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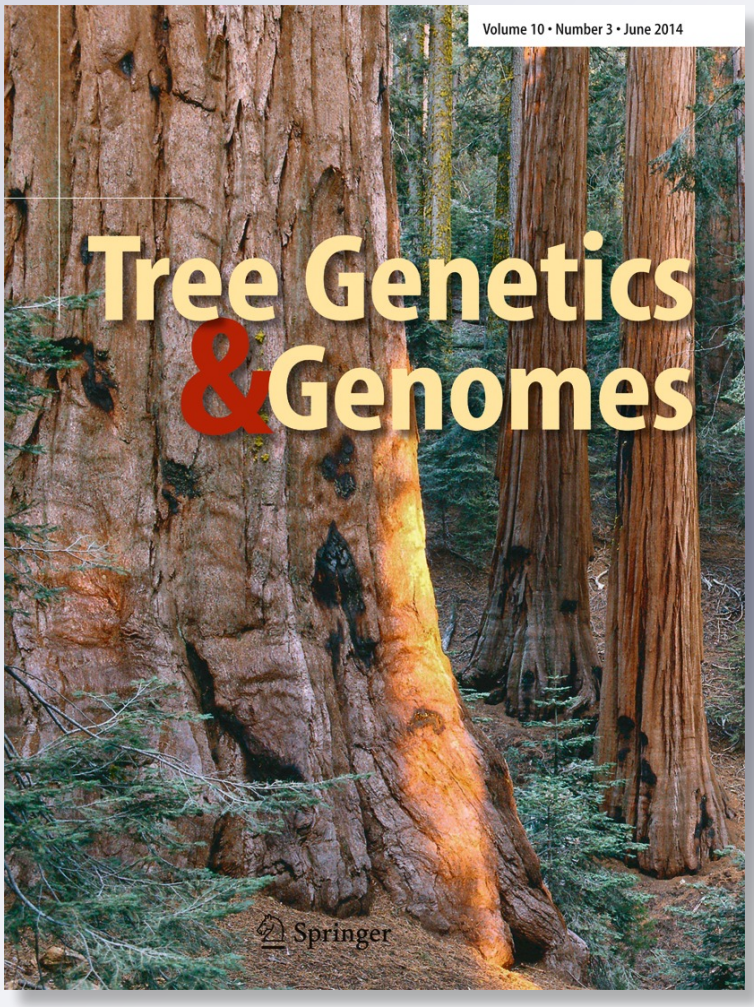

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ORIGINAL PAPER

Molecular analysis of natural root grafting in jack pine (*Pinus banksiana*) trees: how does genetic proximity influence anastomosis occurrence?

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Abstract Natural root grafting has been observed in more than 150 tree species where up to 90 % of trees could be interconnected within a stand. Intraspecific root grafting was previously found in *Pinus banksiana* stands, ranging from 21 to 71 % of trees grafted with one another. It is not known why root grafting is frequent in some species and not in others, or why not all roots that cross form root grafts. We investigated genetic diversity of grafted and non-grafted trees to determine if there was a relationship between genetic distance and the probability of forming natural root grafts. Seven plots were hydraulically excavated in four natural forest stands and three plantations of *P. banksiana* in the western boreal forest of Quebec, Canada. At pairs scale, we studied the effect of geographic and genetic distances on root grafting occurrence. At stand level, we analysed the effect of tree density, soil type, stand type and mean pairwise relatedness on the mean number of grafts per tree and on the percentage of grafted trees per plot. At pairs scale, our analysis revealed that root grafting presence was influenced by spatial distance between trees and less importantly, by genetic distance between individuals. At stand level, root grafting frequency was correlated with stand type (greater in naturally regenerated stands), but not with genetic diversity between individuals. In conclusion, root grafting appears to be principally linked to tree proximity and slightly to genetic proximity between individuals.

Keywords Root grafting · Forest ecology · *Pinus banksiana* · Anastomosis · Genetic distance · Microsatellites

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Introduction

The concept of trees forming discrete physiological entities is still widely accepted among scientists. A number of tree species, however, have been shown to be interconnected through their root systems (Newins 1916; LaRue 1934; Fraser et al. 2005; Tarroux and DesRochers 2010; Tarroux et al. 2010). This phenomenon is especially common in clonal species such as aspen (*Populus tremuloides* Michx.) that produces stands where many trees are interconnected through their parental root systems (DesRochers and Lieffers 2001; Jelínková et al. 2009). Natural root grafting is also observed between non-clonal tree species and has been reported in more than 150 species (Bormann 1966), particularly in pines (*Pinus* spp.) from around the world (LaRue 1934; Armson and Van den Driessche 1959; De Byle 1964; Horton 1969; Eis 1972; Thomas et al. 1999; Fraser et al. 2005; Tarroux and DesRochers 2010). Hence, even if trees are traditionally considered as distinct entities competing with each other for resources, root connections imply that trees are not independent of one another (Loehle and Jones 1990). Interconnected trees can share resources (Kuntz and Riker 1956; Bormann 1961; Stone and Stone 1975a; Fraser et al. 2006) and, in turn, enhance the survival of suppressed trees, which are supported by their connected neighbours (Bormann 1966; Graham and Bormann 1966; Fraser et al. 2006; Tarroux and DesRochers 2010, 2011). Overlapping and joined root systems can also give trees better wind stability (Coutts 1983a; Kumar et al. 1985; Keeley 1988; Basnet et al. 1993).

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The idea that root grafting is coincidental or not is still controversial (Loehle and Jones 1990). Some species exhibit this trait to such a marked degree that intraspecific competition concepts would need to be revised to incorporate frequent non-competitive relationships such as root grafting. The degree of genetic proximity between trees could explain the observed differences in root grafting frequency among species (Stone 1974; Loehle and Jones 1990). Lower inter-individual genetic distances are generally predicted to lead to a greater probability of root grafting, as grafts within trees of the same genotype are reported to be far more common than between trees of the same species (Loehle and Jones 1990). In addition, root grafting is reported to be especially frequent in species known for their low genetic diversity (Stone 1974), such as *Pinus resinosa* Sol. ex Aiton (Boys et al. 2005). Yet root grafting has also been frequently encountered in species exhibiting higher levels of genetic diversity. For example, our work in *Pinus banksiana* Lamb. stands revealed a high level of intraspecific root grafting, ranging from 21 to 71 % of trees grafted within each 40–60 m^2 plot (Tarroux and DesRochers 2010). Genetic distance between grafted and non-grafted trees, in fact, has rarely been measured because it requires laborious and costly excavations, as well as suitable molecular markers to determine individual genetic identity. Using microsatellites markers, Jelínková et al. (2009) showed that for a clonal tree species, aspen, between-clone root grafting was just as frequent as within-clone root grafting. Nevertheless, researches on asexual propagation and/or fruit tree grafting showed that graft success was greatest between clones of the same species (due to genetic proximity) and that grafting between plants of different families and genera was rare (Hartmann and Kester 1975; Mudge et al. 2009).

When root grafting occurs, a callosity is produced at the contact point between the two roots (Bormann and Graham 1959; Graham and Bormann 1966; Eis 1972). It is not clear how wood is produced during root graft formation, and whether the wood originates from the cambium of one tree or from both is not known. A study on artificially grafted tobacco plant showed that plant grafting could result in the exchange of large DNA fragments or entire plastid genomes (Stegemann and Bock 2009). In the present work, *P. banksiana* stands were used to test the hypotheses that (1) genetic proximity will be higher for grafted than for non-grafted individuals and (2) tissue around the graft is produced by the two grafted trees, with mosaics occurring at the grafting point. DNA was extracted from trees and from some of the root grafts. Using microsatellite markers, the genotype of each studied graft was compared to those of the two grafted trees. Microsatellites are ideal genetic markers for individual identification because they are abundant, neutrals, codominant, highly polymorphic and spread across the genome (Bennett 2000; Selkoe and Toonen 2006).

Materials and methods

Study area

Sampling was carried out in the western balsam fir–paper birch (*Abies balsamea—Betula papyrifera*) bioclimatic domain (Grondin 1996) of the boreal forest of northwestern Quebec, Canada (Fig. 1). Plots were located between 47°58′ N and 48°44′N and between 77°6′W and 79°25′W (Fig. 1). The climate of the region is cold continental with an average daily temperature of 1.2 °C and average yearly precipitation of 918 mm (rainfall, 670 mm; snowfall, 248 mm; Environment Canada 2010). The region incorporates a large physiographic unit that is characterised by lacustrine deposits from the maximum post-Wisconsonian extension of proglacial lakes Barlow and Objibway (Veillette 1994). While clay is the dominant deposit throughout the plain (glaciolacustrine deposits), sand (eskers) is also encountered (glaciofluvial deposits). We identified and selected four stands that had naturally regenerated following fire (STM, DUP, STMO, STMA) and three stands that had been artificially regenerated following clear-cutting (LOU, LOUV, BER; Table 1; Fig. 1). Spacing in the plantations varied from 1.7×3 m (LOU, LOUV) to 2×2 m (BER). The study plots in each stand ranged in size from 40 to 60 m^2, so that at least 10 trees were included in each plot (Table 1). The plots were located near a water source (pond, lake and river) so that we could carry hydraulic excavation of the root systems (Tarroux and DesRochers 2010).

Field sampling

In summer 2007, trees were felled with a chain saw, and cross-sectional disks were cut at ground level (0 m) and at breast height (1.30 m) for age determination. Height and diameter at breast height (DBH) of each tree were measured. Plots were then excavated with a high pressure water spray using a forestry water pump (Mark III, Wajax, Lachine, QC), to expose the entire root systems and root grafts. All trees (alive or stumps), roots and grafts were carefully mapped, and distances between all trees in each plot were recorded. A cross-sectional disk was taken from each root with a diameter of at least 2 cm for age determination. All suspected grafts were collected and diameter of grafted roots measured. Trees were considered as grafted when there was vascular continuity between their roots, which can only be checked by removing bark and performing a partial dissection to confirm a common wood layer between the two roots. Only true intraspecific grafting that involves the morphological union of cambium, phloem and xylem (Graham and Bormann 1966) was recorded. In a false graft, the xylem and phloem of roots are not anatomically or physiologically merged; roots are surrounded by layers of root periderm seemingly uniting the complex in the form of a true graft, but in fact, roots have partially

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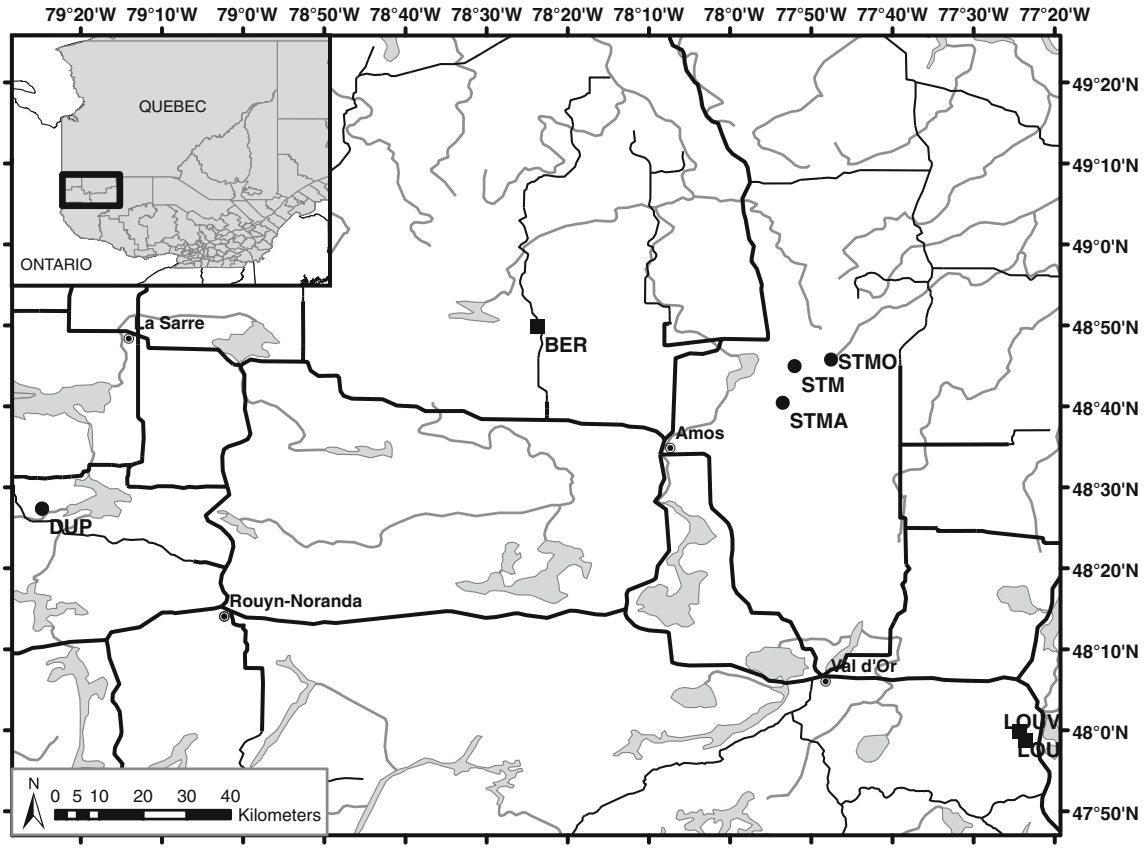

Fig. 1 Map showing the seven populations studied in Abitibi-Temiscamingue, Quebec. Natural stands are represented by a *black circle*, while the plantations are represented by a *black square*

enveloped each other and still separated by a more or less degraded layer of bark (Graham and Bormann 1966). Detailed protocols for hydraulic excavation, field sampling and dendrochronology analysis can be found in Tarroux and

Table 1 Characteristics of the seven excavated plots

	Plots						
	STM	DUP	STMO	STMA	LOU	LOUV	BER
Size of excavated area (m^2)	40	45	50	50	40	40	40
Stand	N	N	N	N	P	P	P
Soil	C	C	S	S	S	S	S
Stand age (years)	45	75	90	65	35	35	35
Density (trees ha^{-1})	6200	3100	4600	5000	3800	4000	5000
Average height (m)	13	20	14	13	16	14	9
Number of trees sampled for genetic analysis	24	10	17	13	11	11	19
Number of grafted trees sampled for genetic analysis	15	1	9	7	8	6	8
Number of excavated trees	24	14	23	26	15	16	20
Number of grafts	20	7	19	18	10	10	12
Number of grafted trees	14	3	14	15	9	9	12
Mean number of grafts per tree	0.87	0.50	0.83	0.72	0.67	0.63	0.6
Percentage of grafted tree (%)	61	21	61	60	60	56	60
r	0.014	0.056	0.002	0.017	0.018	0.044	0.015

Stand type (*N* natural stand and *P* plantation), *Soil* type (*S* sand and *C* clay), *r* mean pairwise relatedness estimates for each plot (Lynch and Ritland 1999)

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DesRochers (2010). Of the 137 excavated trees, we selected 105 for genetic analysis, only including living trees. In the field, we also randomly sampled the wood from callus tissue of 20 suspected grafts for genetic analyses. Using a scalpel, cambial tissue was taken from the graft callus and from the base of each tree trunk. Samples were kept chilled on ice and brought to the laboratory where they were conserved at −86 °C until DNA extraction. However, of these sampled suspected grafts, only six were confirmed as true grafts.

Laboratory work

Wood samples were ground and stored at −20 °C. GenElute Plant Genomic DNA Miniprep Kit of Sigma-Aldrich (product code, G2N350; Oakville, ON, Canada) was used to isolate DNA from cambial tissue. DNA extraction was done following manufacturer's instructions. Microsatellite loci were used to produce genetic profiles for grafted and non-grafted *P. banksiana* trees since the level of variability of these markers was sufficiently high to assess genetic differences between individuals. We used 12 microsatellite primer pairs, which were labelled with fluorescent dyes (FAM-6, NED and VIC), to genotype individuals. The selected primers were designed specifically for pine species: Pde3, Pde5 and Pde7, Pde13, PtTX-3118, PtTX-3020, PtTX-3030, PtTX-2123, PtTX-2090, PtTX-3025, PtTX 4001 and PtTX 4009 (Auckland et al. 2002; Al-Rabab'ah and Williams 2004). Primers pairs were initially tested for amplification and polymorphism with 10 individuals. Despite several attempts, we could not obtain correct amplification for PtTX-3025, PtTX 4001, PtTX 4009 and Pde5. Only microsatellite markers that were successfully amplified were further used in the analysis. Characteristics of the different microsatellite markers (sequence, dye, observed range and annealing temperature) are given in Table 2. PCR reactions were performed using these protocols. Tissue-free extraction and PCR controls were run in parallel. In the negative PCR controls, sterile water was added to PCR reagents and processed using the same PCR protocols. The polymerase chain reaction (PCR) was performed in a Perkin-Elmer 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using a 2.6–3-μL DNA extract and a master mix consisting of 6.6 μL HotMaster buffer (Eppendorf North America, Westbury, NY, USA), 100 μg/μL gelatine, 1 % DMSO, 50 μM for each dNTP, 100 nM of each forward and backward primer, 0.325 U HotMaster Taq DNA Polymerase (Eppendorf NA), and 2.5–3 mM $MgCl_2$ in a total volume of 10 μL. The standard temperature profile was 9 min at 95 °C for Taq activation and 30 cycles of 30 s denaturation at 94 °C, 30 s at the annealing temperature (depending on the primers used) and 1 min at the extension temperature at 63.5 °C. A final extension of 10 min at 72 °C was used. The PCR product (0.6 μL) was mixed with 10 μL Hi-Di formamide and 0.6 μL Tamra 500 size standard (Applied Biosystems, Westborough, MA, USA) and denatured for 5 min at 95 °C. Tubes were placed on ice, and fragments were separated by capillary electrophoresis in 3130 Genetic Analyzer (Applied Biosystems). The resulting microsatellite profiles were examined using GeneMapper v.3.7 (Applied Biosystems). A total of eight polymorphic and unambiguous genotype profile markers were obtained (Appendix 1). Reproducibility of bands scored was tested by rerunning the PCR reactions. At total, 15 PCR plates (combining one, two or three markers) were prepared to obtain good and reproducible results.

Statistical analyses

Data were analysed using Data Collection v3.0 software (Applied Biosystems). Genotypes were inferred from the individual size profiles of the nuclear DNA that we analysed using GeneMapper v.3.7 software (Applied Biosystems). To determine the resolving power of the set of microsatellites that we used, the probability of identity (PI) was estimated (Waits et al. 2001; Peakall and Smouse 2006). It corresponds to the average probability that two independent samples will have an identical genotype. Estimates of PI assume that the population is randomly mating and that comparisons are between unrelated individuals. We also calculated PIsibs estimator as a more conservative estimation of the number of loci necessary to distinguish individuals (Waits et al. 2001; Namroud et al. 2005). Cumulative expected PIs correspond to the probability of identity for multilocus and were calculated as the product of individual locus PI and PIsibs. The Micro-checker v.2.2.3 program of van Oosterhout et al. (2004) was used to test for null alleles and scoring errors due to large allele dropout and stutter peaks. For loci in which we suspected null alleles to be present, we used the program FreeNA (Chapuis and Estoup 2007) to estimate frequencies of putative null alleles ( ). The observed heterozygosity (*Ho*), expected heterozygosity (*He*), unbiased expected heterozygosity (UH_e), average number of alleles (*N*), number of effective alleles (N_e; Kimura and Crow 1964), *F* statistics (F_{IS} and F_{ST}; Wright 1965), mean pairwise relatedness estimates of Lynch and Ritland (r; 1999) were calculated using GenAlEx v.6 (Peakall and Smouse 2006). Mean pairwise relatedness estimates were also estimated excluding loci suspecting null allele presence. GENEPOP program (version 4.2, Rousset 2008) was used to test deviations from the Hardy–Weinberg equilibrium. A global test over all populations was performed to estimate the probability of Hardy–Weinberg Equilibrium (P_{HW}). Genetic differentiation was further analysed using FreeNA (Chapuis and Estoup 2007) for null alleles to describe the partitioning of genetic variation (F_{ST}).

We had previously found a positive relationship between root grafting frequency (number of grafts per tree and percentage of grafted trees) versus soil type, stand type and tree density (Tarroux and DesRochers 2010). In the present work, we added the influence of genetic diversity between individuals as a source

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Table 2 Names, primer sequence, marker dye colour, annealing temperature and observed allele size ranges of the 12 *Pinus banksiana* microsatellite markers that were used (Lian et al. 2000; Al-Rabab'ah and Williams 2004)

Locus	Primer sequences	Dye	Annealing temp. (°C)	Observed range
PtTX-3118	CACGGCCCTTAGCTTTACCTT TTCTGATGGGGCAACTG	B	61	105–305
PtTX-3020	GTCGGGGAAGTGAAAGTA CTAGGTGCAAGAAAAGAGTAT	Y	69	101–197
PtTX-3030	AATGAAAGGCAAGTGTCG GAGATGCAAGATAAAGGAAGTT	G	61	257–326
PtTX-2123	GAAGAACCCACAAACACAAG GGGCAAGAATTCAATGATAA	G	58	182–202
PtTX-2090	CCCGCCTATTCCACCTA CTACACATTTCACCATAAGTCC	G	58	179–330
Pde3	GTTGATACAATCATTGTTGTAACAC CAAATATTTATATTCCCCTACGTG	G	58	70–145
Pde 7	TTGAGTGAGAGGACTCTAGGC AGGTAGACCCTATGGCGATG	Y	58	118–188
Pde13	AATATTCCTAACGACCCTATC TGTTTCTATATGCATGTATGAGTC	G	58	113–327
PtTX-3025	CACGCTGTATAATAACAATCTA TTCTATATTCGCTTTTAGTTTC	Y	*Incomplete amplification*	
PtTX-4001	CTATTTGAGTAAGAAGGGAGTC CTGTGGTAGCATCATC	B		
PtTX-4009	ACCTTGACCTTGTAGAGC CTGTGTCCCTTTAGAGATAG	B		
Pde5	AACGCACCTTTCTCAATGCAC ATAAAGAGGCTACATGGTCCC	B		

Primers are shown in the 5′–3′ orientation. The first primer used is the forward primer, while the second is the reverse primer. Suggested dye labelling *B* blue (FAM-6), *Y* yellow (NED) and *G* green (VIC) for analysis on the Applied Biosystems 3130 Genetic Analyzer

of variation for root grafting frequency. To determine the factors influencing root grafting frequency, the relationships between the number of root grafts per tree (NUMBER model), the percentage of grafted trees per plot (PERCENT model) and stand type (natural stands or plantations), soil type (sand or clay), stand density, and mean pairwise relatedness estimates for each plot (r) were analysed with a mixed linear model using the lme function of the nlme library (Linear and Nonlinear Mixed-Effects Models, Pinheiro et al. 2008) in the *R* statistical environment (v. 2.7.2, R Development Core Team 2008). To avoid 'sacrificial pseudoreplication' error that is incurred when data from different experimental units are treated as independent replicates and pooled in the same analysis (Hurlbert 1984), plots were treated as random effects. A test was done to detect influential observation using Cook's distance function. Due to highly influential observation at the DUP site (Cook distance value= 17.59 while cut-off value was 1), it was removed from the analyses. Models were then compared based on the Akaike information criterion corrected for small sample sizes (Burnham and Anderson 2004) using the aictab function of the AICcmodavg library (Mazerolle 2006). Models with low ΔAICc (<2) and high Akaike weights (ω_i, interpreted as probabilities) were deemed to have the greatest statistical support (Burnham and Anderson 2004). For NUMBER series models, only one model showed ΔAICc<2. In consequence, results were obtained on the selected model. For PERCENT series model, all models showed ΔAICc<2, multimodel inference based on AICc was thus realised using modavg.mer function, and predictions were obtained with the modavgpred.mer function of the AICcmodavg library (Mazerolle 2006).

The full matrices of pairwise distances (geographic and genetic) among each pair of trees within a plot were compared. Inter-individual genetic distances (codominant-genotypic) were calculated using GenAlEx 6 (GD; Peakall and Smouse 2006). This pairwise, individual-by-individual (N × N) genotypic distance matrix is calculated for codominant data, and genetic distances are summed across loci under the assumption of independence. We also estimated the inter-individual relatedness adjusted with null allele presence using *ML*-Relate (Kalinowski-GD; Kalinowski et al. 2006; Wagner et al. 2006). This program calculates maximum likelihood estimates of relatedness and relationship from codominant genetic data (e.g. microsatellites,). It was designed to accommodate microsatellite loci with null alleles. As grafted trees are generally close together, there was a high occurrence of zeros in the presence/absence matrix. We thus reduced the dataset by removing pairs of trees distant by more than 3 m. The relationship between the presence of a root graft (DISTANCE model) and both spatial and inter-individual genetic distances (GenAlEx and *ML*-Relate) between trees were then analysed with a generalised linear mixed-effects model in *R*, using a glmer function in the lme4 library (linear mixed-effects models using S4

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classes (Bates and Maechler 2009). This model can implement logistic regression to test presence/absence of root grafts as a binary variable. Plots were considered as random effects. To determine the most important genetic distance factor explaining root grafting presence, two global models were done. The first global model tested the inter-individual genetic distances estimated with GenAlEx 6 (Peakall and Smouse 2006) and spatial distance, while the second global model tested inter-individual genetic distances predicted by *ML*-Relate (Kalinowski et al. 2006; Wagner et al. 2006) and spatial distance. All models were compared based on the Akaike information criterion (Burnham and Anderson 2004). As two models showed ΔAICc<2, multimodel inference based on AIC was then realised using modavg.mer function (Mazerolle 2006).

To link the root grafts to the corresponding grafted tree, genetic profiles of the six collected grafts were studied: we measured the genetic distance (inter-individual genetic distances estimated with GenAlEx 6) between tissues collected from the grafts' callus tissue and compared it to tissue from the trunks of the two grafted trees.

Results

Genetic diversity of *P. banksiana* stands

The average probability that two individuals presented the same multilocus genotype was very low. Cumulative expected *PI*s using the eight loci ranged from 1.6×10^{-3} (*PIsibs*) to 1.5×10^{-8} (*PI*). Among the microsatellites that we tested, loci PtTX-2090 and Pde13 were the most variable primers (PI=0.022 and 0.016, respectively), while PtTX-3020 and Pde7 were the least variable (PI=0.38 and 0.41, respectively). A total of 79 different alleles were detected in the eight microsatellite loci (Appendix 1). The total number of alleles (*N*) varied from 8 for locus PtTX-3020 to 23 for locus PtTX-2190 (Appendix 1). The effective number of alleles (N_e) varied from 1.648 to 6.074. Levels of genetic diversity, which were measured in terms of average number of alleles, expected (UH_e), and observed heterozygosities (H_o) were moderately high (Appendix 1). We detected significant deviations from Hardy–Weinberg equilibrium in the global test (F_{IS}=0.039; P_{HW}=0.0001). MICRO-CHECKER detected the presence of null alleles at four loci, Pde7, Pde13, PtTX-3030 and PtTX-2090 (Appendix 1). There was no evidence for large allele dropout or for scoring error due to stuttering. We therefore estimated null allele frequencies at each locus and recalculated F_{ST} values accounting for null alleles (Appendix 1). F_{ST} estimates obtained by excluding null alleles were slightly lower than when the presence of null alleles was ignored (Appendix 1). The 95 % confidence intervals ranged from −0.001 to 0.12 for standard F_{ST} and from −0.001 to 0.11 while considering the null alleles presence. In consequence, the 95 % confidence intervals overlap, and null alleles appear to have only small effects on population differentiation in our samples. Because we found no consistent patterns of null alleles across all plots, all loci were retained for analysis. Mean pairwise relatedness estimates (*r*) for each plot varied from 0.002 (STMO) to 0.056 (DUP; Table 1). As mean pairwise values for each plot were always less than 0.125 (third-order relationship), relatedness for individual pairs was very low (Lynch and Ritland 1999).

Individual by individual genetic analysis

Only one model was selected (ΔAICc<2) to explain the number of grafts per tree: the model with stand type (ωi=0.63; Table 3). At stand level, mean number of root grafts per tree was significantly greater in plantations compared to natural stands (*P*=0.048; Table 3). The percentage of grafted trees was not affected by stand type (Table 3). The mean number of root grafts per tree and percentage of grafted trees per plot were not affected by tree density, mean pairwise relatedness estimate or soil type (Table 3). At pairs scale, AIC results showed that the model using spatial distance and genetic distances (ωi=0.55; codominant-genotypic) from GenAlEx 6 was a better predictor of root grafting presence than the model using spatial distance and maximum likelihood estimates of relatedness of Kalinowski et al. (2006; ωi=3.01; Table 3). AIC and multimodel inference showed that root grafting presence was strongly influenced by the spatial distance between trees and marginally by the inter-tree genetic distance (Table 3).

Of the six genotyped grafts, only two matched perfectly with one of the linked trees. For the four other grafts, graft profiles were a combination of the alleles found in both trees involved (Appendix 2). The smallest recorded genetic distance (codominant-genotypic) varied from 0 to 3, and the highest ranged from 3 to 8 (Table 4). Depending on the graft, the genetic distance between the two grafted trees varied from 4 to 10.

Discussion

Our study showed that a greater degree of genetic similarity slightly contributed to increasing the probability of root grafting between individuals (Fig. 2). Interspecific grafts are rare (Bormann 1966), and root grafting was reported to be especially frequent in species showing low genetic diversity (Stone 1974). Moreover, studies on asexual propagation and/or fruit tree grafting showed that graft success was also linked to genetic proximity (Hartmann and Kester 1975; Mudge et al. 2009). Our results partially support this notion that root grafting is more likely to occur between trees having greater genetic similarity (Stone 1974; Loehle and Jones 1990) but also that physic constraints could 'force' tree to root graft. Indeed, in spite of great genetic distances, trees formed root grafts because spatial distance between trees was a much more significant factor

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Table 3 Models selection and multimodel inference according to results of the Akaike Information Criterion corrected for small sample sizes (AICc)

Model	Factors tested	ΔAICc	ω_i	*P* value	95 % confidence interval
NUMBER	Stand	0	**0.63**	**0.048**	n.a
	Soil	2.63	0.17	0.128	n.a
	Density	3.97	0.09	0.218	n.a
	r8	4.45	0.07	0.267	n.a
	r5	5.18	0.05	0.372	n.a
PERCENT	r5	0	0.29	0.262	−171.86/17.62
	r8	0.02	0.29	0.265	−184.32/19.45
	Soil	1.03	0.17	0.422	−6.16/1.75
	Density	1.45	0.14	0.530	−0.001/0.003
	Stand	2.00	0.11	0.792	−3.76/2.63
DISTANCE	Spatial distance + GD	0	0.55	**<0.001** and 0.086*	n.a
	Spatial distance	1.01	0.33	**<0.001**	**−3.15/-1.65**
	Spatial distance + Kalinowski-GD	3.01	0.12	**<0.001** and 0.840	n.a
	Kalinowski-GD	62.94	0	0.478	−0.34/0.42
	GD	63.29	0	0.781	−0.20/0.01*

NUMBER and PERCENT models (lme) were respectively the models relating the number of grafts per tree and the percentage of grafted trees within plots versus the site characteristics and mean pairwise relatedness estimates. DISTANCE is a series of statistical models (logistic regression) relating the presence/absence of root grafting to geographic (m) and/or genetic distances. ΔAIC, akaike weights (ω_i), *P* value and 95 % confidence interval are given for each model/factor tested. Statistically significant values are given in bold

Stand type (natural or plantation), *Soil* type (sand or clay), *Density* number of stems per hectare, *r8* mean pairwise relatedness estimates for each plot (considering eight loci), *r5* mean pairwise relatedness estimates corrected for null allele presence (considering five loci: 3118, 3020, 2123, 2090 and Pde3, Lynch and Ritland 1999), *GD* inter-individual genetic distances estimated with GenAlEx 6 (codominant-genotypic Peakall and Smouse 2006), and *Kalinowski-GD* inter-individual maximum likelihood estimates of relatedness adjusted for null alleles (*ML*-Relate software; Kalinowski et al. 2006; Wagner et al. 2006). *ΔAIC* corresponds the differences in AIC values from the best model; *Akaike weights* (ωi) determine the probability of a model being the best explanatory model considering the data and the suite of candidate models; *P value* is the *P* value of each considered model (lme function). When 0 is excluded of the 95 % confidence interval, factor was statically significant;*Significant at 95 % confidence interval

explaining root grafting (Table 3). We conclude that genetic relatedness between trees had no strong effect on root grafting frequency and that plot characteristics and geographical distance between trees had an overall greater influence on root grafting frequency compared to genetic distance. These results are consistent with those reported by Jelínková et al. (2009) for *P. tremuloides*, where the major contributing factor to root grafting was also proximity of trees. The degree of genetic proximity between trees could explain the observed differences in root grafting frequency among species (Stone 1974; Loehle and Jones 1990) but not necessarily among trees.

The ecological significance of root grafting is still mostly unknown, and it is not clear if root grafting is a beneficial adaptive trait or an accidental event (Loehle and Jones 1990). Since roots are highly intermingled (Brunner et al. 2004; Göttlicher et al. 2008; Lang et al. 2010), but relatively few grafts are formed (i.e. less than one per tree on average, Table 1), one could argue that root grafting is an accidental phenomenon, allowed by favourable site conditions (tree proximity, tree swaying or soil abrasiveness). Nevertheless, it seems that other factors are also at play, since we observed cases where roots of large trees passed through the root system of two or three close trees before forming a root graft with a more distant individual, or a similar occurrence of root grafting in less favourable conditions (e.g. between spatially distant trees in clayey plantations; Tarroux and DesRochers 2010). We also found that root grafts would be preferentially formed with a neighbouring tree if it were smaller (Tarroux and DesRochers 2010). Perhaps, dominant and suppressed trees produce secondary metabolites in different proportions, allowing roots to communicate in the same way that chemical inhibitors are produced to prevent root contact (Reinartz and Popp 1987). Carbohydrate transfers decrease with increasing distance between grafted trees and preferentially travel from large to small trees within a graft (Armson and Van den Driessche 1959; Stone and Stone 1975b; Fraser et al. 2006). The relationship between root grafting and social status of trees within a stand needs to be further studied. Root grafting could also be seen as an adaptive response to stressful environments. Root grafting increases mechanical stability of stands by linking root systems together (Graham and Bormann 1966; Coutts 1983b; Kumar et al. 1985; Keeley 1988; Basnet et al. 1993) and enhances tree growth and chances of stand survival by facilitating resource acquisition (Bormann 1966; Loehle and Jones 1990; Basnet et al. 1993; Tarroux and DesRochers 2011). Root grafting constitutes an evolutionary advantage, and genotypes that tend to form root grafts would be favoured (Loehle and Jones 1990).

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Table 4 Genetic distance (GD) between each graft and the two linked trees

PLOT	GD	GD1	GD2
STMO	8	0	8
	5	0	5
	9	2	8
STM	10	3	6
LOU	5	3	4
DUP	4	1	3

GD the genetic distance between the two grafted trees measured with GenAlEx 6 software (Peakall and Smouse 2006), *GD1* and *GD2* the genetic distance between the graft and one of the two grafted trees, *GD1* the minimum distance recorded and *GD2* to the maximum

In the present study, only two of the six samples collected from graft tissues corresponded to the same genotype of one of the two grafted trees, while the others were a mixture of both genotypes (Appendix 2). This suggests that for four grafts, wood produced around root grafts was synthesised by the two trees. It could be seen as an example of genetic mosaics (Thomas et al. 1999; Pineda-Krch and Lehtilä 2004). The ability of some species (e.g., red algae, fungi) to fuse in the early stages of development and to produce chimeric organisms is not uncommon and raises questions about the validity of the physiological unity and autonomy concept (Santelices 1999, 2004). Intra-organismal genetic heterogeneity (IGH) is often viewed as a biological aberration, but an increasing number of studies have demonstrated that genetically homogenous organisms may be rather rare (Pineda-Krch and Lehtilä 2004). IGH seems less common in terrestrial plants, but genetic mosaics or chimerism has already been reported in a number of angiosperms and gymnosperms (Thomson et al. 1991; Korn 2002; Pineda-Krch and Lehtilä 2004). IGH is often associated with cancerous growths (Pineda-Krch and Lehtilä 2004). The formation of callus tissue around all grafts could be a consequence of the fusion of wood of the two (or more) genotypes from individuals that formed a root graft. Perhaps, the callus tissue is also a response to wounding, when two roots in close contact rub against each other with the swaying of trees in the wind. The wood then needs to be reorganised to allow transfers between the two grafted roots, and tracheids are produced along two axes, which also increase size of the callus (Bormann 1966). Perhaps, the rarity of strong wind events (strong enough to break up the bark between two roots) explains the few numbers of root grafts and the high percentage of grafted trees (a strong wind event would affect most trees in a stand at the same time). Moreover, if significant wind swaying events were regular, they could rather disrupt the delicate processes involved in root fusion and graft formation (Kozlowski and Cooley 1961; Graham and Bormann 1966; Loehle and Jones 1990). In two out of the six examined grafts, the cambium produced wood with the genetic profile of only one of the two trees. Perhaps, a more vigorous tree can «take over» wood production from a union, but a more detailed morphological examination of root grafts would be necessary to understand how wood is genetically organised following graft formation.

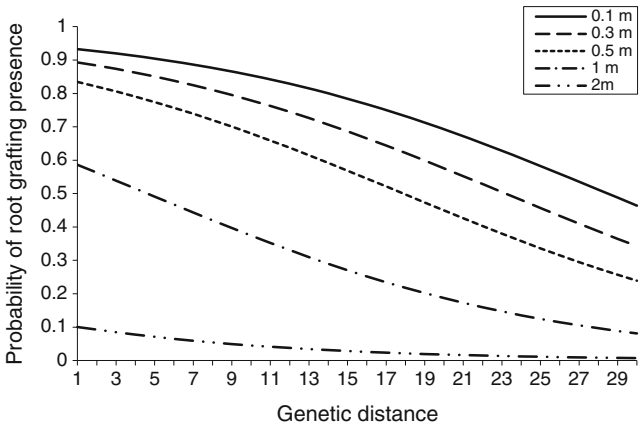

Fig. 2 Probability of grafting presence according to spatial (m) and genetic distances between trees. The genetic distance was measured with GenAlEx 6 software (Peakall and Smouse 2006) using the 'Codom-Genotypic' option

We found the same level of heterozygosity than researches using microsatellites on *P. banksiana* (including PtTX-2123 and PtTX-3030 loci; Cullingham et al. 2011) and *Pinus strobus* (Rajora et al. 2000). We have demonstrated that microsatellite markers developed for *Pinus* species have the potential to produce unique DNA profile in *P. banksiana* trees (Appendix 2). The genetic variability found with four or five microsatellite loci was sufficient to produce unique DNA profiles for trees but with eight loci; the probability of two individuals in the population having the same genetic profile was extremely low. According to Waits et al. (2001), a PI between 0.001 and 0.0001 should be sufficiently low for works that require individual identification. To minimise the cost and time involved in this study, a smaller number of loci could have been used, but we chose to keep the highest resolving power as possible.

The small number of root grafts analysed prevents us to exhaustively explain the complex phenomenon of IGH. However, it would be interesting to make artificial grafting attempts with known lineages of *P. banksiana* or to study other species with varying degrees of genetic diversity, to further verify if genetic proximity better explains root grafting frequency among species rather than among trees of the same species. Finally, excavation of mixed stands would allow us to determine the probability of interspecific root grafting.

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Data archiving statement GPS data and SSR genotype data were submitted to TreeGenes Database. Accession number is TGDR008.

Appendices

Appendix 1

Table 5 Characteristics of eight microsatellite markers in seven stands of *Pinus banksiana*

Marker	*N*	N_e		H_o	H_e	UH_e	F_{IS}	F_{ST}	$F_{ST\ using\ INA}$
PtTX-3118	10.00	2.18	0.01	0.65	0.49	0.50	−0.34	0.03	0.03
PtTX-3020	8.00	1.66	0.00	0.46	0.39	0.42	−0.17	−0.01	−0.01
PtTX-3030	14.00	2.93	0.24	0.38	0.57	0.61	0.29	0.16	0.14
PtTX-2123	9.00	2.97	0.00	0.78	0.63	0.65	−0.21	0.05	0.05
PtTX-2090	23.00	6.07	0.10	0.70	0.80	0.83	0.12	0.06	0.06
Pde3	10.00	2.01	0.05	0.59	0.49	0.51	−0.21	0.12	0.11
Pde 7	10.00	1.65	0.17	0.20	0.36	0.39	0.39	0.00	0.03
Pde13	24.00	3.70	0.28	0.35	0.65	0.70	0.44	0.10	0.09
All loci	13.5 (2.268)	2.895 (0.487)	0.106 (0.037)	0.513 (0.066)	0.55 (0.045)	0.576 (0.049)	0.039 (0.102)	0.064 (0.020)	0.060 (0.016)

N total number of alleles, N_e number of effective alleles, frequencies of null alleles using the EM algorithm (Dempster et al. 1977), H_o observed heterozygosity, *He* expected heterozygosity, UH_e unbiased expected heterozygosity, F_{IS} inbreeding coefficient within individuals relative to the subpopulation, F_{ST} F_{ST} ignoring null alleles, F_{ST} *using INA* estimating global F_{ST} using the INA correction described in Chapuis and Estoup (2007)

Mean for all loci are presented with standard errors in brackets

Appendix 2

Table 6 Genotype profile of grafted trees and grafts obtained from the combination of the eight loci

SITE	TREE	LOCUS															
		3118		Pde7		Pde13		3020		3030		2123		2090		Pde3	
STMO	# 1	107	120	167	167	177	177	176	187	305	314	193	196	194	199	84	84
	# 2	107	123	167	167	NA	NA	176	187	NA	NA	193	202	194	261	84	100
	G1-2	107	120	167	167	177	177	176	187	305	314	193	196	194	199	84	84
	# 3	107	120	167	167	113	113	187	187	305	305	196	199	194	194	84	84
	# 4	107	120	167	167	193	193	187	187	305	305	196	199	194	194	74	84
	G3-4	107	120	167	167	113	113	187	187	305	305	196	199	194	194	84	84
	# 5	120	120	167	167	193	202	167	187	305	319	193	196	194	194	74	84
	# 6	152	152	167	167	193	193	187	187	319	319	196	202	194	194	84	84
	G5-6	120	120	167	167	193	202	167	187	305	319	193	202	194	194	84	84
STM	# 7	107	120	167	167	113	184	176	187	305	319	193	196	194	194	84	100
	# 8	120	120	167	167	193	193	187	187	305	305	193	196	202	268	84	84
	G7-8	107	120	167	167	113	193	187	187	305	319	193	196	194	194	84	84
LOU	# 9	120	120	167	167	NA	NA	187	187	326	326	193	196	199	327	84	84
	# 10	107	120	167	167	157	184	187	193	305	326	193	196	202	268	84	84
	G9-10	107	120	107	167	157	184	187	187	305	326	193	196	NA	NA	84	84
DUP	# 11	120	120	167	167	120	138	187	187	305	305	193	199	194	194	74	84
	# 12	120	120	167	167	120	138	138	187	305	305	196	202	194	194	84	84
	G11-12	120	120	167	167	120	138	138	187	305	305	193	199	194	194	74	84

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