distinguished. These problems can be avoided by the use of more reliable and variable markers such as microsatellites. These markers have proven to be very successful for clone identification in poplar (Rahman and Rajora 2002, Suvanto and Latva-Karjanmaa 2005). Microsatellites (also known as SSR, short sequence repeats) are 1 to 6 bp-long repeat sequences of DNA. They are supposedly selectively neutral, very variable and are randomly distributed throughout the genome. Aside from clone identification, they have proven to be useful for a variety of population-based analyses and for parentage analyses (Lexer *et al*. 2000; Khasa *et al*. 2003).

For clone identification, the resolution is determined by the number of polymorphic loci used, and by the allelic diversity observed at these loci (Parker *et al*. 1998). The more marker loci that are screened the higher the accuracy to identify a clone. Therefore, microsatellites are the markers of choice for population genetic studies and clone identification (Suvanto and Latva-Karjanmaa 2005). Many studies have corroborated the convenience of microsatellites to genotype poplar clones. One example is a study by

Suvanto and Latva-Karjanmaa (2005) where the number of clones that were identified in *P. tremula* with microsatellites was larger than those identified based only on morphological characters.

1.1.4 Pests and diseases

Populus species and interspecific hybrids are increasingly being grown for fuel and fibre in many regions of the world. Susceptibility to damaging diseases is second only to weed competition in limiting the successful establishment and production of high yields in these plantings (Newcombe 1996). The most important pests are two genera of fungi, *Melampsora* and *Septoria*, which greatly affect poplar plantations worldwide.

Leaf rust, caused by species of *Melampsora*, is the greatest threat to poplar plantations in North America (Ostry 2000). The primary hosts of *Melampsora medusae* are poplars, especially *P. balsamifera*, *P. deltoides*, *P. nigra* var. *italica*, *P. tremuloides* and their hybrids and cultivars. *M. medusae* is indigenous to North America and has spread from there to other continents (Walker 1975) because of its high potential for natural spread over long distances by wind (Nagarajan & Singh 1990). Even as far as Australia and New Zealand, where *Populus* has been introduced into a new environment, there is a lot of damage caused by *M. medusae*. Young poplar trees are most susceptible to severe damage by *M. medusae* due to premature leaf drop and loss of vigour. This is why also in western Canada, where the rust is native, extensive damage has been reported to poplars in nurseries and plantations as well as in natural forests.

Septoria spp. is capable of causing a leafspot disease that can result in defoliation. Some species (e.g. *Septoria musiva*) are specially damaging for poplars, due to their ability to form cankers that cause stem breakage (Ostry 2000). *Populus deltoides*, the Eastern cottonwood, is known to be resistant to stem canker (Ostry and McNabb 1985), even though *S. musiva* does cause leaf spots on *P. deltoides*. However, this is seldomly associated with serious damage. In contrast, hybrids of *P. deltoides* with species from

section *Tacamahaca* are typically susceptible to stem canker. In particular, *P. trichocarpa* × *P. deltoides* F_1 hybrids have proven susceptible in many locations in eastern North America (Ostry and McNabb 1985). In 2001, Newcombe and Ostry (2001) proved that the reason for this susceptibility is the recessive character of resistance in *P. deltoides*. The susceptibility of *P. trichocarpa* itself has also been demonstrated in various trials, although in regions such as the Pacific Northwest stem canker of genetically susceptible hybrid poplar and *P. trichocarpa* does not occur, for reasons that are still not understood (Newcombe *et al.* 2001).

Resistance to *Melampsora* and *Septoria* differs greatly among different *Populus* species and their hybrids (Ostry and McNabb 1986). Resistance to these pathogens is a trait that is often selected for in breeding programs. Also, understanding the genetic interactions between hosts and pathogens is useful to improve disease resistance (Han *et al.* 2000). Genes crucial in the defence against pathogens may be clustered together on chromosomes. A study published by Yin *et al.* (2004), attempted to elucidate the molecular mechanisms that make *Populus trichocarpa* resistant to *Melampsora*. The authors mapped two resistance loci, *MXC3* and *MER*, concluding that the *MER* gene was located in such a cluster, making it difficult to identify the resistance gene from among the closely linked candidates. On the other hand, the *MXC3* gene appeared to be outside that cluster, facilitating further functional characterization.

1.2 Population genetics

1.2.1 Gene flow and genetic diversity

Gene flow is the exchange of genes from one population to another. Its effect is capable of counteracting mutation, drift and selection, and has a tendency to homogenize population structure (Slatkin 1987). The rate of migration between populations is inferred from the calculations of the fixation index F_{ST} (or its many analogs, e.g., G_{ST}) which measure the genetic differentiation among populations (Wright 1931; 1965). At the beginning gene flow was measured indirectly studying patterns of genetic diferenciation to deduct long-term levels of gene flow (Neigel 1997; Sork *et al*. 1998). Today the use of DNA markers has became more affordable and parents and progeny can be genotyped allowing gene flow to be measured directly, without the need to make assumptions about the population structure. Even pollen and seed dispersal can be measured directly using paternally or maternally (depending on the species) inherited cytoplasmic markers (Takahata and Slatkin 1984; Ennos 1994; McCauley 1995; Latta *et al*. 1998).

Introgression is the infiltration of the genes of one species into the gene pool of another species through repeated backcrossing of an interspecific hybrid with one of its parents (parental species) (Mayr 1997). The fact that individuals from different species hybridize does not mean that introgression occurs, because all the progeny may be sterile. As mentioned previously, although poplars display hybridization barriers between species, some poplar species hybridize quite easily, even crossing among sections. The sexual reproduction of poplars through a wind-pollination system and the readiness with which they can be propagated asexually facilitates gene flow among species, by perpetuating hybrid plants and prolonging their role as agents of introgression (Stettler *et al.* 1996). The major concern regarding introgression in poplar is the possible reduction of genetic variability, more specifically, the reduction of genetic diversity as a result of the genetic introgression from a limited number of cultivated clones. A related concern is the spontaneous production of recombinant types in natural genetic backgrounds, which might

alter ecological competence and invasiveness (Mullon and Bertrand 1998; Martinsen *et al.* 2001).

1.2.1.1 Intraspecific diversity

Because of the high rates of outcrossing and their continuous geographic distributions, poplars typically display high levels of genetic diversity within populations at molecular loci (Stevens *et al.* 1999; Yeh *et al.* 1995). For example, studies performed on *P. nigra* stands in Europe showed high levels of microsatellite diversity within populations and low but significant differentiation among populations (Imbert and Lefèvre 2003). A low but significant differentiation was found even between nearby populations (F_{ST} ~ 0.043). Nevertheless, the major part of the genetic diversity was found within populations, consistent with the results from studies on other tree species (Adams *et al.* 1992; Hamrick *et al*. 1992).

In North America, a microsatellite study on three wild populations of *P. trichocarpa* in Oregon estimated the average number of alleles per locus to 20 and the observed heterozygosity to 77% (Brunner *et al*. 2004). This study showed that poplars display high level of genetic diversity within populations. Also, four populations of *Populus tremuloides* in Québec showed a high degree of genetic diversity when genotyped at 4 microsatellite loci (Wyman *et al*. 2003). Another example is illustrated by the results obtained for stands of *P. fremontii* and *P. angustifolia* that both displayed a high level of variation within populations (Keim *et al*. 1989).

1.2.1.1.1 Clonal vs. reproductive propagation

Conservation of genetic diversity is deemed important since genetic diversity forms the basis for species to adapt to changing environments. The ability to adapt is particularly important in trees, because they cannot escape adverse environmental conditions. Various

natural disturbances, like fires and insect outbreaks, as well as silvicultural practices (harvesting of plantations and natural stands) all are likely to have an effect on the genetic diversity of *Populus*. These perturbations might affect the biodiversity in different ways, perhaps causing a great deal of variation among ecosystem types, harvesting practices, and scale of disturbance. Following a disturbance, regeneration of trees such as poplar, can take place through clonal propagation or by seed. A study conducted by Wyman *et al*. (2003), analysed the genetic diversity in four populations of *Populus tremuloides* in Québec. Having in mind that following a disturbance (fire, insect outbreak, and cutting) most regeneration is clonal, they used microsatellites to estimate clonal and genetic diversity among and within populations. They concluded that, after stand disturbance, many genotypes will regenerate and different clones will be closely mixed. Finally, they found that there was a high degree of genetic diversity but a small degree of population subdivision. Also, they found that clones that had been identified previously as identical genotypes, based on morphology or on geographical distance, were in fact different genotypes. Another study showed that after a fire, the spatial distribution of aspen trees and their genetic structure evolved from a structured pattern to a more random one (Namroud *et al.* 2005). The studies of Cheliak (1982) and Cheliak *et al.* (1982) also show little population differentiation in aspen.

1.2.1.2 Natural hybridization

Some species from different sections can produce viable seed from natural and artificial crosses (Cagelli and Lefèvre 1995; Rajora 1986; 1989). Poplars from section *Populus* display strong reproductive isolation from the other sections. On the other hand, sections *Tacamahaca*, *Aigeiros*, and *Leucoides* interbreed quite freely among each other. Hybrids are regularly found wherever species from sections *Aigeiros* and *Tacamahaca* are sympatric. One example of this intersectional hybridization is presented by the study of Keim *et al*. (1989). They used RFLPs (restriction fragment length polymorphism) to distinguish genotypes of two poplar species: *P. fremontii* (section *Aigeiros*) and *P. angustifolia* (section *Tacamahaca*). They found that hybrid trees occurred in a zone where

both species overlap. Individual trees were either F_1 hybrids or backcrosses with *P. angustifolia*. No crosses between F_1 hybrids or backcrosses between F_1 hybrid trees and *P. fremontii* were found. The study also showed that interspecific genetic diversity was much greater than intraspecific genetic diversity.

Although intersectional hybridization is often apparently restricted to the F_1 generation (Eckenwalder 1984), examples of introgression are also known (Keim *et al.* 1989). There are examples of intersectional hybridization in poplars in the fossil record. Records dating from the Tertiary were found throughout North America (Eckenwalder 1984). This could indicate that hybrid zones may have played a role in the evolution of poplars (Smith and Sytsma 1990).

Hybrid zones are locations where the hybrid offspring of two divergent populations are prevalent and form a cline. They occur at the area of contact between two closely related but genetically different populations, each regarded as parental form (Barton & Hewitt 1985). In a study by Martinsen *et al*. (2001), the authors demonstrated that there are different selection pressures for different genomic regions. Two species of cottonwoods were used, *Populus fremontii* and *P. angustifolia* (from sections *Aigeiros* and *Tacamahaca,* respectively). They used 35 genetically mapped RFLP markers, diagnostic for the two species and their hybrids, for genotyping. Their results show that hybrid populations can act as evolutionary filters, preventing the introgression of most genes but allowing others to introgress throughout the range of the recipient species (Martinsen *et al*. 2001). In general, genes that confer the organism with a benefit will be the ones allowed to introgress. These results are consistent with the hybrid zone theory (Barton and Hewitt 1985, 1989; Harrison 1990) and may help explain the existence and long term persistence of hybrids.

1.2.2 Poplar as a model tree

As their long lifespan makes trees functionally different from genetic model organisms as rice and Arabidopsis, it is important to also have a tree as a model organism, in order to be better able to study the different aspects of tree biology. Many reasons have been invoked to promote poplar as a genomic system for tree molecular biology. These include easy transformation, rapid growth, small genome size (450-550 Mbp) and considerable genetic variation. Also, poplars can be propagated vegetatively, which in theory will allow mapping populations to be maintained indefinitely and will permit the production of a large amount of clonal material. All these reasons make poplar a very good system for biotech research in forestry (Bradshaw and Strauss 2000; Strauss and Brunner 2004). The interest in conducting genetic analyses in poplar relies in the possibility to identify genes that regulate tree functions such as wood formation and tolerance / resistance to biotic and abiotic stresses or root and shoot biotic interactions. A major step in understanding the molecular genetics of tree functions has recently been achieved with the sequencing of the poplar genome (Tuskan *et al.* 2006).

1.2.2.1 Genomics

Genomics is the field of science that studies the entire DNA sequence and structure of an organism's genome (The American Heritage Dictionary). The goal of genomics is to promote the understanding of the structure, function, and evolution of genomes. Because trees have very distinctive characteristics, genomic studies conducted in poplar are aimed to revealing the genetic mechanisms regulating these tree-specific properties.

1.2.2.1.1 Genetic and physical maps

A genetic linkage map is a statistical representation of the arrangement of genes relative to each other. It is based on the frequencies of recombination between markers. The greater the frequency of recombination between two genetic markers, the farther apart they are assumed to be and vice versa. For plants, the first genetic maps were constructed using visible characters such as seed coat color. Later coding (allozymes; ESTPs, expressed sequence tag polymorphisms) and noncoding DNA markers such as (microsatellites; RAPDs, random amplified polymorphic DNAs); AFLPs, amplified fragment length polymorphisms), have been used. To date, genetic maps have been already constructed for many different forest tree species using a variety of genetic marker types (Neale and Sederoff 1996; Krutovskii *et al*. 1998; Cervera *et al*. 2001; Gosselin *et al*. 2002; Pelgas *et al*. 2005, 2006). Availability of reference maps and marker sets will contribute directly to elucidating gene function via comparative mapping and quantitative trait loci (QTL) detection. Loci controlling quantitative characters are called QTLs.

Genetic linkage mapping is central to genomics since it is a starting point for any genome sequencing project. It allows the assignment and positioning of genes and genetic markers on a specific linkage group (a statistical representation of a chromosome). The first molecular genetic linkage map of *Populus* was developed by Bradshaw (1996) from an original interspecific cross made between a *P. trichocarpa* mother and a *P. deltoides* father in the 1970s at the University of Washington, USA. This cross led to the development of the family 331, a reasonably large F_2 progeny, and a molecular genetic map that now has several hundred molecular markers (Bradshaw *et al*. 1994). After the initial maps of Bradshaw, several additional molecular genetic maps of *Populus*, based on an assortment of marker types, have been developed, including a cross using *P. nigra* and *P. deltoides* (reported by Cervera *et al*. 1997), a cross between *P. trichocarpa*, *P. deltoides*, *P. nigra* (Cervera *et al*. 2001), a map for *P. deltoides* (Wu *et al*. 2000) and a map for *P. alba* (Yin *et al*. 2001).

Work on poplar genetic maps has confirmed the presence of 19 linkage groups, which correspond to the haploid number of chromosomes present in the poplar genome (Bradshaw *et al.* 1994; Cervera *et al.* 2001). These 19 linkage groups have similar genetic / physical map distances to that of *Arabidopsis* (Bradshaw *et al.* 2000). This similarity helps facilitate comparisons between both species, for example, how genomes have evolved through duplication events.

Physical maps provide the exact location of genes or genetic markers on chromosomes. These maps are either assembled from the complete genome sequences, BAC (Bacterial Artificial Chromosome), contigs (a group of clones representing overlapping regions of a genome), or based on *in situ* hybridization or other methods. The Genome BC Forestry genomics (Treenomix) project has constructed a physical map of the poplar genome by sequencing approximately 46,000 BAC clones from *P. trichocarpa*. This accomplishment facilitates the linking of the physical map to the genetic linkage maps (Treenomix project, www.treenomix.ca/contribution-poplar-genome).

1.2.2.1.2 QTL studies

The major purposes of map construction are the study of genome structure, the study of functional relationships, and the identification of QTL (quantitative trait loci) affecting economically important traits (Yin *et al*. 2002). Such studies are necessary as a complete sequence alone is not sufficient to understand the genetic control of adaptive and sylvicultural traits, which are usually complex. Quantitative traits such as height and frost tolerance are controlled by many genes and influenced by the environment. Genetic maps can be used to study the number, location and distribution of QTLs in a genome. For example, QTL detection has indicated that bud burst in poplar species was determined by a few loci with major effects. Bradshaw and Stettler (1995) concluded that only 5 QTLs were responsible for 85% of the heritable genetic variation in the timing of spring bud flush in *Populus*. However, no specific genes were shown to correspond with these QTLs. The genetic control of bud phenology in hybrid poplar was studied further by Frewen *et al.*

(2000), by mapping quantitative trait loci (QTL) affecting the timing of autumn bud set and spring bud flush. Two parental trees, a female *P. trichocarpa* originally from Washington State and a male *P. deltoides* from Texas were used to produce a second generation mapping pedigree. Bud set and bud flush timing was measured on this F_2 generation. Using a linkage map constructed of AFLP and microsatellite markers, three QTL controlling bud set and six QTL controlling bud flush were detected. In addition, five candidate genes believed to be involved in the perception of photoperiod (*PHYB1*, *PHYB2*) and transduction of abscisic acid response signals (*ABI1B*, *ABI1D*, and *ABI3*) were placed on the QTL map. Of these 5 genes, *PHYB2* and *ABI1B* were indeed found at the map locations of QTL affecting bud set and bud flush.

Rae *et al*. (2004) used 300 F_2 individuals of the same family to identify and map the quantitative traits that would make an ideal tree for short rotation coppice. They also looked at the possibility of incorporating these traits into commercial poplar by marker-aided selection. Both microsatellite and AFLP markers were used to produce a molecular linkage map. This enhanced map was used to look more closely at QTL for improved yield and other important correlated traits. The end result was the identification of QTLs for stem, leaf and cell traits (Rae *et al.* 2004).

In another study, Jorge *et al*. (2005) mapped QTL for qualitative and quantitative resistance to leaf rust (*Melampsora*) in a *Populus deltoides* x *P. trichocarpa* F_1 progeny. They noted that analogs of NBS-LRR resistance genes were in the vicinity of a *Melampsora* resistance locus previously identified in another hybrid poplar pedigree (Zhang *et al*. 2001).

1.2.2.1.3 Genome sequence and physical maps

The genome size of *Populus* is small (2C = 1.2 pg) compared to that of other trees, i.e. the poplar genome is much smaller than that of *Pinus* (19.94 to 24.91 pg/C, Hall *et al*. 2000).

This small genome is one of the reasons why *Populus* is a very useful model for plant research. The recent sequencing of *Populus trichocarpa* (clone Nisqually-1) is an important milestone. This important accomplishment was achieved by an international consortium including the U.S. Department of Energy (DOE), Genome Canada, and the Umeå Plant Science Centre in Sweden (Tuskan *et al.* 2006).

The genome of *Populus trichocarpa* contains 19 chromosomes and is estimated to be approximately 480 Mbp. All sequence data is freely available for the scientific community (http://genome.jgi-psf.org). The genetic and physical maps of *Populus trichocarpa* are being integrated to provide researchers with a valuable resource for future studies. The availability of the *Populus* genome sequence allows researchers to compare the genome of an herbaceous plant with that of a perennial plant. The comparison of the *Populus* genome to that of the *Arabidopsis*, will help to identify a gene of interest that may represent a class of genes that is unique to trees. Sterky *et al.* (2004) analyzed 102,019 *Populus* ESTs and showed that the coding content of *Populus* and *Arabidopsis* genomes shows very high similarity, indicating that differences between these tree and herb life forms result primarily from differences in gene regulation and genome duplication.

1.2.2.1.4 Transformation system

The term transformation is used to describe mechanisms of DNA and RNA transfer. The desired result is the introduction, uptake and expression of foreign genetic DNA in the transformed cell (Genetics, 2003). The different transformation systems allow the insertion of genes that confer desired traits not readily available in sexually accessible gene pools. In contrast to sexual breeding, transformation allows new genes to be added while the genotypes of elite clones are preserved, and can therefore reduce the time required to produce a new elite line (Han *et al*. 1996). There are many characteristics targeted by breeders, such as insect resistance, herbicide tolerance, accelerating flowering or wood quality (reviewed by Strauss *et al*. 2001).

Poplars can be transformed with a gene of interest with minimal disruption of its genomic organization (Bisoffi and Gullberg 1996). Also, because they are easily propagated *in vitro*, poplars are the preferred candidates to investigate possible applications of recombinant DNA technology in trees. Poplars were the first forest trees to be transformed with an agronomically important gene for resistance to glyphosate (Fillatti *et al.* 1988; Sellmer and McCown 1989).

A number of methods, both biological and physical, are available to transfer DNA into an organism. Of those methods, *Agrobacterium*-mediated transformation has been the method of choice for many studies on *Populus*, although physical methods like biolistics and electroporation have also been applied successfully (Han *et al*. 1996; 1997).

Agrobacterium-mediated transformation is the easiest type of plant transformation. The soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* are used as living vectors to transfer a segment of DNA from their large endogenous Ti (tumor-inducing) or Ri (root-inducing) plasmid. Plant tissue, often leaves, is cut in small pieces, e.g. 10x10 mm, and soaked for 10 minutes in a medium containing *Agrobacterium*. Some cells along the cut will be transformed by the bacterium that inserts its plasmid into the cell. The main advantage of *Agrobacterium*-mediated gene transfer is that only a fragment of the transformation vector within the T-DNA (transfer DNA) border sequences are transferred, and that only a single or a few copies of the fragment are integrated into the host genome. After placement on selectable rooting and shooting media, the plants will grow. Under optimal conditions the transformation frequency achieved by this method is high, though the efficiency is dependent on bacterial and plant genotype (Binns 1990). The first transgenic poplar was a *P. alba x P. grandidentata* hybrid into which the *aroA* and *nptII* genes were inserted (Fillatti *et al*. 1987). Since then, a number of genes have been used to transform different *Populus* species. To date, *Agrobacterium*-mediated transformation has accounted for the large majority of published reports (Jouanin *et al*. 1993).

Biolostics is a physical transformation method, based on the direct delivery of naked DNA. Small gold or tungsten DNA coated particles are shot at high velocity into young plant cells or plant embryos. Some genetic material will stay in the cells and transform them. This technique has generated many transgenic organisms, including poplar (McCown *et al*. 1991). The transformation efficiency is lower than in *Agrobacterium*-mediated transformation; nonetheless, direct DNA delivery techniques may be useful for transformation of elite clones that are highly resistant to *Agrobacterium* infection.

Electroporation is a transformation method consisting of making holes in the cell walls using electric discharge particle acceleration, allowing the entrance of DNA constructs into the cell. This physical method has been used for transformation of protoplasts (plant, fungal or bacterial cell that had its cell wall partially or completely removed) from several species. In poplar, the successful transformation of *P. tremula x P. alba* by electroporation was achieved by Chupeau *et al*. (1994).

1.3 Populiculture

1.3.1 Overview

Poplar culture (also known as populiculture) spread across the world at the beginning of last century. In 1947 the International Poplar Commission (IPC) was founded under the patronization of the FAO of the United Nations. The work of the Commission has led to important agreements on nomenclature, registration of clones, varietal control (Zsuffa *et al.* 1996). The significance of populiculture is expected to increase in the near future as it can provide wood products for fibre, fuels and chemicals while at the same time contributing to a more favourable carbon balance (Klass 1998). Poplar plantations will provide a source of timber at a time when the demand increases year after year and new legislation in several countries will further restrict tree cutting in existing natural forests.

Breeding work in *Populus* relies very much on the capacity of species from different sections to hybridize. Two of the most important hybrids that are used for poplar culture, date from the 18th century: *P. canescens* (*P. alba* x *P. tremula*) and *P.* × *canadensis* (*P. deltoides* x *P. nigra*) also known as *P. euramericana*. In North America there is another hybrid that has been grown for centuries: *P.* ×*jackii* (*P. balsamifera* x *P. deltoides*) (Eckenwalder 1996). Several cultivars or varieties have been developed through selection and intensive breeding over the last years and are widely planted mostly in the northern hemisphere, but also in other parts of the world (Stettler *et al.* 1996). In North America, there are about 50-80 clones, mostly interspecific hybrids with exotic components, that are currently being used in poplar culture (Dickmann *et al.* 2001).

1.3.2 Production of hybrids

Poplar hybrids occur naturally throughout Canada and the US wherever compatible species come into close proximity. Examples are the hybridization between *P. balsamifera* and *P. trichocarpa* that occurs in the interior of southeastern Alaska and in the Cook Inlet region as well as the trihybrid among *P. deltoides*, *P. balsamifera*, and *P. angustifolia*, reported in southern Alberta (Rood *et al.* 1986). Also, in eastern Canada, natural hybrids are formed between *P. deltoides* and *P. balsamifera* (Rood *et al.* 1986; Floate 2004; Hamzeh *et al*. 2007). Most poplar hybrids, however, result from artificial hybridization and subsequent planting. An unknown number of hybrids also form between native species and introduced clones, cultivars, and species (Eckenwalder 1996). The first large-scale hybridization project with poplars in the United States was initiated in 1925 (Stout 1927; 1933).

There are several reasons why poplar hybrids are so important in populiculture. First of all, crossability among many of the 29 poplar species is high (Zsuffa 1975; Willing and Pryor 1976). Another significant advantage of hybrid poplars is that superior material is quickly available for operational use, because species of sections *Aigeiros* and *Tacamahaca* are easy to propagate through asexual means, usually by vegetative propagation of unrooted dormant stem cuttings or sets. This allows mass-propagation of selected varieties. Another advantage is the superiority of some clones in an F_1 compared to the best parent

(heterosis). The existence of such hybrid vigor has been confirmed by different studies (Stettler *et al.* 1988; Bradshaw and Stettler 1995). This superiority seems to be the result of complementation of dominant or partially dominant genes carried separately by two parent species at different loci, a phenomenon also observed in crops (Jinks 1983; Stettler *et al.* 1996). While these hybrids might face considerable fitness challenges from their parental species in the wild, they can develop very well under artificial conditions and display very attractive commercial features (Stettler *et al.* 1996). It is important to mention that although hybrid vigor can allow many trees to be superior to their parents, breeders still have to carry out a strong selection among the F_1 progeny in order to obtain well performing trees for production.

1.3.2.1 Plantation of clones with exotic components

The use of exotic species in forestry is regarded as a way to complement the native genetic resources available. Hybrids between native and exotic *Populus* species can answer needs that are not fulfilled by the native species therefore exotic species are commonly used in genetic improvement programs and in short rotation intensive forestry. For example, certain characteristics like rootability, stem growth, branching, and disease resistance can be significantly improved by crosses with exotic species (Stettler *et al.* 1996). In North America, native *P. deltoides*, whose rootability varies widely, is crossed with the well-rooting *P. maximowiczii* and *P. nigra* to significantly improve this important trait (Zsuffa 1975; Dickmann and Stuart 1983; Wu *et al.* 1992). In Europe, *P. nigra* is hybridized with *P. deltoides* and other exotic *Populus* species to provide rooting ability, high resistance to bacterial infections, and adaptability to various soil and climate conditions. Frequently, exotic species of *Populus* are resistant to native pathogens (Newcombe 1998), and resistance is often simply inherited in F_1 interspecific hybrids. However, sometimes resistance may not necessarily be expressed in F_1 hybrids between susceptible and resistant parents because it may be under the control of recessive genes.

Plantations of hybrids with exotic components could be problematic because spontaneous hybridization and / or introgression from exotic into native species could have short- and long-term impacts on the genetic diversity of indigenous species. Gene flow from plantations with exotic components to native poplar stands could affect the genetic composition of natural forest and result in a loss of genetic variability or changes in fitness.

In Europe, the potential genetic introgression was analyzed between plantations involving the North American *P. deltoides* or the hybrid *P.* x *canadensis* (a cross between *P. deltoides* and *P. nigra*) and stands of the native *P. nigra*. The problem was not considered to be of significant concern (Heinze 1997; Benetka *et al.* 1999; Fossati *et al*. 2003; Tabbener and Cottrell 2003). However, things could prove to be different in Canada, where crosses between *P. nigra* as the exotic pollen parent and *P. deltoides* have reported to occur more easily (Zsuffa *et al.* 1999).

1.3.2.2 Plantations of genetically modified trees

Before establishing plantations of genetically modified trees, it is important to conduct studies to determine the potential gene flow from genetically engineered trees into natural populations, the long-term stability of introduced genes, and the potential long-term effects of genetically engineered trees in the ecosystem. Field tests are currently being conducted in several countries, with the majority of these field tests occurring in the US in the past 5–7 years (reviewed by van Frankenhuyzen and Beardmore 2004).

One of the first reported field trials with genetically modified forest trees was established in Belgium in 1988. The trait evaluated was herbicide tolerance in poplars. Since then, there have been more than 200 reported trials, involving at least 15 different tree species. More than 50% of those field trials were done with *Populus* species and the main targets were herbicide tolerance (31%), followed by marker genes to evaluate the transformation method (23%), and insect resistance (14%). Until today, there is only one report of

commercial-scale production of transgenic forest trees: a plantation of *Populus nigra* with a *Bt* (*Bacillus thuringiensis*) gene. *Bt* is a soil bacterium that produces proteins (Cry toxins) that act as a natural insecticide. Inserting these *Bt* genes in crops results in the death of insects that eat the leaves. Such *Bt* trees were released in China in 2002 and established on commercial plantations in 2003 (Valenzuela *et al.* 2006).

The constant advances in genomics and gene cloning techniques have allowed researchers to identify and select for attractive traits such as insect resistance or wood quality. The targets for forest product development can be divided broadly into sylvicultural traits and product quality traits. The former are traits that are important to productivity issues such as growth rates and stress or insect tolerance, while product quality traits are more concerned with an improved end use of the material produced, such as alterations in lignin content and strength of wood. An example of an sylvicultural trait is the ability to resist to an herbicide. This trait will result in an increase in growth and yield by limiting weed competition in the first few years of growth. Resistance to the herbicide Roundup has been demonstrated in field tests of transgenic poplars (Meilan *et al.* 2002). An example of a genetic transformation targeting a product quality trait is the modification of lignin content. This trait is important because it can affect the end products through its manipulation (Baucher *et al.* 2003). Lignin removal is a costly process. The possibility to modify the expression of certain enzymes in the biosynthetic pathway to alter the lignin composition will result in easier lignin removal. One example is 4-coumarate ligase (4CL), which was tested in transgenic poplar. Hu *et al.* (1999) produced transgenic *P. tremuloides* trees, in which expression of the gene for 4-coumarate ligase is suppressed. This enzyme is part of the lignin biosynthetic pathway and results showed up to a 45% reduction of lignin in transformed trees. Nevertheless, the overall lignin-cellulose mass remained practically unchanged, suggesting that level of lignin and cellulose could be regulated in a compensatory fashion.

In Canada, researchers from the Canadian Forest Service are studying the environmental impacts of GMT (genetically modified trees). In 1997, the first field test of transgenic

poplars in Canada was established at the Valcartier experimental station, in Québec. The used poplars have been genetically modified with two marker genes (*NPT* and *GUS*). These markers work in two ways; on one hand they allow researchers to check the success of the genetic transformation. On the other hand, they are use to determine if the transgene ends up in the soil by the decomposition of dead leaves and branches (Hay *et al*. 2002). The plantation respects all the safety requirements of the Canadian Food Inspection Agency and is formed by rows of non-transgenic poplars with some transgenic poplars scattered in between. The study by Hay *et al*. (2002) showed that no traces of genetically modified DNA were detected in the field after 4 months, indicating that a possible transfer of DNA from transgenic material to soil microorganisms is unlikely.

Another study involving genetically modified poplars was set up in Alberta in collaboration between the Alberta-Pacific Forest Industries Inc. (Al-Pac) and the Tree Genetic Engineering Research Cooperative of Oregon (TGERC). This trial had transgenic hybrid lines expressing glyphosate tolerance and engineered sterility. Unfortunately, trees did not survive winter conditions. As genetically improved trees are introduced into both small- and large-scale field tests and into commercial operations, it is important to have an infrastructure to control and assess the safety of these trees. Different types of controls and regulations are being either implemented or developed in many countries around the world (CFIA, Canadian Food Inspection Agency, www.inspection.gc.ca)

1.3.3 Risk assessment

The introduction of alien genes from exotic species into native forests is being analyzed because of its potential negative effects. To avoid undesirable effect to the environment, clear guidelines must be established before allowing exotic or transgenic species into the environment, independent of the method used to produce them (Mullin and Bertrand 1998). Gene escape from an exotic species into a wild relative could lead to a loss in the genetic diversity in the recipient species and to possible fitness changes.

Also, because of its enormous potential, recombinant DNA technology has raised many concerns regarding the potential environmental impacts of widespread cultivation of genetically engineered hybrid poplar. These include the possibility that the crop itself will become an invasive pest, the possibility that the competitiveness of wild relatives will be altered through transfer of the transgene by hybridization, and the possibility that the transgene product will have negative impacts on natural populations and ecosystems. Trees in particular, present difficult challenges for risk analysis because of their long generation times, large population size and potential for long distance dispersal of pollen and seeds (Strauss *et al*. 1998). The study of gene flow from already established plantations to natural populations will provide data about the rate of spontaneous hybridization. Simulation models can incorporate these data to assess the consequences of introducing new genes into the environment.

1.3.3.1 Horizontal transfer

Horizontal gene transfer involves exchange of genes between genetically unrelated organisms, without the involvement of sexual reproduction. All known examples of horizontal gene transfer are related to bacteria. Attempts to observe horizontal gene transfer in experimental systems have concluded that their role is essentially irrelevant to any realistic risk assessment involving transgenic plants (Prins and Zadoks 1994, Schlüter *et al*. 1995).

1.3.3.2 Vertical transfer (gene flow)

The essential pre-requisite for vertical gene flow is sexual reproduction between the transformed plant and its wild relative. In most tree species, gene flow is facilitated by the fact that they are wind-pollinated and pollen can fly great distances. Physical isolation barriers can be useful to control gene escape but it seems certain that some level of vertical gene flow will occur.

Genetically engineered sterility has been proposed as an attractive mechanism to eliminate gene flow, but there are many examples of transgenes whose stability changes over generations (Rogers and Parkes 1995) so it is unlikely that transgenic trees could be approved solely on the presumption of permanent sterility (Mullin and Bertrand 1998). Testing transgenic sterility will take rather a long time for most forest trees species, as stability must be demonstrated over many years, through dormancy cycles and during times of various environmental stresses (Strauss *et al*. 1995).

1.3.3.2.1 From plantations (exotics) to surrounding wild populations

Genetic swamping and introgression by related nonindigenous *Populus* taxa may pose a threat to the species' genetic and evolutionary integrity (Vanden Broeck *et al*. 2002). For example, in Europe, hybridisation and potential genetic swamping is seen as a threat to the *in situ* conservation of *P. nigra,* which is considered to be an endangered species in many countries. The amount of introgression of *P. deltoides* genes into the *P. nigra* gene pool is however debated (Benetka *et al*. 1999; Vanden Broeck *et al*. 2002). Fossati *et al*. (2003) studied a region in Italy where hybrid poplars and *P. deltoides* commercial clones are cultivated as monoclonal stands close to an area where black poplar has its natural habitat. SSR analysis was performed to detect introgression between the natural population and the monoclonal plantations of hybrids and *P. deltoides* clones cultivated in the surrounding area. Four microsatellite loci found to have alleles which were species-specific to *P. deltoides* were used as markers to measure the introgression of *P. deltoides* into *P. nigra*. They did not find evidence of hybridization events between *P. nigra* and *P. deltoides* commercial clones and concluded that introgression events in that area are extremely rare or absent (Fossati *et al.* 2003).

Several other studies have measured the rate of introgression from *P.* x *euramericana* to *P. nigra* (Rajora 1985; Heinze 1997; Benetka *et al.* 1999; Benetka *et al.* 2002). *P. nigra*

offspring was analysed in all cases, and the observed rate of introgression was surprisingly small or no introgression was detected. The fact that hybridization in controlled conditions between *P. deltoides* and *P. nigra* is only possible when *P. deltoides* is the female parent (Zsuffa 1974) has been invoked as a possible reason for the lack of introgression of genes of *P. deltoides* in open pollinated progenies of *P. nigra* (Benetka *et al.* 1999). It has been proposed that genetically based stigma-pollen interactions favouring conspecies pollen may play an important factor in determining the mating system of *P. nigra* in the presence of different compatible species (Vanden Broeck *et al.* 2001).

In North America, a study conducted by DiFazio *et al*. (1999), analyzed the gene flow in the vicinity of two plantations of hybrid poplar clones (*Populus trichocarpa* x *Populus deltoides*). Although they found potential for long distance gene flow, the effective rate of gene flow was found to be very low. The abundance of pollen and seeds from the wild stands of *P. trichocarpa* may provide a powerful dilution effect, even very close to hybrid plantations (Brunner *et al*. 2004).

1.3.3.2.2 Potential risks of transgene escape by gene flow

The occurrence of gene flow among compatible species has been proven by many studies (Ellstrand *et al.* 1999). Genes can move from one species to another, so the potential of a transgene escape by gene flow is real. Before releasing genetically modified trees in the environment, it is necessary to understand what will be the impact of an escaped transgene in the natural environment. Genetically engineered trees present a particular challenge because traits must be expressed over a long period of time, while exposed to dramatic environmental stresses and regular changes in developmental status (Mullin and Bertrand 1998). Also because forest trees grow over long periods in relative remote areas, gene flow is not as easily confined in trees as in their agricultural counterparts and “problematic” plantations cannot readily be removed.

There are several biosafety issues to be considered when evaluating the risks of transgenic release. One is the introgression of a transgene into the wild via gene flow. Of greatest concern is the change in invasiveness that accompanies a selective advantage conferred by the transgene and the possibility that the plant or one of its relatives will become a weed (Dale 1992; Raybould and Gray 1994). Another concern is the impact that this novel trait will have in the ecosystem, because traits that may be desirable in a tree (e.g. resistance to drought, insects) if transferred to relatives might turn these into a pest (Mullin and Bertrand 1998). Transformations that aim to confer pathogen resistance to a tree are also under scrutiny because they might encourage the coevolution of the pest which may lead to reduced efficacy or failure of control products (Mullin and Bertrand 1998). In a recent article, Strauss (2003) advocates the use of different levels of confinement in order to allow safe field testing of genetically engineered plants. It follows that the need for biological and / or geographical confinement of small- and large-scale transgenic field trials should be assessed depending on the expressed trait.

1.3.4 Silviculture

Silviculture is the science of controlling the establishment, growth, composition, health, and quality of forests to meet diverse needs and values of landowners and society on a sustainable basis (Smith *et al.* 1997). In silviculture, natural ecological processes are guided to produce forests that are more beneficial to the landowner than nature can provide, in a shorter period of time.

Growing poplars in plantations is challenging. Poplar plantation culture depends on three aspects: planting the best quality stock, high quality sites and timely and appropriate cultural treatments (Stanturf *et al.* 2001). It is critical that clones be carefully selected to fit the sites on which they are to be planted, and that appropriate mixtures of clones be used to maintain diversity and reduce the chances of pests or other problems affecting monoclonal blocks.

Poplars perform best in soils that are well-aerated, have sufficient moisture and nutrients, are sufficiently deep, have a medium texture and have a pH in the range of 5.0 to 7.5 (Baker and Broadfoot 1979). Poplars require full sunlight, adequate water and nutrients for maximum growth potential (Demeritt 1990). Any kind of competition will affect poplar plantation growth and survival. For example, weeds will compete with poplar for nutrients and sunlight, resulting in decreased growth and increased mortality. In North America, the general strategy to minimize weed competition is the application of herbicides. Only in the province of Québec mechanical methods are used, because of a ban in the use of herbicides established by the Ministère des resources naturelles et de la faune du Québec (MRNFQ) (Forest Protection Strategy, MRNFQ, 1994). Large animals such as deer, elk and moose can also cause important losses and for that reason plantations have to be protected using repellents or fencing (for small plantations) (Stanturf *et al.* 2001).

Another aspect of poplar plantations is that they require a nutrient management program in order to maximize plantation growth. Nitrogen (N) is the main element limiting poplar growth in all regions. Providing an adequate N supply and maintaining other nutrients balanced with N is very important (Stanturf *et al.* 2001). Finally, spacing is a crucial factor in the growth of poplars in plantations. DeBell *et al.* (1997) established 6.2 m^2 to be the minimum growing space needed per tree to yield a stand with mean tree diameter at harvest of 15 cm, which is the economic minimum.

1.3.5 Populiculture in Canada

The Canadian forests are among the most extensive in the world and represent one of Canada's most valuable natural resources. Poplars represent one of the top components of this resource, particularly the stands located in the boreal region of the country. Canada's poplar resource is formed mostly by natural populations, pure or mixed, *P. tremuloides* being the major component. The estimated volume of poplar stands in Canada in 2001 was approximately 1.794.000 m^3 (Canada's Forest Inventory 2001).

Despite an increasing demand for wood, Canada is decreasing the harvesting of natural and first growth forests and putting in place various programs towards sustainable development. This means that they will attempt to meet the present need for wood, without compromising the future of such a valuable resource, as stated in the “Brundtland Report” («Our common future») published by the World Commission on Environment and Development in 1987 (WCED 1987). Since 1990, the Canadian Council of Forestry Ministers (CCFM) has had a national strategy in which it has adopted the practice of sustainable forest management. The policy will work toward significantly increasing the yield per hectare in order to produce more wood from less land.

The culture of poplar is imperative to achieve Canada’s demand for timber and fibre. Therefore, several private companies have established plantations of hybrid poplar. In addition, plantations of hybrid poplars are also managed as short rotation intensive culture (SRIC). These plantations are almost exclusively grown on existing farmland or newly-cleared agricultural class lands, using agronomical methods. Except for a few small private poplar stands, most SRIC in Canada are destined for pulp fiber production. Table 1.2 shows the approximate hectares of hybrid poplar cultivated in short rotation in Canada.

Table 1-2 Approximate area of hybrid poplar plantations in Canada. (PCC Report. van Oosten 2004).

	Reported in 2000	Reported in 2004
Company	Hectares	Hectares
Scott Paper Ltd. New Westminster, BC	2300	3000
Norampac Inc. QC	57	265
Louisiana Pacific Canada Ltd. Chambord, QC	158	1420
Tembec Inc. Maurice, QC	-	250
Norbord Inc. QC	-	not available
NorskeCanada Vancouver, BC	1361	1317
Alberta-Pacific Forest Industries Inc. Boyle, AB	70	2617
Domtar Inc. Cornwall, ON	2200	2000
Domtar Inc. Windsor, QC	140	2400

In Québec, the Ministry of Natural Resources has been actively breeding and selecting hybrid poplars for use in the province. The program of genetic improvement of poplars started in 1969 and is run by a team under the Direction de la recherche forestière (DRF) of the Ministère des resources naturelles et de la faune du Québec (MRNFQ). This program has produced improved populations using five main parental species: *P. balsamifera*, *P. deltoides*, *P. maximowiczii*, *P. nigra* and *P. trichocarpa*. To date the DFR has produced a number of hybrid clones recommended for different ecological regions of the province (Périnet *et al.* 2001). The program has been mainly oriented to selecting clones resistant to *Septoria* and towards the production of *P. maximowiczii* hybrids, which are better adapted

to the more northern areas and the areas southeast and away from the St. Lawrence River Valley. The 44 hybrid poplar varieties used in Québec are shown in Table 1.3.

Table 1-3 Hybrid poplar varieties currently distributed in Québec (PCC Report. van Oosten 2004).

Hybrid type	Number of selected clonal varieties	Area of distribution
D x N	11	Southern Québec, Hardwood forest, St.Lawrence Valley
D x B	5	Mixed forest and Boreal forest (balsam fir-yellow birch and balsam fir-paper birch domains)
T x D	2	Southern Québec, Hardwood forest, St.Lawrence Valley
M x B	13	Mixed forest and Boreal forest (sugar maple-yellow birch, balsam fir-yellow birch and balsam fir-paper birch domains)
D x M	2	Mixed forest (sugar maple-yellow birch domain)
N x M	1	Southern Québec, Hardwood forest and Mixed forest
M x DT	6	Transition zone between hardwood forest and boreal forest (sugar maple-yellow birch and balsam fir-yellow birch domains)
B x T	2	Mixed forest and Boreal forest (balsam fir-yellow birch and balsam fir-paper birch domains)
DN x M	2	Southern Québec (Hardwood forest) and transition zone (Mixed forest)
TOTAL	44	

Note; D = *P. deltoides*, N = *P. nigra*, B = *P. balsamifera*, M = *P. maximowiczii*, T = *P. trichocarpa*

1.4 Objectives

There is no empirical information about the level of spontaneous hybridization between poplar plantations and adjacent natural populations in Canada. The goal of this study was to measure the rate of hybridization between two native poplar species (*Populus balsamifera* and *P. deltoides*) and three exotics (*P. nigra*, *P. trichocarpa*, *P. maximowiczii*) grown in plantations using microsatellite markers specific for these species. Two sites in the province of Québec were sampled during three different years for this study, and thousands of leaves and seed samples from dozens of maternal naturally-occurring trees have been collected and genotyped.

Our results provide information about hybridization and introgression between native and exotic *Populus* species, to better evaluate the short- and long-term consequences that introduced varieties with exotic components could have on the genetic constitution of native species.

CHAPTER 2

MATERIALS & METHODS

2.1 Sampling

Seeds from the two native species, *Populus baslsamifera* and *P. deltoides* were collected from female poplar trees surrounding plantations with male trees harboring exotic components. The two sites sampled are located in the province of Québec, Canada. Plantations were established and are managed by the Ministère des Ressources naturelles et de la Faune du Québec (MRNFQ, collaboration of P. Périnet *et al*.). They were at least 20 years old and found to contain sexually mature male trees producing pollen.

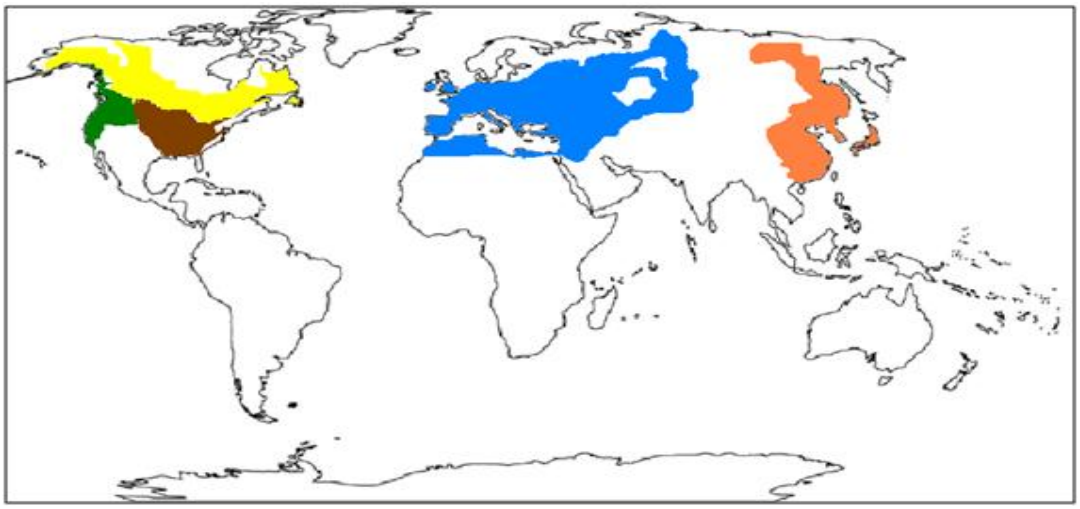


Figure 2-1 Natural distribution of the five species under study: *Populus balsamifera* (yellow), *P. deltoides* (brown*), P. trichocarpa* (green), *P. nigra* (blue) and *P. maximowiczii* (orange).

Site 1, Sorel (Lat: 45°80'N; Long: 73°10'W) includes a plantation of hybrids between *P. deltoides* and exotics (*P. nigra*, *P. trichocarpa,* and *P. maximowiczii*) and pure exotic species (*P. nigra*, *P. trichocarpa,* and *P. maximowiczii*). This plantation is located well within the natural range of *P. deltoides* and nearby natural populations of this species in an agro-forestry landscape. A few scattered individuals of *P. balsamifera* were observed and sampled in the vicinity, which is at the southern most tip of the natural range of the species (Fig. 2.2). Site 2, Matane River Valley (Lat: 48°53' N; Long: 67°13' W), consists of a plantation of pure exotic species (*P. nigra*, *P. trichocarpa*) and hybrids between *P. balsamifera* and exotics (*P. nigra*, *P. trichocarpa*). It is located well within the natural range of *P. balsamifera* and is surrounded by natural populations of this species (Fig. 2.3). Both sites were sampled in 2003, 2004, and 2005. All female trees were sampled in a 200-

1000 m radius according to the direction of dominant winds in order to maximize the pollen flow coming from the plantation. For the Sorel natural stands, a total of 29 *P. deltoides* female trees and 3 *P. balsamifera* female trees (all available trees) were sampled for seeds. For the Matane natural stands, a total of 19 *P. balsamifera* female trees were sampled for seeds.

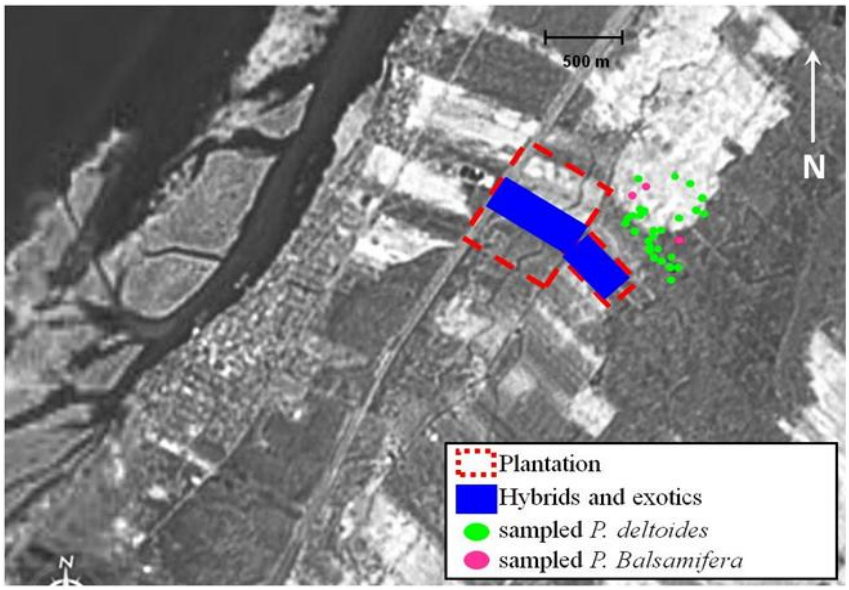


Figure 2-2 Aerial view of site 1 (Sorel).

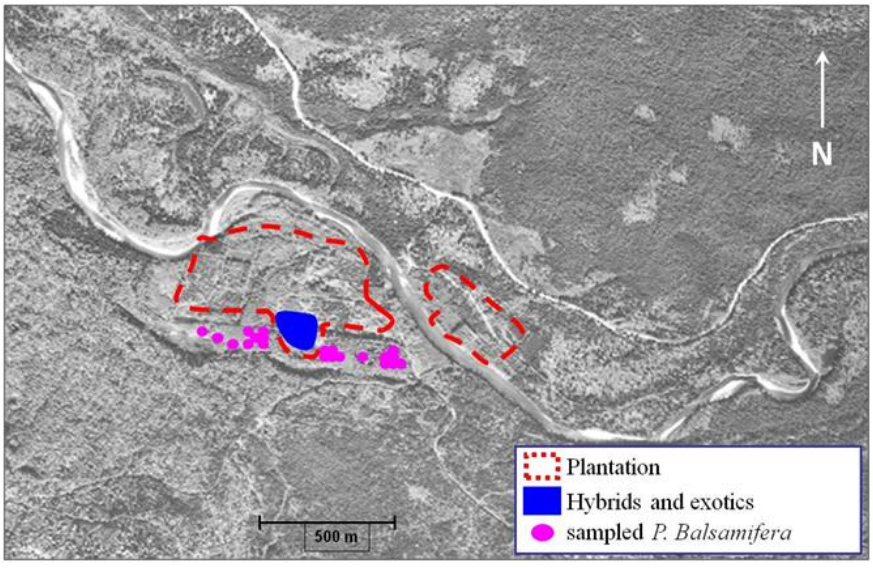


Figure 2-3 Aerial view of site 2 (Matane).

2.2 DNA extraction

For each of both sites, leaves from the native female trees were collected in order to ascertain their identification. Fifty seeds were collected directly on every female tree for each sampling year except for 2003, where seedpods were collected on the ground on site 1 (Sorel). Due to late frost damage in June 2004, no seed was collected on site 2 (Matane) for that year.

Genomic DNA from the female (leaves) and from 48 offspring per female tree (seeds) for each available year of sampling was extracted using the MagAttract 96 DNA Plant Core Kit (Qiagen) following the instructions of the manufacturer. The concentration and quality of the resulting DNA extracts were assessed using a quantitative GeneSpec I UV absorbance spectrometer (Hitachi Genetic Systems) and by comparing the fragment intensity of samples to a known amount of DNA standard on a 1% agarose gel.

2.3 Genotyping and data analysis

2.3.1 Microsatellites or SSRs

Three of the primer pairs from Khasa *et al.* (2005): *PTR6*, *ORNL104,* and *WIN3* were used as species-specific diagnostic microsatellite markers to genotype the offsprings and the maternal trees sampled (see below for marker diagnostic value). The PCR conditions given by Khasa *et al.* (2005) were slightly modified: 10 µl reactions contained 1 µl DNA preparation (1-20 ng), 10× PCR buffer (Invitrogen), 2.0 mM $MgCl_2$, 200 µM of each dNTP, 2 µg BSA, 1-3 pmol of each primer and 1 U of Platinum *Taq* polymerase (Invitrogen). PCR amplifications were carried out with a Peltier Thermal Cycler PTC-200 (MJ Research). Amplification for all primer pairs included an initial denaturation cycle of 1 min at 94 °C followed by 5 cycles with denaturation at 94 °C, annealing at 55-60 °C (depending on the primer used) and extension at 72 °C, each step for 1 min, then followed by 25 to 30 similar cycles with steps of 30 seconds each. The forward primers were labeled

with dye IRD700 in 5' (MWG Biotech) and fragments were sized with an IRD 50-350 bp molecular marker (Li-Cor).
Fragment separation was performed on denaturing polyacrylamide gels containing 6.5× Long Ranger acrylamide: bis-acrylamide solution (Cambrex Bio Science), 7 M urea and 5× TBE buffer (Khasa *et al.* 2005). Gels were run on Li-Cor 4200 DNA sequencers (Li-Cor). The software program SAGA^{GT} version 3.0 (Li-Cor) was used to score the SSR allele sizes.

DNA from each female was migrated next to the 48 samples of DNA from the offspring. For every seed of each native maternal tree sampled, we determined and scored the type of alleles (conspecific (C), interspecific-native (IN), interspecific-exotic (IE)) detected at each one of the three loci. Because the genotype of the female was known (it was determined using the DNA extracted from the collected leaves and poplar is allogamous), all interspecific alleles, either IN or IE, present in the progeny were assumed to be of paternal origin. The embryo was considered a hybrid when at least one interspecific-native or exotic paternal allele was detected at one of the three microsatellite loci surveyed.

To provide an example, when analyzing the progeny of *P. balsamifera*, the expected allele sizes for the species were 180-182 bp for locus *PTR6*, 178 bp for locus *ORNL104* and 248-250 bp for locus *WIN3* (Table 3.1). Once the progeny was genotyped, each particular seed was classified. A seed was considered as the product of a cross with an interspecific native (IN) if, for example, a *P. deltoides* allele (194 bp for locus *PTR6* or 168 bp for locus *ORNL104*) was detected. Also, a seed will be classified as an IE if at least one of the three loci displayed an allele of exotic origin: for example, a fragment of 162 bp for locus *WIN3* (corresponding to *P. trichocarpa*) or a fragment of 192 bp for locus *PTR6* (corresponding to *P. nigra*).

For practical reasons involving the analysis, we considered as F_1 the progeny detected as hybrids, even if some of the plantation trees were advanced generation hybrids. The three loci were used simultanoulsy to disciminate between the species, because individually;

each locus presented some limitation. For example, the locus *ORNL104* was not capable of discriminating between *P. balsamifera* and the exotics. Thus, when *P. balsamifera* was the recipient species analyzed, only interspecific contributions from *P. deltoides* could be safely identified with this locus, and no exotic contributions could be assessed with certainty. A similar situation occurred for the locus *WIN3*, in which case we were not able to discriminate between both native species (due to diagnostic allele size overlap), thus preventing the identification of all spontaneous hybrids between the two native species and possibly underestimating the level of interspecific gene flow.

2.3.2 Statistical Analysis

The proportion of con-specific (*vs* inter-specific, native and exotic combined) hybridizations was analyzed through a generalized linear model. More specifically, if we let π_{ijk} denote the probability that hybridization is con-specific at locus *k* (*k* = 1 for PTR6, 2 for ORN104, 3 for WIN3), in year *j* (*j* = 2003, 2004, 2005), in site-species combination *i* (*i* = 1 for *P. balsamifera* at Matane, 2 for *P. balsamifera* at Sorel, and 3 for *P. deltoides* at Matane), it was initially assumed that the number r_{ijk} of con-specific hybridizations in locus *k*, year *j* and site-species *i* follows a binomial random variable with parameters π_{ijk} and n_{ijk}, the number of seeds in this (*i*, *j*, *k*) combination, and that π_{ijk} depends on site-species combination, year of sampling, locus and their interactions through its logit transform: $\log\left(\frac{\pi_{ijk}}{1-\pi_{ijk}}\right)$. The initial model included all main effects and interactions, but the analysis reported herein is based on a reduced model which depends on site-species combination, year, site-species×year interaction, locus, and site-species×locus interaction only.

Frequencies in the exotic inter-specific hybridization category were generally too small to maintain the two inter-specific categories distinct in the analysis, and the two were therefore pooled.

Specific hypotheses were tested through appropriate contrasts among the parameters. To confirm qualitative observations a logistic model was used. This analysis was performed with the GENMOD procedure of SAS (SAS Institute Inc., Version 9.1, Cary, NC, USA). Generalized linear models are described by McCullagh and Nelder (1989).

CHAPTER 3

RESULTS

3.1 Diagnostic microsatellite markers

Six species-specific microsatellite loci (Khasa *et al*., 2005) were screened on an array of 20 to 60 individuals per species, for each parental species, to identify and validate allele/s specific for each species. Every sample used for the screening was considered as representative of the natural genetic background of each species, and was obtained from allopatric populations remote from contact zones potentially involving natural hybridization. Table 3.1 shows the expected fragment size for each marker among the five *Populus* species. From this screening, three out of six original microsatellite loci were retained for this study, based on their combined ability to discriminate among the 5 species studied (*Populus balsamifera*, *P. deltoides*, *P. trichocarpa*, *P. nigra,* and *P. maximowiczii*). For the locus *ORNL104,* because microsatellite alleles for *P. deltoides* and *P. balsamifera* had very similar size and gel results did not consistently show clear differences of expected fragment size, leaves from 20 *P. deltoides* trees from various parts of North America were genotyped to further assess the specificity of the various diagnostic alleles (data not shown).

Table 3-1 Microsatellite markers used to discriminate among the five poplar species under study.

Species	Microsatellite locus (bp)		
	PTR6	*ORNL104*	*WIN3*
P. blasamifera	180-182	178	248-250
P. deltoides	194	166-168-170	250-252
P. nigra	188-192	176	160-166
P. trichocarpa	182	178	162-164
P. maximowiczii	182-188	178-200	160-164

The three microsatellite primers used (*PTR6, ORNL104,* and *WIN3*) produced clear scorable and fixed differences among the species (Figs. 3.1A and 3.1B).

Primers for the loci *PTR6* and *ORNL104* allowed to discriminate between both native species (*P. balsamifera* and *P. deltoides*) while the locus *WIN3* was used to discriminate both native species from the exotic ones (*P. trichocarpa*, *P. nigra,* and *P. maximowiczii*).

When results from the three loci were combined, the infiltration rate of interspecific paternal alleles in the progeny could be estimated.

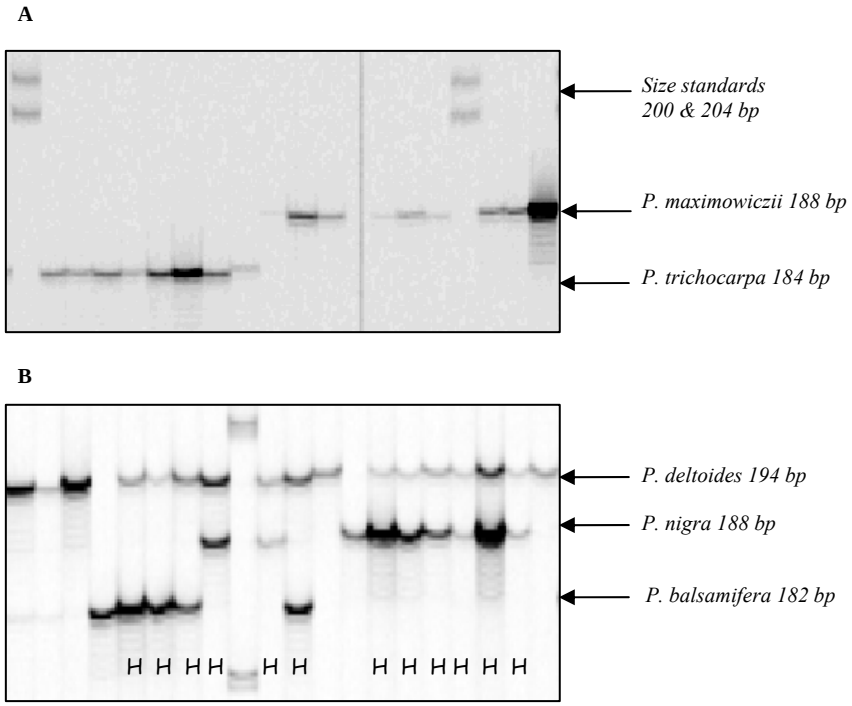


Figure 3-1 A) and B) Examples of polyacrylamide gels showing species-specific microsatellite fragments obtained with primer PTR6. All five species studied are represented, including hybrids (H).

3.2 Detecting interspecific gene flow

To estimate the rate of spontaneous hybridization between native poplars in the natural stands and exotic components in adjacent plantations, we quantified the proportion of exotic alleles present in the progeny.

Table 3.2 shows the total number of seeds genotyped per site per year. Once the females and their offsprings were genotyped for the diagnostic microsatellite loci, the paternal allelic contribution could be determined. Establishing the paternal donor as either native (C or IN) or exotic (IE)) let us determined whether poplars in the natural populations were pollinized by trees from the adjacent plantations, thus detecting and quantifying spontaneous interspecific hybridization that could potentially lead to introgression from exotic alleles into the genetic background of native species.

Table 3-2 Total number of progeny genotyped at loci (PTR6, ORNL104 and WIN3) in 2003, 2004 and 2005, in the northern (Matane) and southern (Sorel) study sites.

Site	*P. balsamifera*			*P. deltoides*		
	2003	2004	2005	2003	2004	2005
Sorel	**22**	**142**	**140**	**101**	**1410**	**1040**
Matane	**341**	**-**	**842**	**-**	**-**	**-**

3.3 *Populus balsamifera* as a recipient species of interspecific gene flow

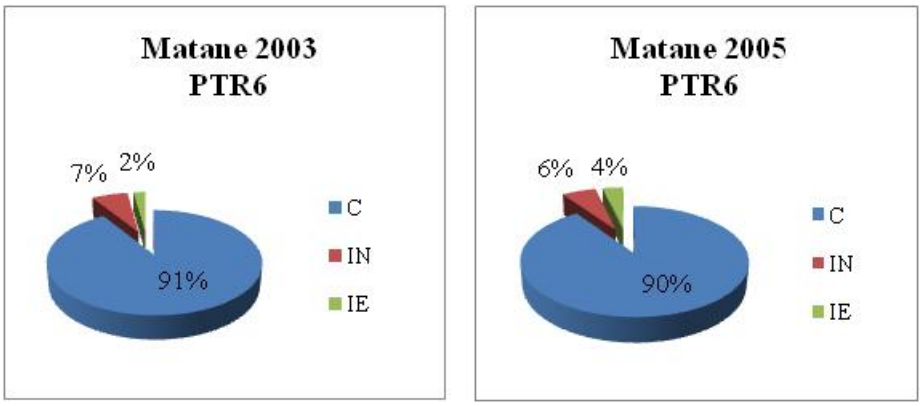
For site 2 (Matane), in both years, interspecific-native (IN-deltoides) and interspecific exotic (IE) alleles were detected among the progeny collected from native *P. balsamifera* females (Table. 3.3).

Table 3-3 Distribution of alleles detected in the *P. balsamifera* progeny at loci *PTR6*, *ORNL104*, and *WIN3* in 2003 and 2005, in the northern site of Matane.

Locus	2003			2005		
	C	IN	IE	C	IN	IE
PTR6	289	21	7	729	48	29
ORNL104	303	31	-	747	75	-
WIN3	265	-	5	702	-	22

In 2003, a total of 13 females were sampled and 341 seeds were collected and genotyped. In 2005 for the same site, 19 females were sampled and a total progeny of 842 seeds was genotyped. Figure 3.2 shows the genotyping results for each locus for each of both years.

A



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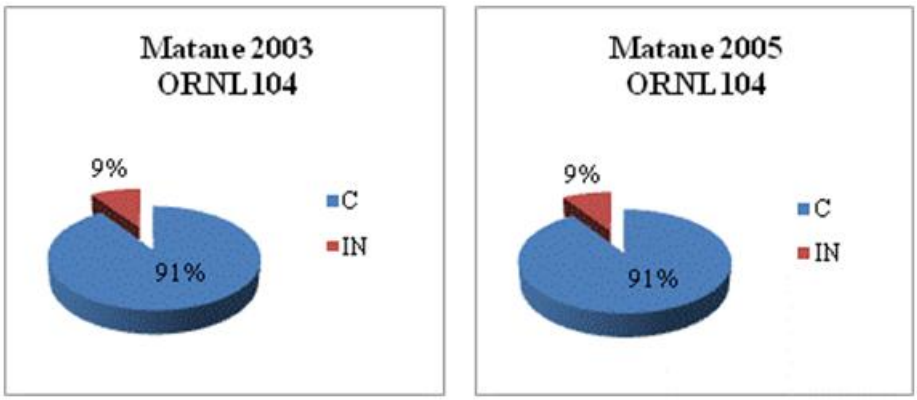


C

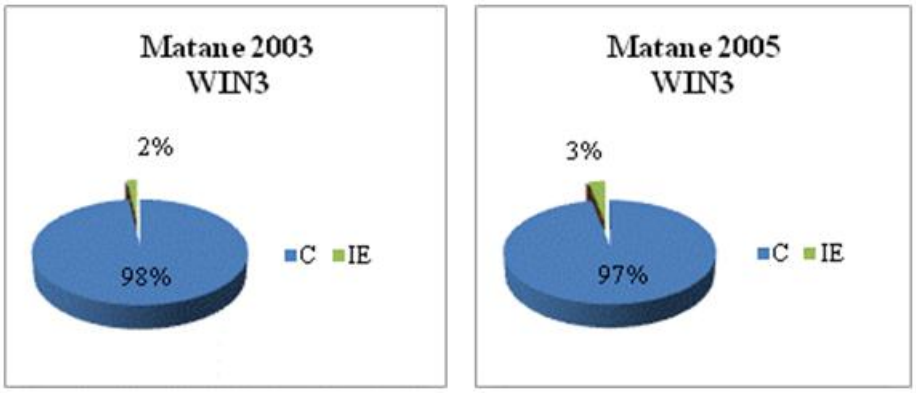


Figure 3-2 Species origin of paternal alleles found within seed array for locus *PTR6* (A), *ORNL104* (B), and W*IN3* (C) of *P. balsamifera* in the northern site of Matane. (C: conspecific, IN: interspecific-native, IE: interspecific-exotic).

When *P. balsamifera* was the recipient species, locus *PTR6* allowed to identify paternal conspecific, interspecific-native (IN), and interspecific-exotic (IE) alleles. The percentage of paternal IN and IE alleles found in the progeny was similar for both years. Around 10% of the genotyped seeds displayed non conspecific paternal alleles and could be classified as hybrids. Locus *ORNL104* allowed to identify interspecific-native alleles from the rest, and for both years the percentage of the progeny displaying an IN paternal allele was 9%. Locus *WIN3* allowed discriminating native alleles from exotic ones. For both years, the percentage of progeny for which an exotic paternal allele was detected was between 2% and 3%.

When the results of the three diagnostic loci were combined, we could observe that around 90% of the progeny resulted from a conspecific cross. Approximately 6-7% of the seeds displayed interspecific native deltoides paternal alleles as the result of a cross between the native *P. balsamifera* female and a native *P. deltoides* male tree. A paternal interspecific exotic allele was detected in 2% to 3% of the offspring, allowing identifying paternal contributions as either a pure exotic or a tree with exotic components.

For site 1 (Sorel), a larger number of paternal non conspecific alleles (IN- deltoides and IE) were detected through the years 2003 to 2005 in the *P. balsamifera* progeny analyzed (Table 3.4).

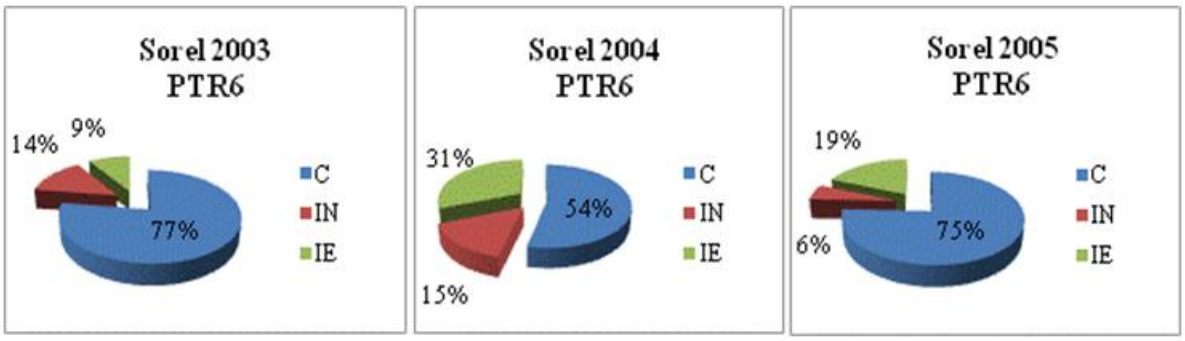
Table 3-4 Distribution of alleles detected in the *P. balsamifera* progeny at loci *PTR6*, *ORNL104*, and *WIN3* in 2003, 2004 and 2005, in the southern site of Sorel.

Locus	2003			2004			2005		
	C	IN	IE	C	IN	IE	C	IN	IE
PTR6	17	3	2	66	19	38	93	7	23
ORNL104	20	2	-	75	62	-	118	17	-
WIN3	18	-	2	73	-	25	110	-	25

A smaller number of *P. balsamifera* maternal trees was found and could be sampled at Sorel, an area located more south and where *P. deltoides* is naturally abundant, but not *P. balsamifera*. One *P. balsamifera* female was sampled in 2003 with a total of 22 seeds

genotyped and three *P. balsamifera* females were found and sampled in 2004 and 2005, with a total of 142 and 140 seeds genotyped, respectively. The percentage of paternal alleles of interspecific origin detected in these years was much higher than for the site located more north (Fig. 3.3), especially in 2004, where the number of progeny exhibiting alleles other than conspecific accounted for approximately 45% of the genotyped progeny.

A



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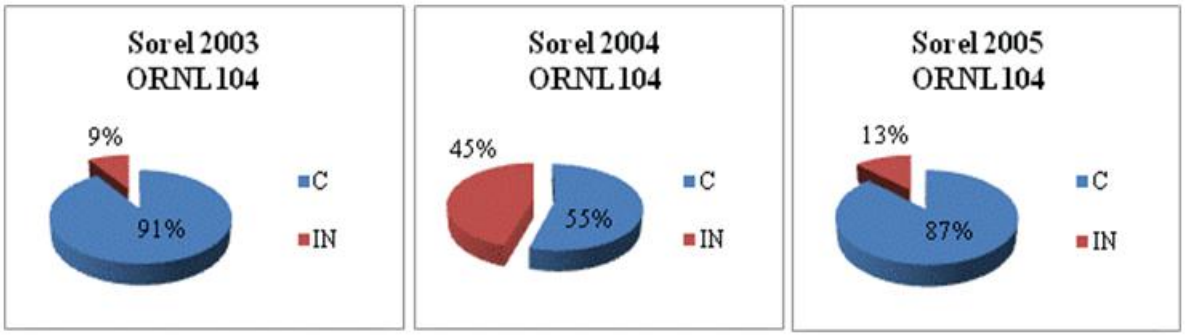

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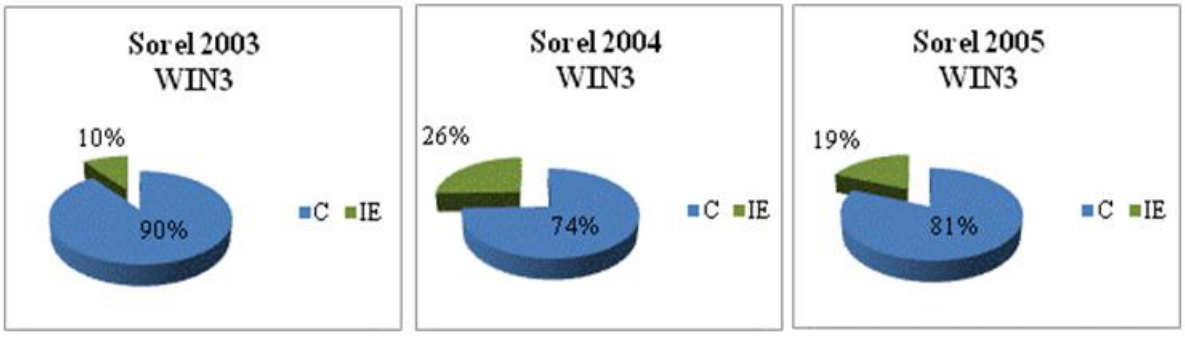


Figure 3-3 Species origin of paternal alleles found within seed array for locus *PTR6* (A), *ORNL104* (B), and *WIN3* (C) of *P. balsamifera* in the southern site of Sorel. (C: conspecific, IN: interspecific-native, IE: interspecific-exotic).

For the locus *PTR6*, the percentage of paternal interspecific alleles found in the *P. balsamifera* progeny varied among the three years, with between 6% and 15% of interspecific-deltoides (IN) alleles, and between 9% and 31% of interspecific-exotic (IE) alleles. The largest difference was among years, where the combined percentage of paternal interspecific native and exotic alleles detected in the progeny for 2003 and 2005 was around 25%, while in 2004 the number of paternal interspecific native and exotic alleles detected accounted for almost 50% of the progeny. The same situation was observed for the locus *ORNL104*, where the percentage of interspecific exotic alleles detected in tthe *P. balsamifera* progeny was is significantly higher for 2004 than for the two other years. Locus *WIN3*, capable of discriminating native alleles from exotic ones, also revealed a higher amount of paternal interspecific exotic alleles for the year 2004. While in 2003 and 2005, more than 81% of the progeny were of conspecific origin, in 2004, this number was reduced to less than 75%. The three markers combined showed that the offsprings of *P. balsamifera* in the more southern location of Sorel had a much higher rate of infiltration of interspecific alleles than in the more northern site of Matane.

3.4 *Populus deltoides* as a recipient species of interspecific gene flow

In the case where *P. deltoides* was the recipient species, the same approach was used once again, except that the sampling was conducted only on the site of Sorel located more south and well within the natural range of the species, between 2003 and 2005. The number of maternal trees genotyped in this case was 10, 29 and 21 for the years 2003, 2004 and 2005, respectively. A total of 101 seeds were genotyped in 2003, 1410 in 2004 and 1040 in 2005. The amount of non conspecific paternal alleles present in the genotyped seeds was much less than that for the progeny of *P. balsamifera* sampled on the same site (Table 3.5).

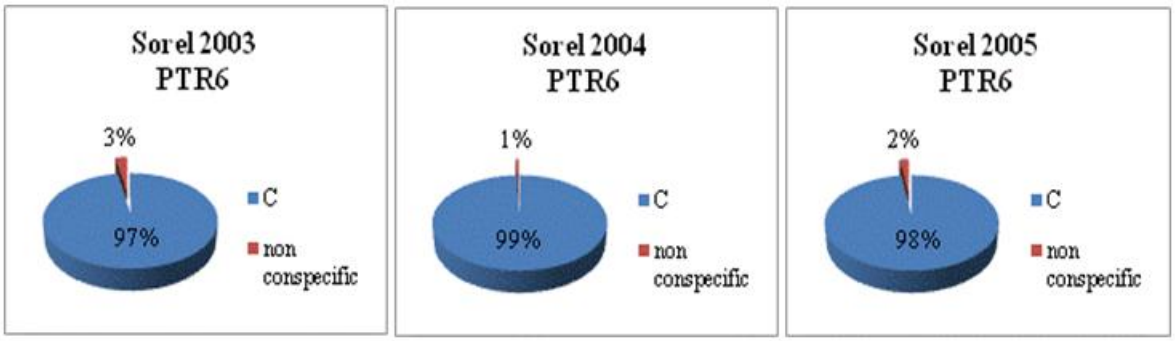
Table 3-5 Distribution of alleles detected in the *P. deltoides* progeny at loci *PTR6*, *ORNL104*, and *WIN3* in 2003, 2004 and 2005, in the southern site of Sorel.

Locus	2003		2004		2005	
	C	interspecific	C	interspecific	C	interspecific
PTR6	73	2	1283	9	936	21
ORNL104	92	6	1342	21	915	43
	Natives	IE	Natives	IE	Natives	IE
WIN3	86	1	1291	15	932	16

When *P. deltoides* was the recipient species, overlapping of allele size limited the discriminating capabilities of loci *PTR6* and O*RNL104*. They were capable of discriminating between conspecific paternal alleles and non conspecific ones but the locus *WIN3* was the only one capable of identifying exotic paternal alleles among the progeny (Fig. 3.4).

The presence of non-conspecific alleles detected within the progeny using loci *PTR6* and *ORNL104* (Fig. 3.4) varied between 1% and 6% for the three years analyzed. For the locus *WIN3*, the quantity of paternal exotic alleles detected was also very low, between 1% and 2%. Even though their potential of discrimination is limited, the results of the three loci combined show that in Sorel, the infiltration of paternal alleles in the *P. deltoides* progeny was much more limited than what was observed for the offsprings of *P. balsamifera*.

A



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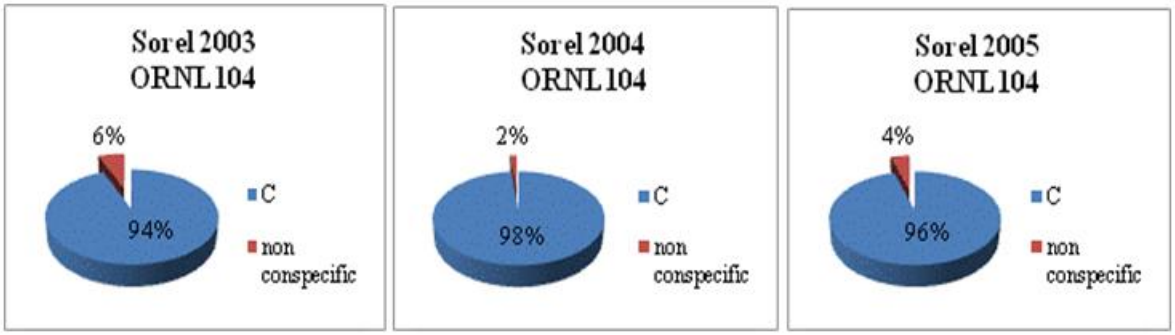

C

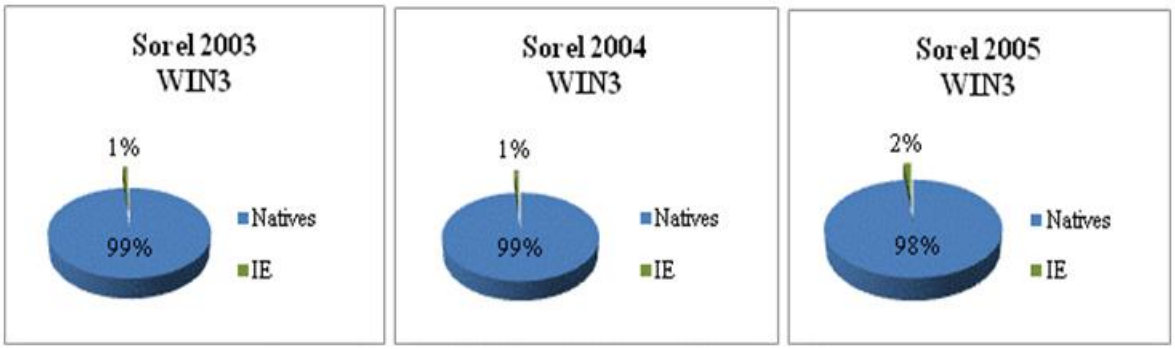

Figure 3-4 Species origin of paternal alleles found within seed array for locus *PTR6* (A), *ORNL104* (B), and W*IN3* (C) of *P. deltoides* in the southern site of Sorel. (C: conspecific, IN: interspecific-native, IE: interspecific-exotic).

3.5 Statistical analysis of hybridization rate

The results obtained using the GENMOD procedure are shown below in Table 3.6.

Table 3-6 Results from the generalized linear model performed with the GENMOD procedure from SAS.

Species	Contrast	d.f.	X^2	*P*
P. balsamifera	2005-2003 at Matane	1	0.2	0.5575
P. balsamifera	2003, 2004, 2005 at Sorel	2	33.04	<.0001
P. deltoides	2003, 2004, 2005 at Sorel	2	18.75	<.0001
P. balsamifera	Sorel / Matane 2003	1	3.22	0.073
P. balsamifera	Sorel / Matane 2005	1	32.83	<.0001
P. balsamifera / P. deltoides	Matane / Sorel 2003	1	2.4	0.1216
P. balsamifera / P. deltoides	Matane / Sorel 2005	1	26.5	<.0001

For *P. balsamifera* at Matane the proportion of conspecific alleles detected in the progeny is not significantly different from year 2003 to year 2005 (X^2 = 0.2, d.f. = 1, P = 0.5575) while at Sorel, there is a significant difference in the proportion of conspecific alleles detected among the progeny between the three years (X^2 = 33.04, d.f. = 2, P < 0.0001).

For *P. deltoides* there is a significant difference in the proportion of conspecific alleles detected among the progeny between the three years studied (X^2 = 18.75, d.f. = 2, P < 0.0001) at the site of Sorel.

For *P. balsamifera* the proportion of conspecific alleles found in the progeny is not significantly different for the two sites in 2003 (X^2 = 3.22, d.f. = 1, P = 0.073), but there is a significant difference for year 2005 (X^2 = 32.83, d.f. = 1, P < 0.0001) between the sites of Matane and Sorel.

The proportion of conspecific alleles detected among the progeny for each species in its natural range (*P.balsamifera* at Matane and *P. deltoides* at Sorel) shows no significant diference for 2003 (X^2 = 2.4, d.f. = 1, P = 0.1216), but a significant difference for year 2005 (X^2 = 26.5, d.f. = 1, P < 0.0001).

CHAPTER 4

DISCUSSION

4.1 Seed genotyping and detection of hybrids

In this study, microsatellite markers were used to genotype thousands of seeds collected on native maternal trees of *P. balsamifera* and *P. deltoides*, respectively, in order to detect and quantify the amount of interspecific spontaneous gene flow from paternal trees with native (*P. balsamifera* and *P. deltoides*) or exotic (*P. trichocarpa*, *P. nigra* and *P. maximowiczii*) components.

In Québec as in other parts of Canada, exotic poplar species have been introduced in genetic improvement programs for intensive forestry, as ornamentals in city parks, and as wind breaks in the agro-forestry landscape. Poplars have weak interspecific reproductive barriers between species, allowing them to hybridize even between sections Eckenwalder 1996). Hybridization and introgression have been previously studied in three poplar species in Canada (*P. balsamifera*, *P. deltoides,* and *P. angustifolia*), but the study was conducted using morphological characters (Floate 2004). There are no previous genetic studies on the levels of interspecific gene flow in poplar species in Canada even though there are for other species, such as *Larix* spp. where a very low rate of interspecific natural hybridization was found (Gros-Louis *et al.* in prep.) and *Picea*, where extensive hybrid zones between black spruce and red spruce were detected using RAPDs (Perron and Bousquet 1997).

Our three microsatellite loci were able to detect the presence of interspecific-native and exotic alleles in the offspring of native *P. balsamifera* and *P. deltoides* trees. Their individual power of discrimination had some limitations depending on the recipient species, such that the combined results of the three loci were necessary to classify each seed. Loci *PTR6* and *ORNL104* have some diagnostic alleles of closely similar size. For example, in the case of locus *ORNL104*, the 176-bp fragment was specific to *P. nigra* while the 178-bp fragment was specific to *P. trichocarpa* and *P. balsamifera*. Thus, the result obtained using locus *WIN3*, capable of discriminating native alleles from exotic ones

in the *P. balsamifera – P. trichocarpa* species complex, was necessary to verify the presence of an exotic allele from *P. trichocarpa*.

Obtaining the genotypes of the maternal trees was very useful to identify the paternal contributions to seeds, but it was difficult to accurately identify second and later generation hybrids. The limitation in identifying F_2, backcrosses and latter generation hybrids using a few nuclear markers was previously mentionned by Boecklen *et al.* (1997). Particularly, in the case of *P. deltoides,* it was not possible to use *PTR6* or *ORNL104* to identify the different types of hybrid offsprings like we did with the progeny from *P. balsamifera* maternal trees. Each of these microsatellite loci had one allele that was present in two of the four remaining species (*P. balsamifera*, *P. trichocarpa*, *P. nigra,* and *P. maximowiczii*), so the locus *WIN3* was necessary to quantify the amount of paternal exotic alleles present in the progeny.

4.2 Observed and effective rate of hybridization

Single sequence repeats (SSRs) or microsatellites are highly polymorphic codominant markers previously used in similar research studies in other parts of the world. Generally in previous studies, only two species needed to be discriminated at a given time. In Europe, where *Populus nigra* is the native species and *P. deltoides* the exotic, few hybrids were found between the 67 sampled trees and the offspring of three female trees genotyped (Fossati *et al.* 2003). In another study conducted in North America (USA), Slavov *et al.* (2003) detected only low amounts of intraspecific hybridization between *P. trichocarpa* populations. In our case, where offsprings from two native species were genotyped, the proportion of alleles from non conspecific paternal trees was variable, between 1% and 46%, depending on the site, the species or the year analyzed.

Our results clearly show a variation in interspecific gene flow between the two native species analyzed, the two sites and even the multiple years of sampling. In the progeny of *P. deltoides* at Sorel, there was a clear difference in the percentage of interspecific alleles

detected in 2004, compared to 2003 and 2005 when lower rates of interspecific gene flow were observed. Spatio-temporal factors are known to play an important role in the levels of gene flow (Ellstrand *et al.* 1999). We could postulate that late spring frosts may have had consequences on the phenology at Sorel, as they prevented us to collect seeds at the northern Matane site in 2004. For example, a change in the flowering time allowing more overlap or synchronicity between *P. deltoides* and the exotic species may have occurred in 2004, resulting in a higher proportion of interspecific paternal alleles in the *P. deltoides* progeny for that year.

For any given year, the percentage of interspecific paternal alleles detected when *P. deltoides* was the recipient species was lower than the percentage of interspecific paternal alleles observed when *P. balsamifera* was the recipient species, especially for the southern site of Sorel where both species could be sampled. Nonsynchronous flowering between *P. deltoides* and exotics could be a reason for this difference, a situation that may not be as favourable between *P. balsamifera* and the exotics. Other studies also suggested that asymmetrical natural hybridization between *P. balsamifera* and *P. deltoides* is possible and could explain variation in rates of hybridization (Hamzeh *et al.* 2007), as also shown between *P. alba* and *P. tremula* (Lexer *et al.* 2004). Other studies conducted by Rajora (1989) and Vanden Broeck *et al.* (2003) concluded that the low frequency of interspecific mating relative to conspecific mating could be due to a higher competitive ability of the conspecific pollen.

In addition, one apparent confounding factor must be examined closely in our study. On the Sorel site where the two native species could be sampled, the large differences observed between the two species in rate of interspecific hybridization might simply reflect the relative species abundance in the pollen cloud. There was also a significant difference in the proportion of interspecific (native and/or exotic) alleles detected in the progeny of *P. balsamifera* between both sites, Matane and Sorel, with much less frequent evidence of interspecific hybridization in the northern Matane site where *P. balsamifera* was highly abundant in the natural landscape. Thus, the difference observed between the two species

at the Sorel site likely reflects the scattered presence of *P. balsamifera* in the landscape and most likely, its weak occurrence in the pollen cloud. Consequently, there were more pollen donors from *P. deltoides* surrounding the site of Sorel that could pollinate *P. balsamifera*, than that in the northern site of Matane, which could explain the high rate of hybridization in *P. balsamifera* progeny in the southern site of Sorel. When one compares the level of interspecific hybridization for each species in its habitat of high occurrence, *P. balsamifera* in the northern study site, and *P. deltoides* in the southern site, the rate of interspecific hybridization did not differ much, reflecting more likely a pollen cloud dominated by conspecific components.

For both sites, most spontaneous hybrids were the results of crosses between the two native species. Although interspecific hybridization was higher between both natives than between each other with exotics, it does not necessarily suggest a stronger pre-zygotic reproductive barrier between the native and the exotic species studied herein. It could simply reflect the fact that for each site, exotic trees were simply fewer than native ones, hence reflecting again a difference in relative abundance in the pollen cloud. Because of the limited size of exotic plantations compared to the wild populations, the exotic pollen was likely "diluted" in a sea of native conspecific pollen and despite the proximity of exotic plantations and potential for high gene flow, the effective rate of spontaneous interspecific hybridization from exotics appeared to be limited on both sites, echoing the predictions from simulation (DiFazio *et al.* 1999) and field studies (Imbert and Lefevre 2003).

The abundance of conspecific native pollen has been reported as a key limiting factor for interspecific crosses under natural conditions (Vanden Broeck 2003). In Matane, where there was no *P. deltoides* in the natural forest because the site is located too far north for the species to establish itself, more hybrids were detected between *P. balsamifera* and the exotic *P. nigra* found in plantation. This further supports the hypothesis that the relative contribution of species to the pollen cloud is the key determining factor of the rate of

spontaneous interspecific hybridization rather than prezygotic reproductive barriers between species.

4.3 Hybridization versus introgression

It is necessary to emphasize that the hybridization rate is not the same as introgression. While we found evidence of offsprings harboring non-conspecific alleles, suggesting some possible effects on the genetic constitution of the local native population within a few generations (Meirmans *et al.,* submitted), there are other important factors to take into account for effective introgression to take place. Infiltration was detected but mating among conspecifics was predominant. For the observed rate of hybridization to become an effective rate of hybridization, several other conditions have to concur, such as adequate viability and fitness of hybrid seeds. Some studies suggest that hybrids are equal or could even be more fit than their parental species. For example, Schweitzer *et al.* (2002) found in artificial conditions that the hybrid offsprings of *Populus fremontii* x *P. angustifolia* (especially later backcross generations) were at least as fit as one of the parental species in both sexual and asexual reproductive parameters. But even if germination tests probe these hybrid seeds to be viable, they have to survive and strive in nature. Post-zygotic reproductive barriers, such as hybrid breakdown, could be limiting factors for the successful establishment of hybrids in natural conditions (Rieseberg and Carney 1998). And still, if they survive and develop into mature trees, they could be sterile and in that case, would not contribute to introgression. In conclusion, if hybrids are not fertile or fit enough to establish themselves successfully in nature, introgression would not occur. Germination tests and long-term studies of fitness in controlled environments and natural conditions would be necessary to evaluate these different factors.

The genetic markers used in the present study allowed detecting the infiltration of interspecific alleles in the progeny of native species but they were not sufficiently powerful to detect introgression. Although natural hybridization and introgression have been cited as potential causes for loss of biodiversity, studies conducted in several crops (Ellstrand *et al.*

1999) showed that in most cases, when alleles have introgressed in natural populations; the consequence is an enhancement of the local genetic diversity in the wild population (Ellstrand 2003). However, whether the enhanced genetic diversity is conducive of higher or lower fitness is poorly documented. Further research is necessary to clearly assess the possibility of hybridization and introgression between poplar trees with exotic components and native species in the natural forest. Yet, based on our results, useful conclusions can be drawn; we are able to attempt giving a response to this question. Considering that *P. x euramericana* (also called *P. x canadensis*, *P. deltoides* x *P. nigra*) was introduced 250 years ago and is established throughout Canada, if introgression of *P. nigra* exotic alleles in the native *P. deltoides* had been ecologically significant, we would expect some of the *P. deltoides* maternal trees sampled in the present study to display non conspecific alleles. Although the number of gene loci assayed was limited, the entire set of *P. deltoides* maternal trees sampled in this study failed to show interspecific components, although old *P. x euramericana* mature trees planted as windbreaks were disseminated throughout the agro-forestry landscape in the Sorel region. This observation indicates that there has not been significant introgression from *P. euramericana* into the native *P. deltoides.* Long-term studies using a larger number of markers have to be conducted to draw more accurate conclusions.

Even though our study main goal was to detect and quantify the existence of spontaneous hybridization between planted poplar trees with exotic components and native species in the natural surrounding stands, our results could be extrapolated to other situations. More specifically, our findings could provide a first glance at the potential risks of establishing plantations with genetically modified fertile trees in terms of potential gene flow in the natural environment. However, one should also consider the potential fitness value of the foreign gene (Meirmans *et al*. submitted) as it may or may not alter the fitness and ecological competence of the spontaneous hybrids (Difazio *et al.* 1999; Strauss *et al.* 2001).

4.4 Long-distance pollen migration versus close population hybridization and introgression

Our results indicate that all five poplar species are capable of naturally hybridizing with one another. The next phase of this research should be to integrate our results into a population model to estimate the risks of gene flow between populations following a number of key parameters. Most models developed until now rely on the interpretation of patterns of pollen dispersal. Generally the distance of pollen dispersal is viewed as a fundamental factor in the extent of hybridization and introgression, and most modeling studies focus on this parameter and the physical factors affecting it (Imbert and Lefevre 2003). Our results suggest that this modeling of long-distance pollen dispersal is not compulsory for hybridization and introgression to occur at a large scale. Other factors should also be considered, such as the actual fitness of the hybrids and introgressants, and the rate of hybridization between close populations. If the rate of spontaneous hybridization is high between a plantation and a recipient adjacent natural population, then at the next generation, the new hybrids could hybridize with trees from other adjacent stands and so on. This metapopulation scenario of stepwise gene dispersion, without long-distance pollen dispersal, could be very effective in disseminating alleles over long distances when many generations are taken into account (Meirmans *et al.,* submitted). However, the long generation time in trees such as poplar would slow down the rate of spread per year. Although potentially significant on the evolutionary scale, such effects would take hundreds of years to materialize at the landscape level in species such as poplars, unless most indigenous forests are lost (Merimans *et al*., submitted). Therefore, stand conversion and climate change are likely to be greater treats to the local natural gene pools, in the near future, than introgressive hybridization from exotic plantations.

4.5 Conclusion and perspectives

The microsatellite markers developed in the present study were capable of identifying first generation hybrids resulting from spontaneous crosses between native poplar and poplar with exotic components, as well as between different native poplar species. Our results provide a general picture of the occurrence of interspecific spontaneous hybridization among poplars in eastern Canada. Although the detected infiltration rate suggests the possibility of introgression even in the short term, these results have to be assessed carefully and in a broader context. Hybridization and introgression may pose a threat, but it is likely to remain low in areas of high natural abundance of native species, as indicated by our results.

As we stated previously, although spontaneous hybridization was present, other factors must be considered that contributes to reducing the ecological significance of the hybridization rate observed, in particular pollen dilution effects, seed survival and establishment as well as the relative fitness of hybrids in the natural environment. For the pollen dilution effects to be significantly reduced, a large proportion of the natural landscape would have to be substituted for exotic plantations, and the occurrence of natural populations in the landscape would have to be severely reduced, at least to a level seen in the agro-forestry landscape of the most southern study site, in Sorel.

Thus, the results of the present study give a first assessment of the potential interspecific gene flow arising from exotic plantations in eastern Canada, and provide useful information that should be interpreted within the limits of the study, and taking into consideration other factors that the present study did not assess. Once all key factors are reasonably considered, it will be possible to make fully informed decisions on the acceptable risks of large-scale populiculture in eastern Canada.

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