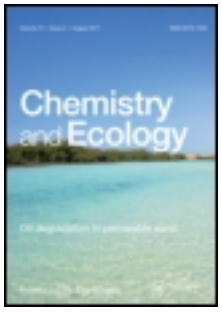

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Genetic analysis of *Pinus banksiana* and *Pinus resinosa* populations from stressed sites contaminated with metals in Northern Ontario (Canada)

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Genetic analysis of *Pinus banksiana* and *Pinus resinosa* populations from stressed sites contaminated with metals in Northern Ontario (Canada)

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The Sudbury region in Canada is known for the mining and smelting of high-sulphide ores containing nickel, copper, iron and precious metals. Although reports provide information of metal levels in soil and plants, knowledge of genetic effects on plants growing in contaminated areas is limited. The main objective of this study was to characterise the level of genetic diversity in *Pinus banksiana* and *Pinus resinosa* populations from the Sudbury (Ontario) region using microsatellite markers. Soil samples were analysed for concentrations of metals. High levels of metal contents in soil were observed within short distances of the smelter compared with control sites. The level of genetic diversity was very low for *P. resinosa* populations and moderate for *P. banksiana* samples. Observed heterozygosity was fivefold higher in *P. banksiana* populations than *P. resinosa* populations studied. Overall, 17 and 24% of the total genetic diversity were attributed to differences among populations for *P. banksiana* and *P. resinosa*, respectively. In general, the inbreeding was significantly higher in *P. resinosa* populations than *P. banksiana* populations and gene flows were relatively low in both species. No significant trend of the levels of genetic diversity for metal contaminated and uncontaminated sites was found.

Keywords: microsatellites; *Pinus banksiana*; *Pinus resinosa*; genetic diversity; metal contamination; Sudbury (Ontario, Canada)

1. Introduction

Several environmental factors and human activities can significantly reduce the level of genetic diversity in plant populations. Evidence of loss of genetic variation based on isoenzymatic analysis at the population level caused by pollution has been reported for some species such as *Pinus sylvestris* and *Daphnia longispina* [1,2]. However, Dobrzeniecka et al. [3] found no association between metal accumulation in soil and genetic diversity in *Picea mariana* populations based on genetic analysis using inter simple sequence repeat (ISSR) dominant markers. Microsatellites have been proven to be powerful tools for studying genetic diversity in tree species. Their inheritance follows a codominant medelian manner.

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Elsik et al. [4] described the usefulness of low-copy microsatellite markers in large complex pine genomes. Other studies on microsatellite analysis of conifer species such as *Pinus strobus* and *Pinus taeda* have been conducted by Echt et al. [5], and Marquardt and Epperson [6]. These studies provide the framework for mapping and for genetic diversity studies in other pine species. No microsatellite analysis of *Pinus banksiana* and *Pinus resinosa* populations has been conducted to compare the level of genetic diversity in those species with other pines. Fowler and Morris [7] and Mosseler et al. [8] found no polymorphism in their studies on isozyme genetic markers in *P. resinosa*. This is in contrast to data reported from other gymnosperms, where 71% of allozyme loci were found to be polymorphic at the species level and 58% at the population level [9]. The low level of genetic variation was confirmed by random amplified polymorphic DNA (RAPDs) analysis [8]. Other studies conducted by Echt et al. [10] and Walter and Epperson [11] revealed population genetic diversity in *P. resinosa* based on chloroplast microsatellite analysis. In general, chloroplast microsatellites mutate at a quicker rate than nuclear loci and are highly variable in conifer species [12]. The amount of variation found in *P. resinosa* using chloroplast microsatellite markers is still much lower than values reported in other pine species using the same markers [13].

Cross-species amplification of microsatellite markers has also been recently analysed in genetic studies of conifers. Echt et al. [5] reported the isolation of microsatellite loci from *P. strobus* and characterised these markers based on their level of polymorphism and abundance throughout *P. strobus* genomes. Echt et al. [5] suggested that the *P. strobus* SSR markers developed could also be used in other conifer species, based on the conservation of SSR loci in mammalian and citrus genomes [14,15]. Echt and May-Marquardt [16] also developed and characterised microsatellite markers from *P. taeda* and assessed these markers, along with markers developed from *P. strobus* in soft and hard pine genomes. Echt et al. [17] reported the evaluation of 21 SSR primer pairs developed from *P. strobus* and six SSR primer pairs developed from *P. radiata* in two other soft pine species, seven hard pine species (one of which was red pine), and two non-pine conifers. Results from this last study showed no trans-specific amplification across pine subgenera. Karhu et al. [18] later reported improved trans-specific amplification using more stringent PCR protocols. Devey et al. [9] also reported that 9 of 20 microsatellite markers derived from *P. radiata* amplified *P. taeda* DNA. Kutil and Williams [19] evaluated the trans-specific recovery of triplet repeat microsatellites in conifers using 15 *P. taeda* SSR primers.

The main objective of this component of the present study was to characterise *P. banksiana* and *P. resinosa* populations from metal contaminated areas in the Sudbury (Ontario) region using heterologous microsatellite primers developed from *P. strobus* and *P. taeda*.

2. Materials and methods

2.1. *Soil analysis*

Soil samples were analysed in collaboration with TESTMARK Laboratories Ltd (Sudbury, Ontario, Canada). The laboratory is ISO/IEC 17025 certified, a member of the Canadian Council of Independent Laboratory (CCIL) and the Canadian Association of Environmental Analytical Laboratories (CAEAL). It is accredited by the Standards Council of Canada (SSC). The laboratory employs standard QA/QC procedures, involving blank and replicate analyses and with a recovery rate of $98 \pm 5\%$ in analyses of spiked samples depending on element selected, in their inductively coupled plasma mass spectrometry (ICPMS) analyses reported here.

The data for the metal levels in soil samples were analysed using SPSS 7.5 for Windows. All the data were transformed using a $\log_{10}$ transformation to achieve a normal distribution. ANOVA

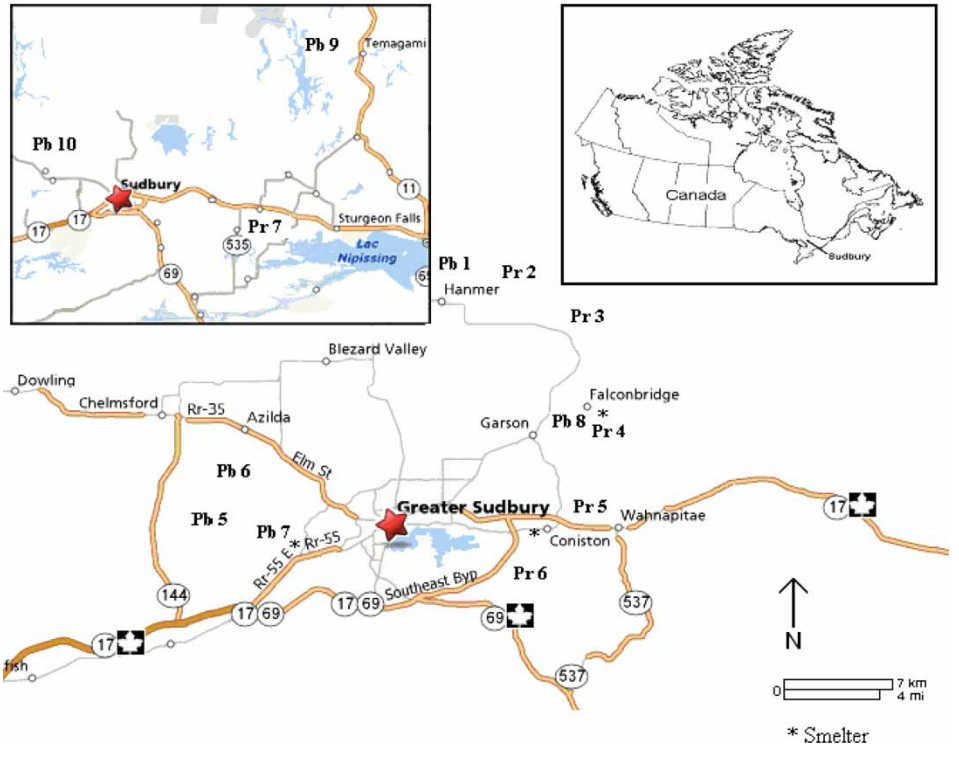

Figure 1. Map of Sudbury (Ontario) showing sampling sites. *Pinus banksiana* populations: Pb 1 represents Val Caron; Pb 2, Introduction 1 (not on map); Pb 3, Introduction 2 (not on map); Pb 4, INCO 3 (not on map); Pb 5, INCO 1; Pb 6, INCO 2; Pb 7, INCO Tailing; Pb 8, Falconbridge; Pb 9, Temagami (control); Pb 10, Low Water Lake, Cartier (control); Pb 11, Introduction 3 (not on map); Pb 12, Introduction 4 (not on map). *Pinus resinosa* populations: Pr 1 represents Introduction 1 (not on map); Pr 2, near Falconbridge; Pr 3, very near Falconbridge; Pr 4, Falconbridge; Pr 5, Coniston/Wahnipitae; Pr 6, Daisy Lake (near highway 17); Pr 7, Verner; Pr 8, Introduction 2 (not on map).

followed by Tukey HSD multiple comparison analysis was performed to determine significant differences ($p < 0.05$) among the five sites.

2.2. *Tree sampling*

Needles were collected from 12 *P. banksiana* and 8 *P. resinosa* populations from the Sudbury, Ontario region (Figure 1). In general, 10% of each population were surveyed as a sampling size representing 100–200 trees. Upon collection, ∼5 g of needles from each individual sample were weighed, wrapped in labelled aluminium foil, flash frozen in liquid nitrogen and stored at −20 °C until further use.

2.3. *Molecular analysis*

2.3.1. *DNA extraction*

The total cellular DNA from individual samples was extracted from the needles using the method described by Nkongolo et al. [20], with some modifications. The concentration of each bulk sample was determined using the fluorochrome Hoechst 33258 fluorescent DNA quantitation kit from Bio-Rad (cat. # 170-2480). Samples were then standardised to a 1 ng·μL^{-1} volume using 1 × TE.

2.3.2. *Amplification of DNA samples from* P. banksiana *and* P. resinosa *populations*

Twenty-two microsatellite primers, synthesised by Invitrogen, were chosen for amplification of 12 populations from *P. banksiana* and 10 populations form *P. resinosa*. DNA amplification was performed following the procedure described by Nkongolo et al. [20] with some modifications. In a 25 μL volume, 5 ng of template DNA, 0.3 μM of each primer pair and 200 μM each of dATP, dCTP, dGTP and dTTP were mixed with 10 × reaction buffer II (Perkin–Elmer), 1.5 mM $MgCl_2$ and 0.625 units of *Taq* DNA polymerase (Perkin–Elmer). Samples were amplified on a DNA thermal cycler (Perkin–Elmer). The PCR protocol for microsatellite analysis was performed as follows: 95°C for 5 min followed by one cycle of 2 min at 85°C. This was followed by 42 cycles of 95°C for 30 s, 46.7°C for 1 min 30 s and 72°C for 30 s. with a final extension step of 72°C for 7 min. Initial amplification was verified on a 1% agarose gel in TBE buffer. The remaining PCR product was diluted with an equal volume of denaturing loading buffer consisting of 10 mM sodium hydroxide, 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. Six microlitres of this mix was boiled for 5 min and then snap-cooled on ice before being loaded on to a 6% denaturing polyacrylamide gel containing 8 M urea and 1 × TBE buffer on a SEQUI-GEN GT Nucleic Acid Electrophoresis Cell apparatus (Bio-Rad) at 45 W for 2 h. Following electrophoresis, the gel was silver-stained using the Silver Sequence DNA Sequencing System (Promega). Stained gels were dried and documented.

2.4. *Statistical analysis*

Popgene software, version 1.32 [21] was used to assess the intra- and interpopulation genetic diversity parameters such as the mean number of alleles (N_A) across loci, the total number of alleles (N_T) per locus and Shannon's information index (I) [21]. Further, the observed and expected heterozygosities (H_O and H_E respectively) were calculated using Genepop software, Version 3.4 [22]. The probability test was computed using the Markov chain method (1000 iterations) in order to determine populations in Hardy–Weinberg equilibrium (Genepop). Hardy–Weinberg equilibrium (HWE) deviations were tested using alternative hypotheses, deficiency and excess of heterozygotes, for each locus, and across loci and populations using Fisher's method. Chapuis and Estoup [23] categorised null allele frequencies into three classes; negligible ($r < 0.05$), moderate ($0.05 \leq r < 0.20$) or large ($r \geq 0.20$). A test for null alleles was also perfomed using the EM algorithm of Dempster et al. [24]. Wright's F statistics, such as among population differentiation (F_{ST}) and inbreeding within populations (F_{IS}), were determined for both species by the method described in Weir and Cockerham [25] using Genepop. The average effective number of migrants exchanged between populations in each generation, or gene flow (N_M) is estimated from F_{ST} (subdivision among populations).

The relationship among the 12 *P. banksiana* and 10 *P. resinosa* populations based on the genotypes generated by the loci was determined by calculating the Cavalli-Sforza and Edwards [26] chord's distance using PowerMarker software Version 3.25. FSTAT software was also used to test the significance of F_{ST} and F_{IS}. The scale used for the genetic distance runs from 0 (meaning no genetic difference) to 1 (different for all conditions–criteria).

3. Results

3.1. *Metal content*

Soil samples were analysed for concentrations of metals including aluminium, arsenic, cadmium, cobalt, copper, lead, manganese, magnesium, nickel and zinc. The metal content in all the samples

Table 1. Metal concentrations in soil from the Sudbury region.

Locations	Depth (cm)	Cadmium ($mg \cdot kg^{-1}$)	Cobalt ($mg \cdot kg^{-1}$)	Copper ($mg \cdot kg^{-1}$)	Lead ($mg \cdot kg^{-1}$)	Magnesium ($mg \cdot kg^{-1}$)	Nickel ($mg \cdot kg^{-1}$)	Zinc ($mg \cdot kg^{-1}$)
1. Xstrata	0–5	0.1	10.0	93.0	29.0	207.0	96.0	23.0
	5–20	0.1	6.5	75.5	15.5	219.0	42.5	19.0
2. Xstrata	0–5	1.3	29.0	866.0	202.0	129.0	841.0	38.0
	5–20	0.1	6.0	45.5	12.0	206.5	48.5	17.5
3. Xstrata	0–5	0.7	20.0	673.0	180.0	232.0	410.0	60.0
	5–20	0.1	9.0	43.5	13.5	373.5	33.5	29.5
4. Vale	0–5	0.3	7.0	146.0	33.0	464.0	128.0	40.0
	5–20	0.1	9.0	34.5	17.0	477.0	43.5	59.5
5. Vale	0–5	1.2	29.0	808.0	158.0	697.0	675.0	83.0
	5–20	0.3	13.5	175.0	29.5	877.5	96.0	66.5
6. Vale	0–5	0.8	16.0	528.0	85.0	181.0	306.0	52.0
	5–20	0.2	7.5	90.0	22.0	271.5	39.5	73.5
7. Vale Tailings	0–5	0.3	37.0	245.0	46.0	361.0	229.0	38.0
	5–20	0.1	23.5	200.5	28.0	396.0	144.0	40.0
8. Xstrata Tailings	0–5	0.3	31.0	355.0	53.0	1032.0	356.0	80.0
	5–20	0.2	30.5	302.5	52.0	1096.0	327.	78.0
9. Control	0–5	0.2	15.0	48.0	29.0	516.0	42.0	56.0
	5–20	0.1	19.0	46.0	13.5	542.0	56.5	47.0
10. Control	0–5	0.2	3.0	35.0	4.0	129.0	26.0	22.0
	5–20	0.1	5.0	9.0	9.5	232.0	14.5	18.5
LSD		1.23	15.3	98.1	49.0	98.5	99	21

Notes: Concentrations are given in $mg \cdot kg^{-1}$, dry weight. LSD, least significant differences ($p > 0.05$). Sites 1, 2 and F are located around Xstrata Nickel (formerly Falconbridge Ltd.) smelter; sites 4, 5 and 6 are located around Vale (Inco Ltd.) smelter; site 7 is Vale (Inco Ltd.) tailing; site 8 is located on Xstrata (Falconbridge Ltd.) property; site 9 is Temagami (control site), and site 10 is Low Water Lake (control site).

analysed is summarised in Table 1. High levels of metal content in soil were observed within short distances of the smelter in Sudbury compared with control sites [27–29]. As in previous studies, the highest level of metal accumulation in soil was recorded in samples from populations near the Falconbridge and Vale (previously INCO) smelters in Sudbury, Ontario [23,28,29].

3.2. *Genetic diversity*

The microsatellite loci analysed in *P. banksiana* and *P. resinosa* populations are summarised in Table 2. Eleven of 22 primer pairs generated moderate to good amplification products. For *P. banksiana*, the mean number of alleles per locus was 9 and the mean effective number of alleles was 3.5 (Table 3). The mean number of alleles across loci per population ranged from 3.00 to 4.67, with the samples from the INCO 3 (site 4) and introduction 3 (site 11) population having the highest allelic diversity. The lowest allelic diversity was observed in samples from the INCO tailing (site 7) populations (Table 4).

For *P. resinosa*, the mean number of alleles per locus was 6 and the mean effective number of alleles was 2.50. The mean number of alleles across loci per population ranged from 2.33 to 3.00 for the *P. resinosa* populations (Table 3). The highest allelic diversity was observed the samples from site 2 near Falconbridge and site 7 located in Verner. The lowest allelic diversity was found in the samples from site 4 in Coniston/Whanipitae and the newly introduced population (introduction or site 2) from nursery 2, identified as population or site 8 (Table 4).

3.2.1. *Heterozygosity and homozygosity*

For *P. banksiana*, the observed heterozygosity (H_O) at the population level ranged from 0.26 to 0.67 and the expected heterozygosity (H_E) varied from 0.46 to 0.72. The samples from INCO 3

Table 2. Simple sequence repeat (SSR) loci analysed in *Pinus banksiana* and *Pinus resinosa* populations from the Sudbury, Ontario region.

Locus	Motif	Primer sequence	Amplification	Range
PtTX 3013	(GTT)	F: GCTTCTCCATTAACTAATTCTA R: TCAAAATTGTTCGTAAAACCTC	Moderate	134
PtTX 3025	(CAA)	F: CACGCTGTATAATAACAATCTA R: TTCTATATTCGCTTTTAGTTTC	Poor	266
PtTX 3030	(TA)...(GGT)	F: AATGAAAGGCAAGTGTCG R: GAGATGCAAGATAAAGGAAGTT	Moderate	287
PtTX 3020	A(CAA)	F: GTCGGGGAAGTGAAAGTA R: CTAGGTGCAAGAAAAGAGTAT	Poor	211
PtTX 3098	(GTT)	F: TTTGCACTATGGCATAAGTCCT R: CCCTGTTTCTACCCTTGATGA	Good	187
PtTX 3019	(CAA)	F: AAGAATATCAAGCACTCC R: CAAAGGCATAAAGAAACT	Moderate	223
PtTX 2123	(AGC)	F: GAAGAACCCACAAACACAAG R: GGGCAAGAATTCAATGATAA	Good	202
PtTX 3118	(CAT)CGT(CAT)CAC(CAT)	F: CACGGCCCTTAGCTTTACCTT R: TTCTGATGGGGCAACTG	Moderate	212
PtTX 3017	(GAT)	F: GGCCTCTCCAGTTGCTCAT R: AAGATAAAGAAAGTTGGAAGAC	Poor	212
PtTX 3088	(GAT)	F: TTTGCACTATGGCATAAGTCCT R: CCCTGTTTCTACCCTTGATGA	Good	253
RPS 1	(AC)	F: GCCCACTATTCAAGATGTCA R: GATGTTAGCAGAAACATGAGG	Poor	193–207
RPS 2	(AC)	F: CATGGTGTTGGTCATTGTTCCA R: TGGAGGCTATCACGTATGCACC	Good	149–171
RPS 6	(AC)	F: TTTTCTAATCAGTGTGCGCTACA R: CACCGCTGCCCTATTTTACA	Poor	159–164
RPS 12	(AC)	F: TCAATGTGGAGATGGTGATT R: ACTTCTGACCTAACCAGAAACC	Poor	163–209
RPS 20	(AC)(AT)	F: ACTTCCCCACAGGTTAACACA R: AACAAGATAGGCGGGATTCA	Good	138–174
RPS 25b	(AC)(AT)	F: CACATATGGCAGAACACACA R: GATCGTCGCACTATCGAAC	Moderate	97–115
RPS 34b	(AC)	F: CAGTGTTCTCTTATCACAGCG R: GCACTATAATGAAATAGCGCA	Poor	145–149
RPS 39	(AC)	F: GCCAGCTCCAACCAGAATC R: GGCTCGCTGACCCAATAA	Poor	172–174
RPS 50	(AC)	F: CCCAGAAATCTGTTTTAGAGC R: ACACATGAAATGTCAGAATGC	Poor	160–188
RPS 84	(AG)(AC)	F:CCTTTGGTCATTGTATTTTTGGAC R: CTTCCTTTTCCTTCTTGCTCCAC	Moderate	145–163
RPS 90	(AC)	F: ACCCATTGTGGTGTGTTTGTG R: CCTCCGACCATAAACCTTAATG	Poor	138–164
RPS 118b	(AC)	F: CATTGTGGTGTGTTTGTGAA R: CCACCTCCGACCATAAAC	Poor	148–164

Note: The PtTX series are derived from *Pinus taeda* and the RPS series from *Pinus strobus*.

(site 4) produced the highest H_O values and the samples from Low Water Lake (site 10) used as control showing the lowest observed heterozygosity (Table 4). The degree of population differentiation (FST) was 17% for *P. banksiana* (Table 3).

For *P. resinosa*, the observed heterozygosity (H_O) at the population level, ranged from 0.07 to 0.55. Samples from site 2 located near Falconbridge produced the highest heterozygosity and the samples from nursery 2 (introduction 2) called population or site 8 showed the lowest values (Table 4). H_E values ranged from 0.28 to 0.43. The degree of population differentiation (F_{ST}) was 23.9% for *P. resinosa* (Table 3).

After the correction for null alleles, the exact test for HWE revealed that the majority of the populations for both species deviated significantly from the HWE. The results revealed that the

Table 3. Mean total alleles, effective number of alleles, and genetic differentiation and gene flow for 10 populations of *Pinus banksiana* and 10 populations of *Pinus resinosa*.

	N_T	N_E	F_{IS}	F_{ST}	N_M
Pinus banksiana					
Mean over all populations	9	3.5	0.18	0.17	1.21
SE	1.7	0.17	0.19	0.09	0.80
Pinus resinosa					
Mean over all populations	6	2.50	0.75	0.24	0.79
SE	4.0	1.20	0.17	0.10	0.03

Note: N_T is the total mean number of alleles over all populations; N_E is the effective number of alleles; F_{IS} is the individual inbreeding coefficient relative to the subpopulations; F_{ST} is the inbreeding coefficient relative to the total population; N_M is the gene flow estimate from $F_{ST} = 0.25(1 - F_{ST})F_{ST}$.

Table 4. Genetic diversity estimates for 12 *Pinus banksiana* and 8 *Pinus resinosa* populations from the Sudbury, Ontario region.

Species/Population	N_A	N_{Ap}	H_O	H_E	I
Pinus banksiana					
Val Caron (site 1)	4.3333	3.3604	0.6667	0.6983	1.2967
Introduction 1 (site 2)	4.0000	2.7951	0.4667	0.6096	1.1051
Introduction 2 (site 3)	4.0000	2.6056	0.4000	0.5850	1.0650
Inco 3 (site 4)	4.6667	2.9764	0.7333	0.6133	1.1734
Inco 1 (site 5)	4.0000	2.9054	0.5000	0.6500	1.1727
Inco 2 (site 6)	3.6667	2.7763	0.6333	0.5367	0.9716
Inco tailing (site 7)	3.0000	2.1221	0.3333	0.5129	0.8551
Falconbridge (site 8)	3.6667	2.1973	0.5852	0.5421	0.9443
Temagami (site 9)	4.0000	2.6277	0.6333	0.6050	1.1054
Low Water Lake (site 10)	3.6667	2.7987	0.2593	0.6235	1.1098
Introduction 3 (site 11)	4.6667	3.7420	0.4000	0.7283	1.3936
Introduction 4 (site 12)	3.6667	2.0854	0.2583	0.4554	0.8578
Mean	4.0000	2.7422	0.4912	0.7194	1.5155
SE	0.4678	±0.1723	±0.3679	±0.0133	±0.0939
Pinus resionosa					
Introduction 1 (site 1)	2.6667	2.0994	0.1000	0.4150	0.7118
Near Falconbridge (site 2)	3.0000	2.1847	0.5506	0.4258	0.7809
Very Near Falconbridge (site 3)	2.6667	1.8039	0.1667	0.3267	0.5926
Falconbridge (site 4)	2.6667	1.9750	0.1333	0.3748	0.6653
Coniston/Wahnipitae (site 5)	2.3333	1.7365	0.0667	0.3431	0.5497
Daisy Lake (site 6)	2.6667	1.7188	0.0667	0.3346	0.5914
Verner (site 7)	3.0000	1.5541	0.1000	0.3017	0.5459
Introduction 2 (site 8)	2.3333	1.7060	0.0667	0.2783	0.5041
Mean	2.6667	1.8473	0.0892	0.4606	0.9477
Standard error	0.2520	±1.3076	±0.0941	±0.3992	±0.828

Note: N_A, mean allele number per locus; N_{Ap}, mean number of polymorphic alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; I, Shannon's information index.

null allele frequency estimates were negligible for all populations (data not shown). The HWE deviation for these populations might be the result of other factors than null alleles. The global tests revealed significant heterozygote deficiency for most populations.

3.3. *Gene flow*

The gene flow estimates were considered low for both species, $N_m = 1.21$ for *P. banksiana* and $N_m = 0.79$ for *P. resinosa* (Table 3) based on Slatkin [30]. There was also no significant difference

in the inbreeding coefficients among stands within the same species. The mean inbreeding coefficients were considered high for *P. resinosa* and low for *P. banksiana* (Table 3).

3.4. *Genetic relatedness*

The coefficient of genetic distance was calculated for pair-wise comparisons of the 12 *P. banksiana* and 8 *P. resinosa* populations using Cavalli-Sforza and Edwards [26] chord distance coefficients (D_c). The scale for genetic distance ranges from 0 (indicating no genetic difference) to 1 (indicating difference from all conditions/criteria). The genetic distance among the *P. banksiana* populations ranged from 0.04 to 0.27 (Table 5). For *P. banksiana*, the most closely related populations were INCO 2 (site 6) and INCO 3 (site 4), whereas the most distantly related populations were the Low Water Lake (site 10) and INCO tailing (site 7) (Table 5).

The genetic distance among the *P. resinosa* populations ranged from 0.02 to 0.42 (Table 6). The most genetically closely related populations were from Daisy Lake (site 6) and Verner (site 7) while the most distantly related populations were from site 4 (Falconbridge) and nursery 2 or introduction 2 (site 8) (Table 6).

Table 5. Distance matrix generated from simple sequence repeat (SSR) data used in neighbour-joining analysis of *Pinus banksiana* populations from the Sudbury, Ontario region.

	Site											
Site	1	2	3	4	5	6	7	8	9	10	11	12
1	0.00	0.17	0.11	0.19	0.05	0.23	0.13	0.19	0.14	0.15	0.08	0.26
2		0.00	0.11	0.06	0.15	0.09	0.13	0.10	0.11	0.15	0.09	0.05
3			0.00	0.15	0.07	0.22	0.09	0.13	0.18	0.19	0.14	0.15
4				0.00	0.12	0.04	0.20	0.06	0.10	0.13	0.11	0.06
5					0.000	0.14	0.08	0.11	0.13	0.14	0.08	0.19
6						0.00	0.18	0.08	0.12	0.18	0.12	0.09
7							0.000	0.18	0.24	0.27	0.15	0.17
8								0.000	0.10	0.14	0.11	0.09
9									0.000	0.19	0.11	0.18
10										0.000	0.04	0.20
11											0.000	0.15
12												0.000

Note: Site 1, Val Caron; site 2, Introduction 1; site 3, Introduction 2; site 4, Inco 3; site 5, Inco 1; site 6, Inco 2; site 7, Inco tailing; site 8, Falconbridge; site 9, Temagami; site 10, Low Water Lake; site 11, Introduction 3; site 12, Introduction 4.

Table 6. Distance matrix generated from simple sequence repeat (SSR) data using neighbour-joining analysis of *Pinus resinosa* populations from the Sudbury, Ontario region.

	Site							
Site	1	2	3	4	5	6	7	8
1	0.00	0.11	0.29	0.20	0.15	0.19	0.20	0.20
2		0.00	0.23	0.07	0.12	0.09	0.12	0.26
3			0.00	0.34	0.08	0.25	0.23	0.11
4				0.00	0.15	0.08	0.17	0.42
5					0.00	0.11	0.13	0.14
6						0.00	0.02	0.22
7							0.00	0.15
8								0.00

Note: Site 1, Introduction 1; site 2, near Falconbridge; site 3, very near Falconbridge; site 4, within Falconbridge property; site 5, Coniston/Whanipitae; site 6, Daisy Lake; site 7, Verner; site 8, Introduction 2.

4. Discussion

4.1. *Cross-species amplification of microsatellite markers*

The microsatellite primers used in this study were developed from a large insert genomic library from *P. strobus* by Echt et al. [5] and from a low-copy microsatellite library for *P. taeda* [4]. Echt et al. [17] tested the RPS series of microsatellites in several soft and hard pine species, including *P. resinosa*, as well as non-pine conifers, to assess their transferability across pine species. They found that (AC) dinucleotide microsatellite loci were amplifiable in soft pine species, but were unsuccessful in amplifying DNA in hard pine. Stringent optimisation of PCR conditions improved the transferability of microsatellite loci across species [18]. In our study, of the 12 RPS series microsatellite markers screened, only three generated moderate to good amplification products in *P. banksiana* and *P. resinosa* after several optimisation reactions. Criteria for successful transfer were clear amplification products in the appropriate base pair range and polymorphism. However, 7 of the 10 PtTX microsatellite primers tested generated moderate to good amplification products.

4.2. *Genetic diversity*

In this study, the genetic diversity of *P. banksiana* was fivefold higher compared with *P. resinosa*. The H_O values for *P. banksiana* were within the same ranges of data reported in other species. In fact, Rajora et al. [31] analysed six *P. strobus* populations from Ontario and three from Newfoundland, and identified seven alleles per microsatellite locus and an H_O of 0.51. Echt et al. [5] found a mean number of 5.4 alleles per locus and an average observed heterozygosity of 0.52 for *P. strobus* trees from north–central USA. Mehes et al. [32] reported an H_O value of 0.49 for *P. strobus* and Bruno and Brinegar [33] detected an H_O value of 0.46 in *Sequoia sempervirens*. The level of genetic diversity described in our study for *P. banksiana* was higher than that described for other conifer species such as *Larix lyallii* ($H_O = 0.389$; [34] and *Pinus monticola* ($H_O = 0.36$; [32]). The H_O estimates for *P. banksiana* remain lower compared with other Pinaceae species such as *Pinus radiata* ($H_O = 0.625$; [35]) and *Picea mariana* ($H_O = 0.589$; [3]). However, the level of genetic diversity observed in *P. resinosa* populations in this study ($H_O = 0.089$) is the lowest found in conifer species studied using microsatellite markers.

Previous izozyme, RAPD and ISSR analyses of *P. resinosa* populations revealed a low level of genetic variation [36,37]. The ability of *P. resinosa* to self-pollinate and its fragmented metapopulation structure may have promoted the loss of genetic variation through inbreeding in small populations [36]. Despite the increased population numbers that occurred after glacial periods by range extension and mutation, there has been little detection of an increase in genetic variation at the molecular level. *P. resinosa* is a model example that illustrates the long duration expected for a species with a long generation time to recover from passage through a genetic bottleneck. Simulations presented by Nei et al. [38] indicate that it can take as much as one million years for a species such as *P. resinosa* to recover its genetic variations via a combination of mutations and increases in population number.

High levels of population differentiation and inbreeding values were observed in the *P. resinosa* populations ($F_{ST} = 0.24$), and to some extent in *P. banksiana* populations ($F_{ST} = 0.17$), as compared with other studies. This indicates that 17 and 24% of the total genetic diversity are attributed to differences among populations for *P. banksiana* and *P. resinosa*, respectively. In *P. strobus* and *Pinus monticola* mean F_{ST} values were 0.07 and 0.13, respectively [32]. Previous studies for *P. strobus* showed values of 0.061 [31] and 0.019 [39] for population differentiation (F_{ST}). Ledig et al. [40] reported an F_{ST} value of 0.152 for *P. pinceana*. Possible explanations for high values of F_{ST} are population isolation, small population size, restricted gene flow and young individuals within the populations [31].

Interpopulation differentiation (F_{ST}) values were used to estimate the number of migrants per generation among populations of the same species. The level of gene flow estimates for *P. banksiana* ($N_m = 1.2$) and *P. resinosa* ($N_m = 0.80$) were lower than those reported in *P. strobus* ($N_m = 3.9$, [31] and $N_m = 3.10$, [32]), *P. monticola* ($N_m = 5.9$, [32]) and similar to the N_m value of 1.39 reported for *P. pinceana* [40]. Possible causes for low gene flow are the presence of small fragmented or isolated populations characterised by a restricted gene exchange among stands [30].

The Cavalli-Sforza and Edward [26] chord distance coefficients (D_c) are more appropriate for estimating the genetic distance among *P. monticola* and *P. strobus* populations for two main reasons. The algorithm is relatively unaffected by the presence of null alleles with a low to moderate frequency [23]. Further, D_c is not based on a mutational model; rather it relies on allele frequencies to determine the geometric placement of populations in a multidimensional sphere [34]. Genetic distances revealed that populations from similar regions did not always cluster together for *P. banksiana* and *P. resinosa* (data not shown). For both species, there was no defined trend in site grouping based on microsatellite analysis. Samples from introduced *P. banksiana* and *P. resinosa* populations did cluster together with the existing populations. No correlation was found between the genetic distance matrix and geographic distance matrix. This lack of correlation between the genetic and geographical location has been reported also in other pine species [32].

4.3. *Metal contamination and genetic diversity*

In the Greater Sudbury Ontario region, *P. banksiana* and *P. resinosa* have been exposed to toxic substances for more than 50 years. This exposure did not reduce the level of genetic diversity. Among the sites analysed, the highest level of metal content in soil and plant tissues were observed in samples from populations located near Falconbridge and INCO smelters [28,29]. The level of genetic variation in those sites that were highly contaminated was in some cases found to be higher than the levels observed in populations that were uncontaminated. The levels of genetic variation in populations near INCO and Falconbridge were comparable with that of control populations from Temagami, Ontario and higher than levels found in the Low Water Lake control population. This was also reported in other plant species growing in the Greater Sudbury, Ontario region such as *Deschampsia cespitosa* (herbaceous species) and *Picea mariana* populations [3,41]. Analysis of *D. cespitosa* populations growing in metal residue dumping sites in Cobalt (Ontario) showed a significant reduction in the level of genetic variation. The cobalt content in soils from that region was 20 times higher than that of the Sudbury populations [41]. This would indicate that although the level of accumulation of metals in the Sudbury soils is high, they have not reached a threshold level that can affect the allelic frequency and genetic diversity of conifer and herbaceous species [37].

5. Conclusion

In conclusion, cross-species amplification of microsatellite primers in this study was successful. The level of genetic diversity in *P. resinosa* was low, which is consistent with previous studies using other marker systems. The genetic diversity in *P. banksiana* was moderate. The population differentiation was relatively high for both species. The inbreeding coefficient was high for *P. resinosa* and low in *P. banksiana*. Gene flow was low among populations of the two species analysed. The low level of genetic variation in *P. resinosa* is indicative that the sustainability of its populations is threatened. The moderate levels of genetic variation found in *P. banksiana* populations suggest a sustainability that is less problematic. This study revealed no association between long-term exposure of pine populations to metals and the level of genetic variation.

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