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Repeated unidirectional introgression towards *Populus balsamifera* in contact zones of exotic and native poplars

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Abstract

As the evolutionary significance of hybridization is largely dictated by its extent beyond the first generation, we broadly surveyed patterns of introgression across a sympatric zone of two native poplars (*Populus balsamifera*, *Populus deltoides*) in Quebec, Canada within which European exotic *Populus nigra* and its hybrids have been extensively planted since the 1800s. Single nucleotide polymorphisms (SNPs) that appeared fixed within each species were characterized by DNA-sequencing pools of pure individuals. Thirty-five of these diagnostic SNPs were employed in a high-throughput assay that genotyped 635 trees of different age classes, sampled from 15 sites with various degrees of anthropogenic disturbance. The degree of admixture within sampled trees was then assessed through Bayesian clustering of genotypes. Hybrids were present in seven of the populations, with 2.4% of all sampled trees showing spontaneous admixture. Sites with hybrids were significantly more disturbed than pure stands, while hybrids comprised both immature juveniles and trees of reproductive age. All three possible F1s were detected. Advanced-generation hybrids were consistently biased towards *P. balsamifera* regardless of whether hybridization had occurred with *P. deltoides* or *P. nigra*. Gene exchange between *P. deltoides* and *P. nigra* was not detected beyond the F1 generation; however, detection of a trihybrid demonstrates that even this apparent reproductive isolation does not necessarily result in an evolutionary dead end. Collectively, results demonstrate the natural fertility of hybrid poplars and suggest that introduced genes could potentially affect the genetic integrity of native trees, similar to that arising from introgression between natives.

Keywords: hybridization, introgression, *Populus balsamifera*, *Populus deltoides*, *Populus nigra*, single nucleotide polymorphisms

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Introduction

A major concern with the intentional introduction of organisms with novel traits is the unintentional entry of non-native genetic material into the natural environment through introgression with wild relatives (Anderson & Hubricht 1938; Chapman & Burke 2006). The potential spread of non-native genome regions (including exotic genes, transgenes or any type of heritable genomics-derived modification) into natural populations will depend in part upon the frequency of spontaneous hybridization events, F1 fertility, the establishment and viability of various classes of hybrid offspring, as well as the fitness of the new trait in different ecological contexts (e.g. Burke & Rieseberg 2003; Wilkinson *et al.* 2003; Meirmans *et al.* 2009). Modelling studies, furthermore, indicate that the potential for a new gene region

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to spread across the range of a native species highly depends on the structure of natural populations and the fitness consequences of the trait in question (Meirmans *et al.* 2009). Studies of contact zones that comprise native and exotic species can provide insight into the potential for new gene regions to breach the species barrier and make a foray into native genomes.

Populus species and their hybrids ('poplars') are favoured for emerging biotechnological applications in biofuels, carbon sequestration and environmental remediation (Tuskan *et al.* 2006; Sticklen 2008). As poplars have generally weak reproductive barriers between species, patterns of realized hybridization within the genus have an immediate practical relevance to the sustainable deployment of new poplar cultivars for novel purposes. Here, we focus on hybridization among the European exotic *Populus nigra* L. and two species that are native to North America, *Populus balsamifera* L. and *Populus deltoides* Marsh. The two native species differ ecologically: *P. balsamifera* is found at higher latitudes (42–68°N) across the boreal zone, from lower flood plains to disjunct forested patches among the arctic tundra, while *P. deltoides* is preferentially found near streams and bottomlands from 28–46°N (Burns & Honkala 1990). Although primarily peripatric, a broad zone of natural range overlap extends across most of the continent, from Alberta to Québec, Canada (Eckenwalder 1984). Like all poplars, the three species are wind-pollinated and dioecious (and therefore obligate outcrossers) and the mean dispersal distance of pollen can be substantial (e.g. 7.6 km, Slavov *et al.* 2009). Trees typically reach reproductive maturity after ten years, producing seeds that are adapted to long-distance dispersal (Burns & Honkala 1990). Additionally, these three species are easily crossed (e.g. Willing & Pryor 1976; Stettler *et al.* 1980) and have congruent flowering times in areas of range overlap, allowing for natural hybridization to occasionally occur (Eckenwalder 1984). Traditionally, Populus species have been classified into different sections based on their morphological characteristics and potential for interbreeding (Dickmann 2001), although these sections conflict with phylogenetic evidence in a few cases (e.g. *P. nigra*, Hamzeh & Dayanandan 2004). *Populus balsamifera* has been placed in a different section of the genus (section Tacamahaca) than *P. deltoides* and *P. nigra* (section Aigeiros) and hybridization between these sections has been well documented (e.g. Eckenwalder 1984).

Although several studies have documented cases of hybridization between pairs of these three poplar species (e.g. Eckenwalder 1984; Arens *et al.* 1998; Fossati *et al.* 2003; Floate 2004; Hamzeh *et al.* 2007), both the issue of whether advanced-generation introgressants can naturally establish, and where these hybrids tend to occur have remained unclear. Apparent reproductive isolation may result from the use of a paucity of unlinked markers, a low frequency of backcross events teamed with insufficient sampling of standing trees in natural populations, a short duration of contact or habitat-mediated selection against introgressants in particular study environments (e.g. habitats where parental species have higher fitness). Hybrid formation between *P. deltoides* and *P. balsamifera* has long been inferred based on morphological characters (Eckenwalder 1984; Floate 2004) and has recently been documented with molecular markers by Hamzeh *et al.* (2007). Leaf morphology suggests that of the characters of *P. deltoides* can introgress into a *P. balsamifera* background in southern Alberta, Canada. Through the use of two single nucleotide polymorphisms (SNPs) from the nuclear rDNA internal transcribed spacer and four SNPs from the chloroplast, 23 F1s between *P. deltoides* and *P. balsamifera*, representing selected putative hybrids (*P.* × *jackii*) from natural populations, were genotyped. However, in this study, the SNP sampling strategy was not designed to detect hybridization beyond the F1. In another study, Meirmans *et al.* (P.G. Meirmans, M. Lamothe, M.-C. Gros-Louis, D. Khasa, P. Périnet, J. Bousquet, N. Isabel, personal communication) genotyped thousands of seeds and greenhouse-reared seedlings from female *P. balsamifera* and *P. deltoides* adjacent to two plantations of exotic poplars (including *P. nigra*). Hybrid progeny between cultivated trees and natives were formed, with much higher rates of hybridization on *P. balsamifera* than on *P. deltoides* mothers (P.G. Meirmans, M. Lamothe, M.-C. Gros-Louis, D. Khasa, P. Périnet, J. Bousquet, N. Isabel, personal communication). The potential for gene flow between *P. deltoides* and *P. nigra* has been studied quite extensively within Europe using microsatellite and AFLP markers (e.g. Arens *et al.* 1998; Fossati *et al.* 2003; Vanden Broeck *et al.* 2004). These European studies collectively indicate that gene flow stops at the first generation (but see Vanden Broeck *et al.* 2004) where many exotic poplars have been planted in recent times (e.g. the 1940s, Fossati *et al.* 2003), although the number of informative markers used may limit inferences of advanced gene flow in some of these cases.

In contrast with some European study sites, exotic poplar species and hybrid cultivars (e.g. *P.* × *canadensis*, a spontaneous cross between *P. deltoides* and *P. nigra*) of multiple origins (Zsuffa 1977) have been broadly planted by North American homesteaders since at least 1862 (Richardson *et al.* 2007) allowing ample opportunity for later generation hybrids to form. In this study, we assemble an array of species-specific SNP markers, then broadly sample and genotype trees from natural populations across a long-standing contact zone of

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native and exotic poplar species to address the following questions: (i) Do spontaneous hybrid poplars establish and reach reproductive age? (ii) Are advanced-generation hybrids detected in nature? (iii) How do patterns of introgression compare between introduced trees and the two native species? (iv) Are sites where hybrids are detected more disturbed, as per the classic suggestion by Anderson (1948)? We also use cpDNA polymorphisms to assess the maternal parentage of any detected hybrids. As research on new uses of *Populus* species and their hybrids continues to intensify, the responses to these questions bear not just on our understanding of the maintenance of species boundaries, but also have important implications for the intentional planting of exotic species with novel traits and their potential impact on native biodiversity.

Materials and methods

Marker development

Our broad goal was to develop a high-throughput interspecific SNP assay for markers that are completely fixed within species but differ among species. Seven species-specific SNPs from six gene regions had previously been developed to diagnose *Populus balsamifera*, *Populus deltoides* or *Populus nigra*, as described in Meirmans *et al.* (2007). As the unambiguous detection of advanced-generation hybrids requires multiple loci, additional species-specific SNPs were identified through DNA sequencing. Putative DNA regions were identified by BLAST searches of orthologues (found through literature surveys) against the *Populus trichocarpa* Nisqually-1 genome sequence (version 1.1) in the Joint Genome Initiative database (http://genome.jgi-psf.org/Poptr1_1). Primers were designed using an in-house modification of Primer3 (Rozen & Skaletsky 2000), for discrimination of homologous sequences. Primer sequences used to amplify the gene regions are available in Table S1 (Supporting information).

DNA was extracted from pure provenances of *P. balsamifera* (60 individuals), *P. deltoides* (25 individuals) and *P. nigra* (25 individuals) with a DNeasy plant kit (QIAGEN) according to the manufacturer's instructions (accessions detailed in Meirmans *et al.* 2007). DNA was pooled (as described in Meirmans *et al.* 2007), then regions were amplified by PCR using a MJ Research PT-200 thermocycler. Fifteen-microlitre reactions contained 1× PCR buffer (Invitrogen), 0.13 μM of each primer, 0.17 μM of each dNTP, 2.0 mM $MgCl_2$ and 1 U Platinum *Taq* polymerase (Invitrogen) according to the following conditions: an initial 4 min at 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 45 s at 72 °C; followed by a final 5 min at 72 °C. PCR products were verified and approximately quantified through electrophoresis, then sequenced at the McGill University and Génome Québec Innovation Centre (MUGQIC) on a 3730×l DNA Analyzer system (Applied Biosystems), according to their internal protocols.

Chromatograms were aligned and visually inspected with SeqMan version 7.1.0 (DNAStar, Inc.). SNPs that appeared species-specific were scored and for a subset of these SNPs, their flanking nucleotide sequences were used to develop SNP primers (Table S1, Supporting information) for high-throughput genotyping with a Sequenom iPlex Gold SNP array. SNP positions were localized assuming synteny with the genome sequence of *P. trichocarpa* Nisqually-1 and their positions were illustrated using MapChart version 2.1 (Voorrips 2002).

Field sampling and morphology

A total of 635 samples were collected from 15 populations across the area of sympatry between *P. balsamifera* and *P. deltoides* within Quebec, Canada (Fig. 1, Table S2, Supporting information). Sites were chosen near intentional plantings of the exotic *P. nigra* 'Italica', a male clone, and several cultivars of *P.* × *canadensis* planted in city parks (e.g. Figs 2a, S1, Supporting information). Field work was conducted in 2007 during the winter because poplar architecture could be easily distinguished from other tree species even at great distances (Fig. S1, Supporting information). Trees from different age classes that are relatively remote from each other were randomly sampled within a stand with an effort made to avoid sampling clones. Tree age class was approximately estimated, according to five broad classes (0–2, 2–10, 10–20, 20–40, and >40 years of age), with a nondestructive method. Estimation of age for each of the sampled trees was based on the number of verticils counted from the base to the top of the tree. This nondestructive technique was performed to be able to return to sites where hybrid(s) could be detected and exhaustively sample the entire population in further studies. As field work occurred during the winter, branches were collected from dormant trees. The species were identified based on bud morphology (e.g. number of bud scars), branch colour and architecture, and bark features according to Maini (1968) and Fernald (1970).

The degree of anthropogenic disturbance was indexed for all sites based on a crude scale of 1 (seemingly natural) to 3 (highly disturbed) (Fig. S2, Supporting information). When populations spanned clearly heterogeneous habitats, arithmetic means were calculated to give an average disturbance index for each sample site. Branches were forced in warm glasshouses to provide fresh leaf material for morphological reverification (following Maini 1968) and for the extraction of DNA.

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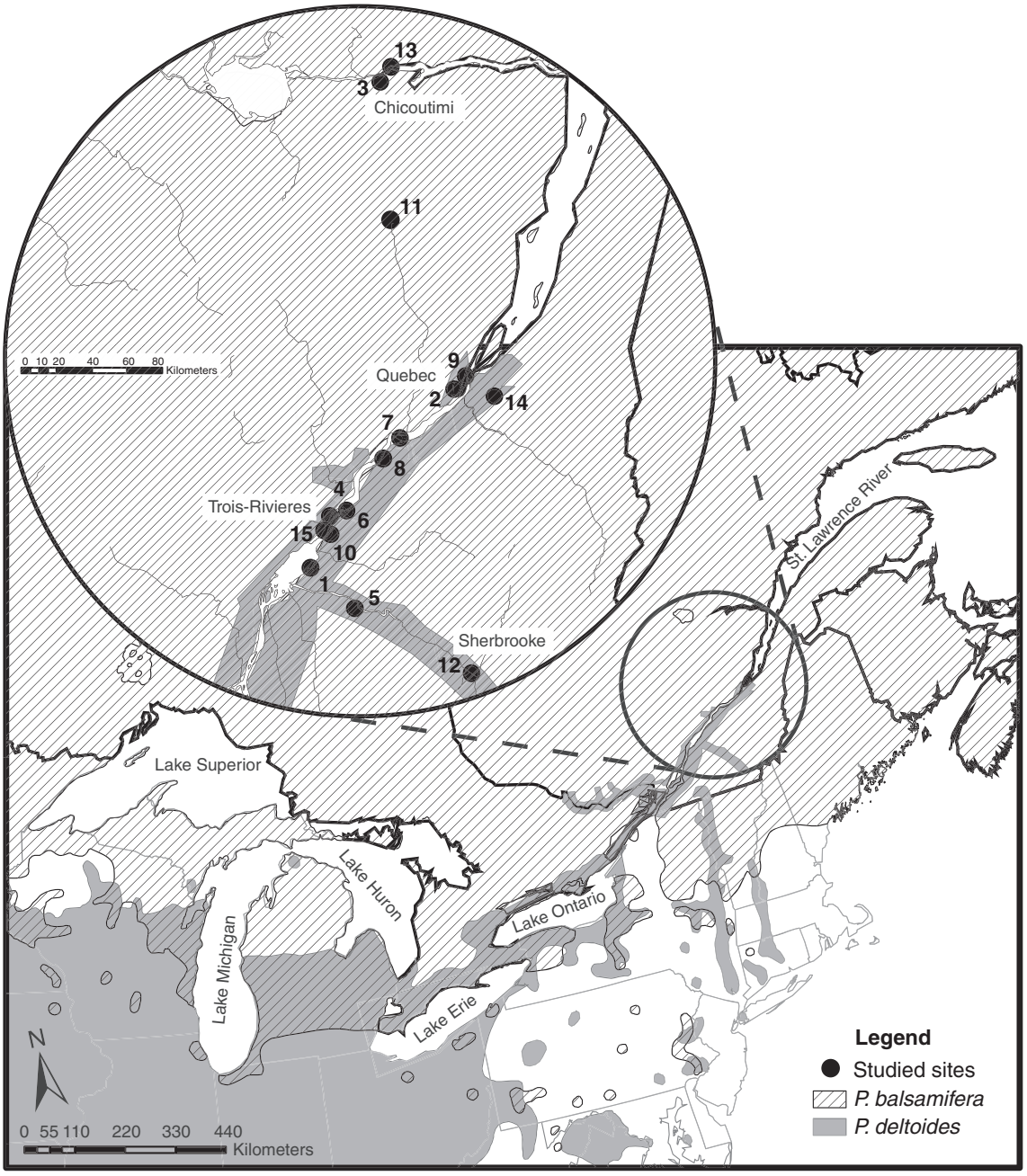

Fig. 1 Range map of *Populus deltoides* and *P. balsamifera* in northeastern North America. Studied populations in Québec are shown in the map enlargement (●). Data used to generate the map for USA and Ontario are based on Little (1971), U.S. Geological Survey (1999) and Rousseau (1974) for Québec. Precise geographic coordinates for each sampled population can be found in Table 1.

High-throughput genotyping

DNA was extracted from dried or fresh leaf tissue using a Nucleospin 96 plant kit with the PL2/PL3 and PC buffers from a Nucleospin II plant kit and a vacuum manifold according to the manufacturer's instructions. SNPs were genotyped with the Sequenom iPlex Gold technology at the McGill University and Génome Québec Innovation Centre (MUGQIC) using their internal protocols.

Admixture analyses

In the event that our putative diagnostic SNPs proved to be polymorphic within species (i.e. occur at frequencies lower than our detection threshold), Structure version 2.3.1 (Pritchard *et al.* 2000) was used to analyse the SNP data. All 635 trees that were genotyped with our SNP array were included, as were the genotypes from 30 sequenced pure individuals used for marker devel-

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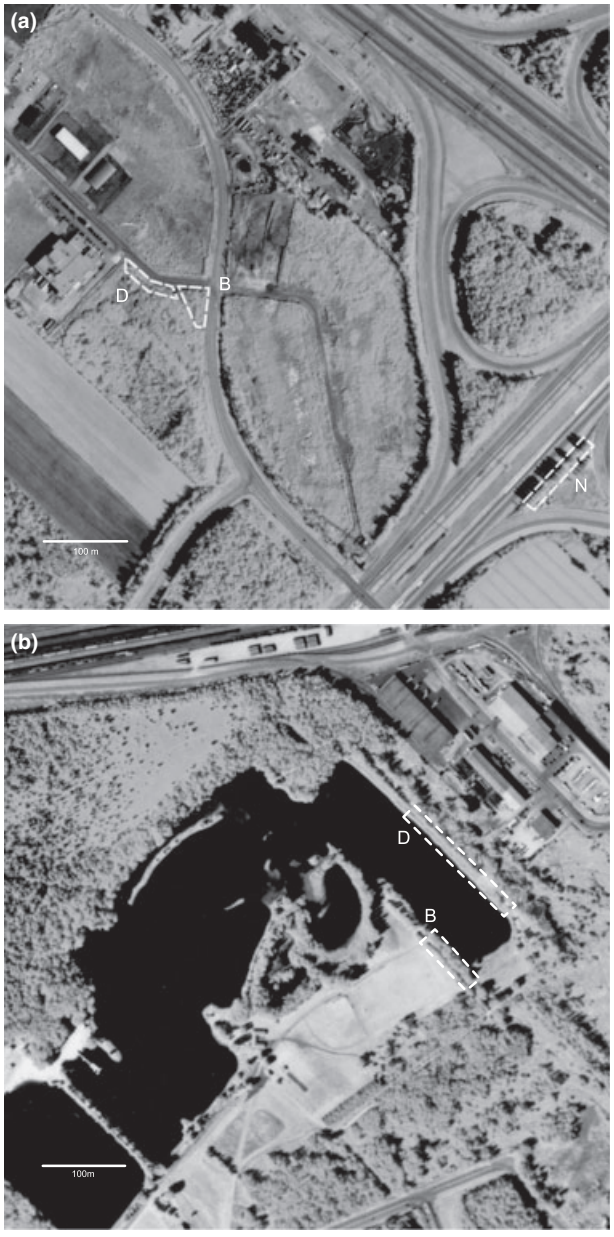

Fig. 2 Aerial photographs of two of the studied sites illustrating different levels of anthropogenic disturbance. (a) One of the Nicolet sites (index 3 – highly disturbed) is located in an industrial zone. Natural regeneration of either *P. balsamifera* (B) and *P. deltoides* (D) were sampled. Some *P. nigra* 'Italica' (N) are present on the southeast side of the site. (b) Base de plein air de Sainte-Foy (nearly 1 – seemingly natural). Natural regeneration of each *P. balsamifera* (B) and *P. deltoides* (D) adjacent to a city park were sampled. *P. nigra* 'Italica' are found within a distance of 500 m (not shown). Dashed lines delineate either areas where *P. balsamifera* (B) and *P. deltoides* (D) were sampled or *P. nigra* 'Italica' (N) was located.

opment (10 from each species). An admixture model was used, alpha (the Dirichlet parameter, α, for degree of admixture) inferred, lambda (the allele frequency prior, λ) was defined as one (i.e. the default value was used), and $K = 3$ was assumed as there are three putative parental species within the sampling area. Markov chain Monte Carlo replicates were run for an initial burn-in of 100 000, followed by 500 000 replicates. Probability intervals for admixture estimates were determined with the ANCESTDIST function. Exploratory analyses at $K = 2$ and 4 were also performed for the same parameter set. Based on the Structure analysis, individual trees were characterized as belonging to one of the three putative pure species (according to an a priori threshold of greater than 90% posterior probability for one of the three classes), or as admixed.

To further validate our classification of admixed individuals, NewHybrids (Anderson & Thompson 2002) was used. This program was developed for analyses of hybrids between a single pair of species, so consequently three data sets were generated for all pairs of species and their hybrids, based on Structure results. This was performed by excluding all individuals with greater than 0.05 posterior probabilities of membership in one of the three clusters (i.e. all pure *P. nigra* and its hybrids eliminated in the first data set, all pure *P. deltoides* and its hybrids in the second data set, and all pure *P. balsamifera* and its hybrids in the third data set). Jeffreys priors were used with a burn-in of 5000 sweeps followed by 10 000 sweeps. Posterior probability of assignment as pure species, F1s, F2s and backcrosses were all noted.

Chloroplast haplotyping

As an indicator of the directionality of the crosses (i.e. the species designation of the maternal line), the *trn*L intron was amplified and sequenced from all admixed individuals (as detected by the above analyses), as well as a subset of individuals identified as pure species. Previous studies (Hamzeh & Dayanandan 2004; Hamzeh *et al.* 2007) had indicated that variation within this region was sufficient to identify the chloroplast contributions by *P. balsamifera*, *P. deltoides* or *P. nigra*. Primers C and D (Taberlet *et al.* 1991) were used in PCR reactions that were identical to those described for nuclear regions above, while the following amplification profile was used: 5 min at 95 °C; 30 replicates of 1 min at 92 °C, 1 min at 55 °C, 3 min at 72 °C; followed by 10 min at 72 °C. Sequencing reactions were also identical to those for nuclear gene regions.

Analyses of age and site disturbance

The distribution of tree age was noted within each pure species and within any detected admixed individuals. A *G*-test of independence evaluated whether any differences between sexual status (i.e. immature vs.

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reproductively mature) were significant for trees in the admixed (hybrid) or parental classes. Average disturbance indices were compared between two classes of sites: those where admixed trees were present and those where admixed trees were absent. A *t*-test was used to determine whether sites with admixed trees were significantly more likely to be disturbed.

Results

Marker development

A total of 35 SNPs that diagnosed one of the three species were selected for use from 24 gene regions. An additional three SNPs that were specific to *P. trichocarpa* were included in the SNP assay for our own internal purposes (i.e. not relevant to this study). These regions spanned 15 of the 19 linkage groups (chromosomes) found throughout all poplar species plus one unassembled scaffold (Scaffold 40, Fig. 3). Included within these gene regions were 11 SNPs from 11 different linkage groups that diagnosed *P. balsamifera*, 11 SNPs from eight linkage groups that diagnosed *P. deltoides* and 13 SNPs from 13 linkage groups that diagnosed *P. nigra* (Fig. 3). The physical distance between each gene region on the same chromosome ranged from 325 kb to 7.5 Mb (Table S1, Supporting information).

Field sampling and morphology

A total of 635 trees were sampled from 15 populations across the contact zone (Fig. 1, Table 1). Because of progressive degradation from expanding human habitation and land use, it was difficult to find natural regeneration of *P. deltoides* in riparian environments (e.g. Fig. S1, Supporting information) for sampling. It was far easier to locate cohorts of natural *P. deltoides* on disturbed sites including gravel pits, industrial parks, commercial districts and along public transportation routes such as roads and railway tracks (e.g. Fig. S2, Supporting information). Ultimately, the sample size within populations ranged from 9 to 82 trees (Table 1). Populations primarily comprised various mixtures of pure *P. balsamifera* and *P. deltoides*. Based on winter morphology, they were easily distinguished. A few hybrid poplars (*P.* × *canadensis*: an intentional cross between *P. deltoides* and *P. nigra*) were also identified in public parks adjacent to native stands. When twigs were forced in the greenhouse, leaf morphology was always consistent with the winter morphology.

High-throughput genotyping

The Sequenom assay originally comprised 42 SNPs, yet four of these SNPs (9.5%) gave consistently poor results across all individuals, so they were excluded from all further analyses [as well as from Fig. 3 and Table S1 (Supporting information); we mention this fact solely as a convenience for those interested in using Sequenom]. Sequenom genotyping based on the remaining 38 loci resulted in a total of 20 395 genotypes and 3773 counts of missing data. This indicates a 84.4% success rate using our samples with Sequenom, which is comparable with other high-throughput genotyping studies conducted using Illumina (e.g. 78.5–82%, Pavy *et al.* 2008).

Admixture analyses

By analysing the 35 relevant loci with Structure, the use of $K = 3$ groups resulted in very clear clustering, with

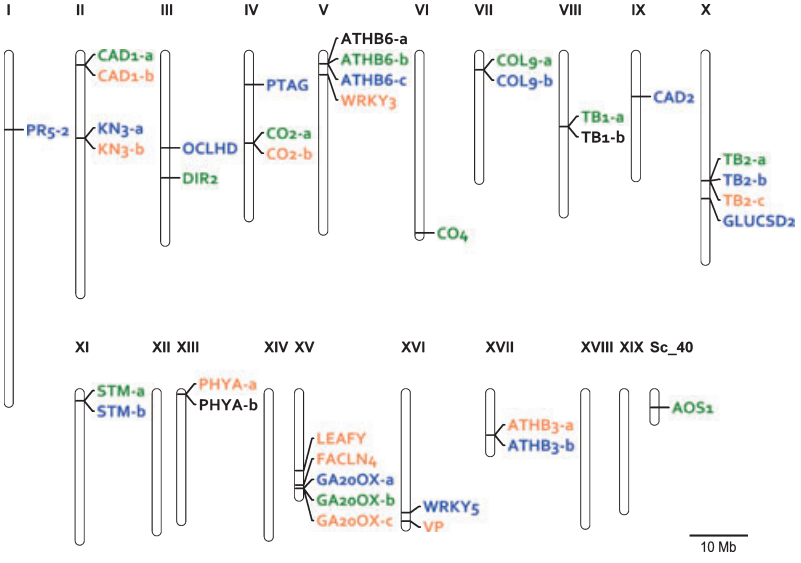

Fig. 3 Physical location of 38 diagnostic SNPs from 24 gene regions on 15 of the 19 *Populus* linkage groups (plus one unassembled scaffold). Map locations were determined assuming synteny with the genome sequence of *P. trichocarpa* Nisqually-1. Green: *P. balsamifera* SNPs; orange: *P. deltoides* SNPs; blue: *P. nigra* SNPs; black: *P. trichocarpa* SNPs (developed for internal purposes and not included in subsequent analyses).

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Table 1 Genetic constitution of sampled trees across a contact zone of exotic and native *Populus*. Variation at 35 SNPs was assessed within 15 populations that varied in their degree of disturbance and comprised the introduced *P. nigra* and native *P. balsamifera* and *P. deltoides*. Hybrids were detected within 7 of the 15 populations and represented 3% of all sampled trees

Pop. no.	Locality	Average Site Disturbance Index	No. samples	No. *P. deltoides**	No. *P. balsamifera**	No. *P. nigra**	Hybrids identified†
1	Baie-du-Fèvre	2.0	30	30	—	—	—
2	Base de plein air de Sainte-Foy	1.5	35	17	15	—	16, 17, 18
3	Chicoutimi	2.5	43	—	40	—	13, 14, 15
4	Cap de la Madeleine	2.0	57	31	25	—	12
5	Drummondville	2.0	61	29	30	—	2‡, 3‡
6	Gentilly	2.0	81	80	—	1	—
7	Joly	2.0	29	—	28	—	11
8	Leclercville	1.0	9	9	—	—	—
9	Domaine Maizerets	1.0	28	28	—	—	—
10	Nicolet	2.8	79	56	21	—	1‡, 4
11	Route 175	1.0	51	—	51	—	—
12	Sherbrooke	2.0	52	5	41	—	5, 6, 7, 8, 9, 10
13	Saint-Fulgence	2.0	29	—	29	—	—
14	Saint-Henri	2.0	22	—	22	—	—
15	Trois-Rivières	2.0	29	29	—	—	—
		Total	635	314	302	1	18

*Posterior probabilities exceeding 92% for one of $K = 3$ in Structure (Pritchard *et al.* 2000).
†Identification of hybrid individuals as given in Fig. 5.
‡Intentionally planted.

most individuals showing a very high probability of membership within one of the three groups (Fig. 4). For a single test run at $K = 2$, we found that Structure categorized the 11 *P. nigra* samples as admixtures between a *P. deltoides* group (70%) and a *P. balsamifera* group (30%), which made little biological sense. The use of $K = 4$ yielded results wherein all of the pure *P. deltoides* individuals were approximately 50% admixed, indicating a K that is too high. As SNPs were intentionally selected to differentiate the three species, $K = 3$ was the most credible K, with no noteworthy additional structure.

Overall, 2.8% of the sampled trees showed some degree of admixture (Table 1), with admixed individuals found in 7 of the 15 sampled populations (53%, Table 1). We designated the 617 sampled individuals with posterior probabilities of greater than 90% for one of the three clusters as pure species, with 314 individuals assigned to the *P. deltoides* cluster, 302 assigned to the *P. balsamifera* cluster and 1 individual assigned to the *P. nigra* cluster. The remaining 18 individuals showed signatures of admixture, with at least 15% posterior probabilities observed in two or more clusters (Figs 4 and 5).

Among the 18 individuals that showed signatures of admixture (Fig. 5), 10 were designated as F1s: those individuals with approximately 50% admixture for each of two species (probability intervals of the 10 estimates ranged from the most extreme lower bound of 0.310 to the most extreme upper bound of 0.689). All three possible combinations of F1s were present. Three of the four *P. deltoides* × *P. nigra* F1s were very likely to have been intentionally planted as hybrid cultivars, which would decrease our rate of naturally occurring hybrid detection to 15/635 or 2.4% of all sampled trees. Seven individuals were designated as advanced-generation introgressants, showing between 18 and 38% admixture

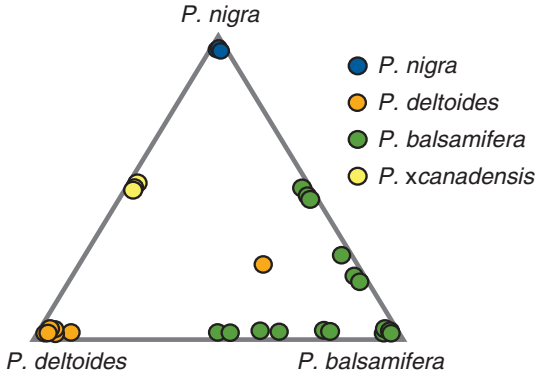

Fig. 4 Morphological species designation (colour) compared with genetic constitution (triangular position) of studied *Populus*. 35 SNPs were used to cluster trees into $K = 3$ groups, comprising two native (*P. balsamifera* and *P. deltoides*) and one exotic species (*P. nigra*). This included 635 trees sampled from 15 populations across a broad contact zone plus 10 remotely sampled individuals from each of the three pure species. Advanced-generation hybrids are consistently biased towards *P. balsamifera* and this asymmetric introgression is not revealed by tree morphology.

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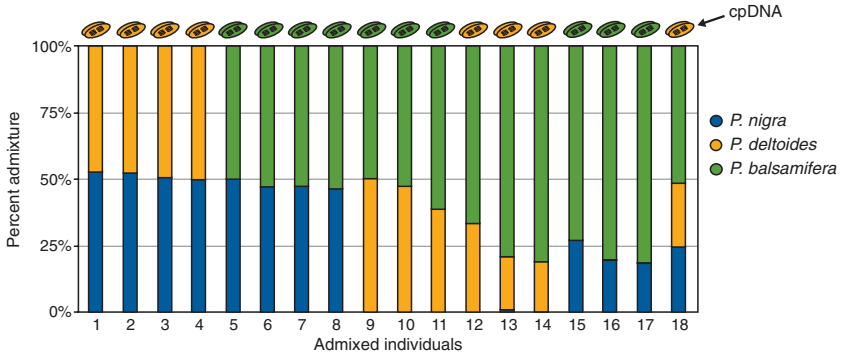

Fig. 5 Detailed genomic constitution of admixed *Populus* inferred by Bayesian clustering based on 35 SNPs. Each individual is shown as a vertical line partitioned into $K = 3$ coloured components representing membership in genetic clusters corresponding to the two native species *P. balsamifera* and *P. deltoides* and the exotic species *P. nigra*.

(most extreme lower and upper bounds of probability intervals of 0.074 and 0.467, respectively) for the *P. deltoides* group, the *P. nigra* group or both using Structure (Fig. 5). Backcrosses were consistently biased towards one of the native species, *P. balsamifera*, with posterior probabilities for membership within the *P. balsamifera* class ranging from 61.0 to 81.4% (probability intervals with an extreme lower bound of 0.523, extreme upper bound of 0.924) (Fig. 5). Interestingly, one trihybrid was detected whose genome contained approximately 50% *P. balsamifera*, 25% *P. deltoides* and 25% *P. nigra*, suggesting that a F1 between *P. deltoides* and *P. nigra* (possibly intentionally planted) had crossed with a pure *P. balsamifera* tree (individual 18, Fig. 5).

The three pairwise NewHybrids data sets further confirmed our classification of pure species and hybrids. The 617 pure species identified by Structure were likewise identified as pure, but with even higher posterior probabilities (range = 0.993–1.000). The same 18 individuals showed signs of admixture. Four *P. deltoides* × *P. nigra* F1s (individuals 1–4, Fig. 5), four *P. nigra* × *P. balsamifera* F1s (individuals 5–8, Fig. 5) and two *P. balsamifera* × *P. deltoides* F1s (individuals 9 and 10, Fig. 5) were identified, with posterior probabilities ranging from 0.936 to 1.000 for all F1 assignments. Additionally, seven backcrosses towards *P. balsamifera* were found (posterior probabilities of assignment ranging from 0.915 to 1.000), with either *P. deltoides* (individuals 11–14, Fig. 5) or *P. nigra* (individuals 15–18, Fig. 5) as the alternate parent. Note that it was not feasible to analyse the trihybrid with NewHybrids.

The morphological assignment was identical to that of the genetic clustering analyses for 620 of the 635 trees (97.6%, Fig. 5), further supporting our designation of pure species, as described above. The morphological classification of *P.* × *canadensis* hybrids (i.e. *P. deltoides* × *P. nigra* crosses) was corroborated by genetic analyses in three out of four cases. All of the remaining 14 hybrids were misdiagnosed based on morphology, with one admixed tree exhibiting a *P. deltoides* phenotype and 13 hybrid trees resembling *P. balsamifera*.

Chloroplast haplotyping

Seven polymorphisms within the *trn*L intron unequivocally diagnosed the chloroplast constitution of each of the three parental species. The admixed individuals exhibited either the chloroplast haplotype of *P. balsamifera* (10 individuals) or *P. deltoides* (eight individuals, Fig. 5). F1s between *P. deltoides* and *P. nigra* had the chloroplast haplotype of *P. deltoides*, F1s and backcrosses between *P. balsamifera* and *P. nigra* consistently contained the chloroplast of *P. balsamifera*, while F1s and backcrosses between *P. balsamifera* and *P. deltoides* exhibited the chloroplast haplotypes of either of the two species (Fig. 5). The chloroplast of the trihybrid matched that of *P. deltoides* (Fig. 5).

Analyses of age and site disturbance

Of the 635 sampled trees, 65 had missing age data, either because of human oversight or inability to assess tree age with confidence (10.2% of sampled individuals). The remaining 570 age-assessed trees comprised both immature juveniles (<10 years of age) and reproductively mature trees (>10 years old, Table 2). The tree age distributions were relatively consistent among the pure native species, and remarkably similar between

Table 2 Distribution of age classes of species of *Populus* and their hybrids sampled across a contact zone of native and exotic poplars. The three oldest hybrids were *P.* × *canadensis*, which is intentionally planted in this region

	Estimated age (years)				
	Immature		Reproductively mature		
	0–2	2–10	10–20	20–40	>40
P. deltoides	5	222	23	61	2
P. balsamifera	0	169	71	1	0
Hybrids (all classes)	0	11	3	1	3
Total	5	402	97	63	5

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hybrid and pure categories. Hybrid poplars do indeed reach reproductive age and were not significantly more likely to be immature as was the case for the pure species (*G*-test, $P = 0.84$, d.f. = 1). Mature trees included three *P. deltoides* × *P. nigra* hybrids, which were likely to have been intentionally planted.

In localities where hybrids were present, sites were significantly more disturbed (*t*-test, d.f. = 13, $P = 0.029$). The mean disturbance index was 1.57 ± 0.17 for sites that only contained pure tree species ($n = 7$), compared with 2.14 ± 0.16 for sites with the presence of hybrids ($n = 8$). These sites with admixed individuals were also more heterogeneous as evidenced by more intermediate values (Table 1).

Discussion

Asymmetric introgression among three poplar species

We determined the frequency (2.4%) and location of naturally occurring hybrids through broad surveys of populations across a contact zone of one exotic and two native poplar species. By SNP genotyping, we demonstrated that F1s form in all pairwise combinations of the three species. All three of these pairings were inferred to be fertile in nature, based on the detection of advanced-generation hybrids (Figs 4 and 5). The observed introgression was consistently biased towards one of the native species, *Populus balsamifera,* regardless of whether the alternate parent was native (*Populus deltoides*), exotic (*Populus nigra*) or exotic hybrid (*Populus* × *canadensis*). Although few advanced-generation hybrids were detected overall, the pattern and frequency of gene exchange between exotic *P. nigra* and *P. balsamifera* was comparable with that between the two native species.

Our detection of advanced-generation introgressants indicates that hybrids can and do establish themselves in natural populations. The admixed individuals that we detected in this study tended to be found in more disturbed habitats (*t*-test, Fig. S2, Supporting information). Anthropogenic perturbations have long been discussed as factors that promote hybrid establishment (e.g. Marie-Victorin 1922; Anderson 1948), as disturbance can provide niches that are distinct from those of the parental forms. In contrast with a 'Bartonian' tension zone governed by dispersal and selection against hybrids (Barton 1979; Barton & Hewitt 1985), hybrid lineages may, in some cases, be maintained by ecological selection against parental genotypes in disturbed habitats (e.g. Rand & Harrison 1989) when the population dynamics are favourable.

As we did not observe any backcrosses of *P.* × *canadensis* with either of its parental species, gene exchange between *P. deltoides* and *P. nigra* could potentially be blocked at the F1. However, it remains quite feasible that backcrosses can and do indeed form between these two species, yet at rates below our ability to detect them through broad sampling. Of note here, our discovery of a three-way hybrid suggests that any putative reproductive isolation between *P. deltoides* and *P. nigra* may not necessarily result in an evolutionary dead end, as crosses with *P. balsamifera* may represent an additional pathway for introgression to occur. Although only a single individual was observed, this is one of the first reports of trihybrid detection in nature (but see *Iris nelsonii*, Arnold 1993), highlighting the importance of multispecies approaches in some complex systems with extensive reticulation such as *Populus*.

This pattern of unidirectional gene flow towards one species has been noted in many other systems (e.g. Bacilieri *et al.* 1996) including other *Populus* species. Genes from *Populus fremontii* × *Populus angustifolia* hybrids preferentially introgress into *P. angustifolia* backgrounds in natural populations (Keim *et al.* 1989; Martinsen *et al.* 2001). Partial postzygotic isolation between these species was first confirmed in a preliminary crossing study, which suggested the action of late-acting genetic incompatibilities in some hybrids. Genotyping studies within *Populus alba* and *Populus tremula* hybrid populations has likewise uncovered unidirectional introgression (towards *P. alba*), yet habitat-mediated selection related to disturbance, as well as differences in the extent of clonal reproduction may be key factors in this system (Lexer *et al.* 2005; van Loo *et al.* 2008).

Why might gene flow be unidirectional?

Although the sources of the observed asymmetry in gene flow remain uncertain, we can speculate about the extent to which a few of the many possible factors may contribute (e.g. prezygotic mechanisms, abundance effects, types of epistatic interactions). First, several lines of evidence suggest that prezygotic barriers are unlikely to be contributing factors to the observed asymmetry in introgression. All three species have congruent flowering times within the study region (April to May) and more phenological variation is generally found within vs. among the native species (P. Périnet, personal observation). Previous studies of relative pollen performance in experimental crosses between *P. deltoides*, *P. balsamifera* and *P. nigra* (among others) indicate a lack of significant differences among these species for per cent pollen germination, length of pollen tube growth and number of micropylar penetrations when pollen was applied from only one species at a time (Guries & Stettler 1976), suggesting a general weakness of prezygotic barriers. However, the effectiveness of

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prezyogtic mechanisms under the application of pollen mixtures from more than one species remains unclear, although the action of mentor effects has been discussed (Stettler *et al.* 1980).

Second, biases in relative abundance can influence the direction of gene flow (Barton & Hewitt 1985; Burgess *et al.* 2005; Lepais *et al.* 2009). Population size differences have been evoked in a study of hybridization rates in seed from native poplars adjacent to plantations (P.G. Meirmans, M. Lamothe, M.-C. Gros-Louis, D. Khasa, P. Périnet, J. Bousquet, N. Isabel, personal communication). Relative abundance may contribute to asymmetric introgression of *P. nigra* genes, as this exotic species is much less abundant and expected to contribute less to the pollen cloud than the native *P. balsamifera* (please note that *Populus* is dioecious and that nearly all planted *P. nigra* are male clones; while *P. nigra* females were not observed, making the abundance differential even more profound for the case of *P. nigra* as the female parent). We may actually be underestimating the potential for *P. nigra* to hybridize with native species due to this density difference. However, the abundances of both native species within sampled populations were comparable (Table 1) and we likewise observe asymmetry of *P. deltoides* alleles into *P. balsamifera*, making it difficult to broadly ascribe the pattern to abundance alone.

Finally, epistatic interactions can render hybrids of particular classes sterile, or be lethal in some cases, resulting in gene flow asymmetry. In this study, we have noted some cursory biases in marker introgression, although we consider analyses of 35 markers across a small number of backcrosses (i.e. seven trees) to be too little data to make strong inferences about the role of genomic architecture in observed patterns. For example, alleles from either alternate species were never observed along LG XV among the *P. balsamifera* backcrosses. Clearly, segregation distortion in advanced-generation hybrids may arise from marker linkage with genes under selection, yet whether these patterns arise from intrinsic genic causes or from habitat-mediated selection remains a question open to debate.

In addition to studies of naturally backcrossed trees with low marker density, this distortion of parental nuclear alleles in advanced-generation hybrids is routinely observed within *Populus* mapping populations with very dense marker placement (e.g. Yin *et al.* 2008). Bradshaw & Stettler (1994) first detected segregation distortion in a *P. trichocarpa* by *P. deltoides* cross, arising from a recessive lethal allele in pollen that was tightly linked to a mapped RFLP marker. Detailed genomic analyses of backcrossed *P. fremontii* into *P. angustifolia* demonstrated segregation distortion in 21% of the mapped markers (Woolbright *et al.* 2008). Additionally, experimental crosses of *P. fremontii* × *P. angustifolia* F1s to derive an F2 met with low success, further implicating negative epistatic interactions. Segregation distortion in advanced-generation hybrids may clearly arise from marker linkage with genes under selection.

Asymmetry could potentially result from sex linkage: in animals, when an F1 hybrid between closely related species is sterile or inviable, it is typically the heterogametic sex ('Haldane's rule', Haldane 1922; Coyne & Orr 1997). Evidence of Haldane's rule has seldom been observed in plant species, where dioecy is a relatively rare phenomenon (D. Charlesworth, personal communication). Mapping studies provide mounting evidence that sex determination in *Populus* is governed by a region of low recombination on chromosome XIX, and that female poplars are the heterogametic sex (Gaudet *et al.* 2008; Yin *et al.* 2008). If negative epistatic interactions were to occur among recessive genes that are linked with poplar's incipient sex chromosome, asymmetric breeding barriers between species may arise across a hybrid zone (Wang & Zhao 2008). The potential applicability of Haldane's rule to *Populus* remains completely unstudied.

SNP selection and analytical issues

The 35-SNP assay reported here has enough power to detect complex hybrid combinations among three different species, including beyond the first generation. The ability to resolve patterns of gene flow largely depends on the discriminating power of the markers (Boecklen & Howard 1997) and this effect is complicated further by the study of more than two species. Our approach was to develop an array of SNPs that are fixed (or nearly fixed) among the three species. The Structure analysis (Pritchard *et al.* 2000) in no way assumes marker fixation to generate posterior probabilities for membership in alternative clusters, so a lack of strict fixation, because of insufficient sampling on our part, rare events of incomplete lineage sorting or even some small degree of genotyping error should have little effect on our results.

It should be noted, however, that Structure does assume independence amongst loci. Based on their physical distances (325 kb to 7.5 Mb), the 24 gene regions used in this study were treated as unlinked, as linkage disequilibrium typically breaks down over a few hundred base pairs for several species of *Populus* (e.g. *P. tremula*, Ingvarsson 2005; *P. balsamifera*, Breen *et al.* 2009) and likewise in undomesticated tree species (e.g. Brown *et al.* 2004). As our SNPs were designed to be species specific and only seven backcrosses were detected, LD cannot be estimated for the markers with

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any degree of accuracy, as adequate polymorphism is required, and it requires many generations of recombination between species to completely break down linkage along chromosomes. Theoretically, only one diagnostic SNP may be informative on a particular linkage group in the absence of local recombination, while there are, for example, many species-specific SNPs on LG XV. Our assumption of independence among diagnostic SNPs on the same chromosome could result in a small upward bias in the confidence that we have in our assignments. However, the assignments themselves would highly probably remain unchanged if putatively linked SNPs were removed from analyses, as our genotyping assay employs numerous species-specific SNPs located on separate chromosomes (8 chromosomes for *P. deltoides* SNPs, 12 chromosomes for *P. nigra* SNPs, 10 chromosomes plus one scaffold for *P. balsamifera* SNPs) for the unequivocal identification of advanced-generation hybrids.

Structure has been frequently used to diagnose hybrids and admixed individuals (e.g. Beaumont *et al.* 2001; Gow *et al.* 2007) and it performs well in simulations, particularly when the markers employed show a high degree of divergence in the parental species (e.g. Vähä & Primmer 2006), as in this study. As F2s should, in theory, be classified as F1s using Structure, we carefully reconfirmed the genotypes of admixed individuals visually. As all or nearly all of the SNPs were heterozygous for individuals with approximately 50% admixture, those individuals can clearly be identified as F1s, as the probability of this condition in an F2 is exceedingly low ($\sim 10^{-7}$ for 22 SNPs, the minimum number of SNPs that differentiated pairs of parental species in this study). Furthermore, F2s are biologically implausible given that hybridization is relatively rare within populations and that poplars are dioecious, with separate male and female trees. Hence, trees with approximately 50% admixture between two species were all considered to be first-generation hybrids.

The program NewHybrids (Anderson & Thompson 2002) has been successfully used to assess gene exchange between exotic and native species (e.g. Mercure & Bruneau 2008) through the Bayesian assignment of putatively admixed individuals to one of six classes (e.g. pure parental types, F1s, F2s, backcrosses). However, this method models hybridization events for two generations between only two putative parental species, as is the typical case. NewHybrids fails to perform if more than two species are involved in hybridization events, as in this study. In an exploratory analysis with all data pooled (data not shown), any individuals with alleles from the third least abundant species (here *P. nigra*) are misclassified as F2s by NewHybrids, be they pure species or admixed. As samples that morphologically resemble a pure native species may contain cryptic foreign alleles from either of the other two species (Fig. 2), there is no way of knowing a priori how to partition individuals into three pairwise data sets to be analysed with NewHybrids. Here, we conducted three post hoc analyses with NewHybrids after an initial analysis with Structure, noting that trihybrids cannot be analysed with this approach. These three post hoc analyses with NewHybrids classified the 17 'bihybrids' as F1s or backcrosses to *P. balsamifera* with extremely high degrees of confidence (0.915–1.000). However, we urge caution in the sole use of NewHybrids for admixture analyses if gene flow between more than two species is a possibility.

Implications and future work

As firm controls are currently in place regarding the planting of trees with novel traits within Canada (Bonfils 2005), we have been limited in our power to directly assess the extent and consequences of gene flow of novel gene regions into wild populations. Our approach has been to evaluate gene flow from historically planted exotics (retrospective tests) as a proxy for any genomic invasion through introgression. Knowledge of the frequency of F1 formation between cultivated trees and natural populations is one important step in the risk assessment process but provides an incomplete picture of potential risk (Wilkinson *et al.* 2003). Even if hybridization is frequent, when gene flow is blocked after the first generation, the introduction of trees with novel traits may have little consequence on the genetic integrity of native species.

Here, we have presented indirect evidence of realized F1 fertility for *Populus* hybrids. The frequency and direction of introgression was comparable between exotic and native species. The risks posed by trees with novel traits on the genetic integrity of natives could more probably be an issue in disturbed or heterogeneous habitats. The potential for introgression also depends on the native species in question. Our data show that *P. balsamifera* is more susceptible than *P. deltoides* to genomic invasions by the DNA of an alternate species. As discussed in a recent commentary by Buerkle (2009) the ecological context of hybridization clearly matters: regions of the genome may cross the species boundary in an asymmetric fashion by neutral processes (e.g. demography *sensu* abundance, Currat *et al.* 2008; Lepais *et al.* 2009, where the rate and pattern of introgression is a function of the frequency of parental alleles in the population of interest) or selective processes (e.g. intrinsic or extrinsic selection against certain hybrid classes, such as backcrosses to one of the two parents). This differentiation should not be confused

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with the neutrality of a marker (related to the marker's direct effect on individual fitness) although it should be noted that even markers with negative fitness effects could reach high frequency if linked with other genome regions under positive selection. A more detailed knowledge of the sources of this asymmetry in gene flow may provide important clues as to mitigation tactics for the successful implementation of the results of tree biotechnology.

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Our team's mandate at the Canadian Forest Service is to assess the risks posed by novel genes to the changing Canadian environment through theoretical modelling and empirical studies of gene flow in natural tree populations. Stacey Lee Thompson synthesizes evolutionary genetic/genomic analyses of plant populations, Manuel Lamothe is an expert in molecular marker development and a budding bioinformatician, Patrick G. Meirmans is a molecular ecologist and popgen modeller, while Nathalie Isabel, our team leader, is a forest and environmental genomicist. We all share a special interest in cultivating diversity in the research community through dynamic management techniques.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Gene regions, diagnostic SNPs and primer sequences (5′ → 3′) used in this study

Table S2 Details of studied populations

Fig. S1 Examples of sampled populations that indicate proximity of the species, disturbance and age: (a) this site, named Chicoutimi, consists of natural regeneration of *P. balsamifera* (B) and is located on an upper terrace next to the ravine of a creek, close to a commercial zone. There is one old *P. deltoides* × *P. nigra* hybrid (DN) within a distance of 150 m; (b) the stand in Leclercville comprises several cohorts of *P. deltoides* (D), 20–50 years old, with a few veteran *P. deltoides* trees still present, and is representative of a typical cottonwood habitat by the St Lawrence River with a long history of human influence.

Fig. S2 Examples of disturbance at collection sites and proximity to exotics. Natural regeneration of *P. deltoides* (D) or *P. balsamifera* (B) can be found after disturbances in industrial (a–c) and agricultural (d) zones, along roads and highways in southern Québec. Site locations are Gentilly (a), Nicolet (b), Sherbrooke (c) and Joly (d). Trees of *P. nigra* 'Italica' (N) are found close to the four sites within a distance of 1 km.

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