

Impacts of forest fragmentation on the mating system and genetic diversity of white spruce (*Picea glauca*) at the landscape level

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We studied the mating system of white spruce (*Picea glauca*) in a landscape fragmented by agriculture in northern Ontario, Canada. We sampled 23 stands that ranged in size from 1 to > 500 trees isolated by 250–3000 m from the nearest other stand. Six polymorphic allozyme loci from four enzyme systems were used to genotype approximately 10 000 embryos from 104 families. We detected no allele frequency heterogeneity in the pollen pool among stands or families ($\Phi_{FT} = -0.025$). Overall, estimates of outcrossing were high ($t_m = 94\%$ and mean $t_s = 91\%$) but significantly different from unity. Bi-parental inbreeding ($t_m - t_s = 3.2\%$) was low but significantly different from zero. Allozyme-based outcrossing estimates did not differ significantly among three stand-size classes (SSCs): small (< 10 trees), medium (10–100 trees) and large (≥ 100 trees). The number of effective pollen

donors was high in all SSCs, but was significantly lower in small stands ($N_{ep} = 62.5$) than in medium-sized and large stands (both $N_{ep} = 143$). The primary selfing rate was significantly higher in medium stands than in large stands. We found no significant difference in genetic diversity measures in the filial (seed) population among SSCs. Overall, these results indicate that white spruce stands in this fragmented landscape are resistant to genetic diversity losses, primarily through high pollen-mediated gene-flow and early selection against inbred embryos. We discuss the importance of using seed data, in conjunction with genetic data, to evaluate the impacts of fragmentation on natural populations.

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Introduction

One of the main objectives of genetic resource conservation is to maintain genetic diversity in natural populations to ensure the continued survival, fitness, and evolutionary potential of a species. Forest fragmentation can affect pollen-mediated gene dispersal, and consequently the mating system of trees, and the genetic diversity and structure of the resultant seed population. The study of the genetic consequences of forest fragmentation in natural plant populations has received increasing attention over the last decade (Hall *et al.*, 1996; Young *et al.*, 1996; Nason and Hamrick, 1997; Collevatti *et al.*, 2001; Cascante *et al.*, 2002; Fuchs *et al.*, 2003; Bacles *et al.*, 2004). Possible negative genetic impacts of fragmentation in plants include increased inbreeding and reduced genetic diversity caused by increased genetic drift and reduced gene flow between fragments (Young *et al.*, 1996). In several herbaceous species, reproductive success decreases with a decrease in population size, either because of pollen-limitation or

an increase in inbreeding in small populations (Vergeer *et al.*, 2003; Reed, 2005).

Much of the research on the impacts of forest fragmentation on tree mating systems has been conducted on tropical species (eg Hall *et al.*, 1996; Nason and Hamrick, 1997; Collevatti *et al.*, 2001; Cascante *et al.*, 2002; Fuchs *et al.*, 2003). Fragmentation affects the mating system of these species by creating genetic bottlenecks (Aldrich and Hamrick, 1998), decreasing offspring fitness (Cascante *et al.*, 2002), or by reducing the number of sires (Fuchs *et al.*, 2003). These tropical species are usually animal-pollinated and occur at naturally low densities. The mutualistic relationship between trees and pollinators may make these species more susceptible to habitat fragmentation than wind-pollinated species. Nevertheless, in wind-pollinated conifers, the mating system can be affected by population structure attributes. For example, in some conifer species, outcrossing rates are correlated with population density (Farris and Mitton, 1984; Rajora *et al.*, 2002), family structure and tree heights (Mitton, 1992; O'Connell *et al.*, 2004). In wind-pollinated trees, surrounding vegetation can also impede pollen dispersal, so that fragmentation can increase pollen and gene flow distances between forest trees (Young and Merriam, 1994; Dyer and Sork, 2001).

White spruce (*Picea glauca* (Moench) Voss), is an ecologically and economically important conifer with a trans-continental range in Canada (Nienstaedt and

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Zasada, 1990). Although white spruce is widespread in Canada, there are concerns about the impact of harvesting and fragmentation on its genetic resources (Rajora, 1999). Natural populations of white spruce have moderate to high levels of genetic diversity (Cheliak *et al*, 1985; Innes and Ringius, 1990; Furnier *et al*, 1991; Rajora, 1999; Rajora and Dancik, 2000). White spruce is a predominantly outcrossing species, but does show significant amounts of self-fertilization in some populations. Population outcrossing rates of 98% (Cheliak *et al*, 1985) and 93% (Denti and Schoen, 1988) have been measured for Ontario populations, but rates as low as 70 and 76% have been estimated for two Newfoundland populations (Innes and Ringius, 1990). Individual family outcrossing rates range from 78 to 100% (Denti and Schoen, 1988). White spruce shows high inbreeding depression, with 80–96% lower seed set following self-pollination compared to cross-pollinations (Fowler and Park, 1983). We have previously found that forest fragmentation negatively affects reproductive success in white spruce, by reducing the number of fertilized ovules and viable seeds per cone in small stands (O'Connell *et al*, 2006). Trees from small stands (<10 trees) set 38% fewer seeds per cone than trees from large stands (>100 trees). The seed viability data strongly suggest that pollen-limitation and increased inbreeding in small stands limit seed set in fragmented stands of white spruce.

Outcrossing rates in plants are usually estimated from filled seeds, and more often from germinated seeds or seedlings. In most conifers, self-fertilized embryos result in empty seeds. Most inbred individuals are eliminated at very early, postzygotic stages of development (eg during embryo development); therefore, the outcrossing estimates based on molecular marker data alone will be inflated compared to actual cross-fertilization rates. By combining empty seed data with molecular marker based estimates of selfing, we can obtain the primary selfing rate, which is the selfing rate at the ovule fertilization stage (ie actual inbreeding rate; Rajora *et al*, 2000a, 2002).

In this study, we used allozyme markers in addition to seed set data, to assess the genetic impacts of forest fragmentation on natural stands of white spruce at the landscape level. Specifically, the objectives were to determine the effects of stand size on the levels of genetic diversity and structure of the pollen pool, inbreeding and correlated matings in the mature parental stands, and genetic (allelic and genotypic) diversity of the resulting filial (seed) population.

Materials and methods

Study site and sampling

The study area is located at the western end of Lake Nipissing, Northern Ontario, Canada (O'Connell *et al*, 2006). The area around Lake Nipissing offers an ideal site to study the impacts of habitat fragmentation on temperate, wind-pollinated species. This area is an old glacial lakebed, interspersed with a number of rocky outcrops of varying sizes, containing residual forests composed, for the most part, of white spruce, eastern white pine (*Pinus strobus* L.), white birch (*Betula papyrifera* Marsh.), and trembling aspen (*Populus tremuloides* Michx.). These isolated patches of trees occur within an

agricultural landscape used largely for corn and dairy production. Small tree stands also occur on islands in Lake Nipissing.

We sampled 23 white spruce stands during August and September 1994. The location map of the stands and details on the sampling procedure are provided in O'Connell *et al* (2006). Briefly, we sampled cones from a total of 104 trees, from 23 stands, varying in size from a single reproductive tree to contiguous forest with thousands of individuals (Table 1). Sampled stands were divided into three stand size classes (SSCs) that differed by an order of magnitude: small (1–<10 trees), medium (10–<100 trees) and large (\geq 100 trees). The total sample area was 378 km². Sampled stands were separated by 250–3000 m from the closest other neighboring white spruce stand (Table 1). Our previous study showed that even at the lowest of these inter-stand distances, seed production was significantly reduced in small stands (O'Connell *et al*, 2006). Sampled trees ranged in age from 19 to 73 years, and in height from 4 to 22 m. Trees did not differ significantly in age and size among SSCs (O'Connell *et al*, 2006). In all 19 stands occurred on rocky outcrops surrounded by agricultural fields, and four stands occurred on similar rocky outcrops on small islands in the lake (A, B, C, and D; Table 1). We sampled cones from all reproductive individuals in small stands, and from six trees per stand in the medium and large stand size classes. There was no significant difference in white spruce tree density among the three SSCs (O'Connell *et al*, 2006).

Genotyping

Seeds were hydrated in Petri dishes on filter paper moistened with distilled water for at least 24 h prior to dissection. A total of 96 embryos were assayed for each family (ie seeds from the same maternal tree), except for two trees from stand C, where only 52 and 36 seeds were available. Of these sampled embryos, the corresponding megagametophytes were assayed separately for 10 seeds per family. Megagametophytes in conifers have the same haploid genotype as the female gamete. Individual embryos and megagametophytes were homogenized in 10 μ l of cold seed extraction buffer (Cheliak and Pitel, 1984). Enzyme electrophoresis was performed using fresh extracts on cellulose acetate gels (Helena Laboratories, Beaumont, TX) following Hebert and Beaton (1989). All gels were run for 25 min at 200 V. Based on a prescreening of enzyme systems and encoding allozyme loci, six polymorphic loci from four enzyme systems were used in this study. These loci met the suitability criteria for mating system analysis. The four enzyme systems assayed were: Fumarate hydratase or Fumarase (FUM; EC 4.2.1.2), Isocitrate dehydrogenase (IDH; EC 1.1.1.42), Leucine aminopeptidase (LAP; EC 3.4.11.1), and Phosphoglucosmutase (PGM; EC 5.4.2.2). The six allozyme loci used for mating system and genetic diversity analyses are as follows: *Fum1*, *Idh1*, *Lap1*, *Lap2*, *Pgm1*, and *Pgm2*. A total of 9880 embryos, and corresponding megagametophytes for 1030 of these embryos, from 104 families were genotyped (Supplementary Table 1). The segregation of the two most common alleles for each locus was verified in megagametophytes of heterozygous mother trees, and they all showed single-locus Mendelian segregation patterns.

Table 1 Stand parameters, sample sizes and percent outcrossing (t_m , multilocus and t_s , mean single-locus) estimates and percent bi-parental inbreeding (t_m-t_s) in 23 stands of white spruce in Northern Ontario, Canada

Stand	No. of reproductive trees per stand	Distance to nearest stand (m)	No. of embryos sampled	t_m (SD)	t_s (SD)	t_m-t_s (SD)
Small						
A	3	400	288	89.5 (0.8)	89.8 (0.2)	-0.3 (0.7)
B	1	2000	96	90.8 (0.7)	90.2 (0.2)	0.6 (0.5)
C	3	3000	183	91.8 (1.2)	90.2 (0.2)	1.6 (1.0)
D	3	1500	288	90.4 (0.6)	90.0 (0.1)	0.4 (0.5)
E	1	300	96	90.3 (0.6)	90.0 (0.2)	0.3 (0.5)
F	4	300	384	90.7 (0.9)	90.1 (0.2)	0.6 (0.8)
G	1	700	96	90.4 (0.4)	90.0 (0.1)	0.4 (0.3)
H	3	500	288	90.4 (0.6)	90.0 (0.1)	0.4 (0.5)
I	1	250	96	89.4 (0.7)	89.9 (0.1)	-0.5 (0.6)
J	8	300	768	89.2 (1.2)	89.7 (0.4)	-0.5 (0.9)
K	4	250	384	91.1 (1.1)	90.2 (0.3)	0.9 (0.8)
Medium						
L	21	300	576	89.5 (0.7)	89.8 (0.2)	-0.3 (0.6)
M	20	250	576	90.3 (0.5)	89.9 (0.2)	0.4 (0.5)
N	56	450	576	91.0 (0.9)	90.2 (0.3)	0.8 (0.7)
O	43	300	576	91.8 (1.0)	90.2 (0.3)	1.6 (0.8)
P	56	300	576	89.9 (0.8)	90.0 (0.2)	-0.1 (0.6)
Q	20	300	576	92.0 (1.3)	90.4 (0.5)	1.6 (0.8)
Large						
R	100	400	576	91.8 (1.0)	90.1 (0.2)	1.7 (0.8)
S	180	400	576	89.6 (0.8)	89.9 (0.2)	-0.3 (0.7)
T	180	300	576	90.6 (0.8)	90.0 (0.1)	0.6 (0.7)
U	> 500	Contiguous	576	91.8 (1.1)	90.3 (0.3)	1.5 (0.9)
V	> 500	Contiguous	576	91.7 (1.2)	90.2 (0.3)	1.5 (0.9)
W	> 500	Contiguous	576	92.4 (0.9)	90.2 (0.3)	2.2 (0.7)

Every reproductive tree was sampled in small stands, and six trees per stand were sampled in medium-sized and large stands.

Ovule and pollen pool genetic heterogeneity

Differentiation among the 23 stands for maternal allele frequencies ($n=104$ trees) were tested with a multivariate analysis of molecular variance (AMOVA) (Dyer and Sork, 2001). The allele frequencies of the pollen pools are estimates of the frequency of the paternal alleles in the sampled seeds from individual families or stands, respectively. To test for allele frequency heterogeneity in the pollen pool among stands, and among families nested within stands, we used the program TwoGener (Smouse *et al*, 2001). TwoGener estimates Φ_{FT} , a measure of pollen pool differentiation among maternal trees (ie families). This measure is analogous to F_{ST} in an AMOVA, but instead of estimating differentiation among populations, it estimates the amount of differentiation in the pollen pool among different mothers. We conducted a two level-analysis of the pollen pool with TwoGener. The effects analyzed were stand ($N=23$), and family ($N=104$), nested within stand. The number of families per stand ranged from one to six (mean of 2.9).

We also used TwoGener to obtain pairwise estimates of genetic differentiation in the pollen pools among the 23 stands. The R-Package (version 4; Casgrain and Legendre, 2001) was used to convert stand x - y coordinates in meters, to pairwise distances in meters. To test for a correlation between genetic differentiation and geographic distances between stands, we used a Mantel's test (Mantel, 1967).

Mating system analysis

Multilocus (t_m) and mean single-locus (t_s) outcrossing rates were jointly estimated with pollen gene frequencies

using the program MLTR (version 3.0; Ritland, 2002) and the Expectation-Maximization method. The pollen pool allele frequencies within a stand were more similar to the overall ovule frequencies, than the ovule allele frequencies within the same stand (results not shown). The TwoGener analysis also indicated that there was no significant differentiation in the pollen pool among mothers, or among stands. Mating system parameters for each group were, therefore, analyzed using the pooled data for all stands. Analyzing the pooled data increases the statistical power (Ritland, 2002). Outcrossing rates were estimated at four different levels: (1) grouped as individual families (ie maternal tree), (2) grouped by stand, (3) as a single pooled population of all stands, and (4) grouped by SSC. SD and 95% confidence intervals for the mating system parameter estimates were obtained from 300 bootstraps with resampling individuals within families for the family and stand level estimates, and resampling of families for the pooled population of all stands and SSC level estimates. Correlation of paternity (r_p), correlation of outcrossing (r_t) and inbreeding coefficients (F) estimates were constrained to equal zero when estimating the outcrossing rates. We also estimated r_p (the probability that two outcrossed seeds from a family share the same father) with the outcrossing rate fixed at 94%, and F and r_t fixed at zero. Biparental inbreeding rates were estimated as t_m-t_s . Significant differences in outcrossing estimates (t_m and t_s), biparental inbreeding (t_m-t_s) and r_p between SSCs were tested using MLTR. Mating system estimates were considered as significantly different from each other when their 95% confidence intervals did not overlap.

The effective number of pollen parents, N_{ep} , can be estimated from both the correlation of paternity estimate (r_p) and the estimate of differentiation among families (Φ_{FT}). Both, $[N_{ep}]^{-1}$ and r_p are defined as the probability that two pollen grains drawn from the same female are from the same male, thus $N_{ep} = r_p^{-1}$ (Ritland, 1989; Smouse and Sork, 2004). In the absence of inbreeding, $2\Phi_{FT}$ should approximate r_p and thus, $N_{ep} \approx (2\Phi_{FT})^{-1}$ (Austerlitz and Smouse, 2001; Burczyk and Koralewski, 2005). We estimated N_{ep} from r_p as well as from Φ_{FT} , calculated first for a single pooled population of all stands, and then separately for the small, medium, and large SSCs.

We estimated the primary selfing rate, by combining empty seed data with allozyme-based estimates of selfing in the filled seeds. In white spruce, seed development requires pollination and fertilization (Owens and Molder, 1979). Empty seeds (postzygotic abortions) have rounded seed coats with an aborted embryo (O'Connell *et al*, 2006). Inbreeding depression is the major cause for embryo abortion in conifers. As discussed in Rajora *et al* (2000a, 2002), we assumed that the 80% of the empty seeds were caused by inbreeding depression following self-fertilization and 20% by environmental factors. Estimates of the percentage of empty seeds were based on 25 cones/per tree (when available) for each of 102 trees (O'Connell *et al*, 2006). The primary selfing rate for each of the 102 trees was calculated by using the following formula for actual inbreeding rate from Rajora *et al* (2000a, 2002) where the primary selfing rate = (percentage of empty seeds \times 80%) + (percentage of filled seeds \times s), where s is the selfing rate and $s = 1 - t_m$, where t_m is the individual family outcrossing rate estimated with allozymes. To test for an effect of SSC on the percentage of empty seeds, the percentage of selfed seeds and the primary selfing rates, we used a nested ANOVA, with SSC as a fixed effect, and stand, nested within SSC, as a random effect. We performed posthoc contrast tests to test for significant pairwise differences among SSC. We conducted all statistical analyses with the software JMP™ (version 3.2.1, SAS Institute, 1997).

A Pearson correlation analysis was used to determine if there was a positive correlation between individual family multilocus selfing rates ($s = 1 - t_m$), and the percentage of empty seeds. Data for both these measures were available for 102 of the 104 trees.

Genetic diversity analyses

Measures of genetic diversity were estimated separately for the filial (ie seed) population of each stand. Sample sizes ranged from one to six families (96 to 576 seeds) per stand, with the exception of one small stand, where 768 embryos from eight trees were genotyped. For each stand, we calculated both allelic and genotypic measures of genetic diversity. We estimated expected heterozygosity (He), observed heterozygosity (Ho), and Weir and Cockerham's (1984) inbreeding coefficient (f) with the software Genepop (version 3.3, an updated version of 1.2, Raymond and Rousset, 1995). We also calculated the number of alleles per locus (A), and the effective number of alleles per locus as:

$$Ae = \frac{1}{1 - He}$$

where $1 - He$ is the expected homozygosity. The program FSTAT (Goudet, 2001) estimates genetic measures that are corrected for samples of different sizes, and thus allows us to compare these quantities among populations. We used FSTAT to calculate the allelic richness (AR), the gene diversity (H , equivalent to He) and the inbreeding coefficient (F_{IS}).

We also calculated latent genetic potential (LGP ; Bergmann *et al*, 1990) and a measure of genotypic diversity, genotypic additivity (G_A ; Rajora *et al*, 2000b), for each stand. The latent genetic potential provides an estimate of new mutations and rare or low frequency allele complement that could provide selection potential for adaptation under changed environment and stress conditions (Stebbins and Hartl, 1988). LGP was calculated as the difference between total number of alleles and effective number of alleles summed over all loci:

$$LGP = \sum_{l=1}^n (A_l - Ae_l)$$

where l is equal to locus 1 to n , A is the number of alleles and Ae is the number of effective alleles at locus l . Genotypic additivity (G_A) estimates are the measures of genotypic diversity reflecting the diversity of allele combinations in diploid genomes. G_A is the observed or theoretical number of single-locus genotypes (G) summed over all loci. Observed [$G_A(O)$] and expected [$G_A(E)$] genotypic additivity were calculated as:

$$G_A(O) = \sum_{l=1}^n G(O) \text{ and } G_A(E) = \sum_{l=1}^n G(E)$$

where l is equal to locus 1 to n , $G(O)$ is the number of genotypes observed at locus l , and

$$G(E) = \frac{a(a+1)}{2}$$

where a is the number of alleles at locus l . As these measures are sensitive to sample size, samples from small stands will likely show lower diversity simply due to smaller sample sizes. One to four families and 96 to 384 seeds per stand were sampled in small stands (except for one stand where eight families and 768 embryos were sampled), *versus* six families and 576 seeds per stand sampled in the medium-sized and large stands. These measures (A , LGP , $G_A(O)$ and $G_A(E)$) can be statistically compared, however, between medium-sized and large stands, because the sample size is identical in both of these SSCs ($n = 576$ seeds per stand). For each measure of genetic diversity, a one-way ANOVA was used to test for differences in stand measures of genetic diversity among SSCs. We performed two analyses for A , LGP , $G_A(O)$ and $G_A(E)$, one including small stands and one excluding small stands.

Outcross pollen pool genetic diversity

We estimated the allele frequency of the outcross pollen pool separately for each of the 23 stands using MLTR with the outcrossing rate fixed at 94%. The pollen genetic diversity ($H_{(pollen)}$) and allelic richness ($AR_{(pollen)}$) of the 23 stands were estimated at each locus with the program FSTAT. We tested for a significant difference in mean stand-level pollen gene diversity and allelic richness among the three SSCs using a permutation test in FSTAT.

Results

Ovule and pollen pool heterogeneity

An AMOVA indicated significant differences in ovule allele frequencies among the 23 stands ($F_{ST}=7.4\%$; $P=0.01$). The TwoGener estimates of pollen pool differentiation among stands ($\Phi_{ST}=-0.01$), and families nested within stands ($\Phi_{FS}=-0.015$) were very near zero, indicating that there was no genetic structure in the pollen pool. The estimate of the percent variation due to within family differences was $>100\%$ (102.6%). Estimates of Φ -values below zero and percent variation above 100% are statistical artifacts due to sampling error, but can be expected when there is little or no pollen pool structure. There was also no significant correlation between pairwise estimates of genetic differentiation in the pollen pool among stands, and geographic distances between stands ($n=253$, Mantel's $r=-0.154$, $P=0.084$, based on 1000 permutations).

Mating system

Outcrossing rates at the stand level had quite a narrow range. Multilocus outcrossing rates, t_m , ranged from 89.2 to 92.4%, and mean single locus outcrossing rates, t_s , ranged from 89.7 to 90.3% (Table 1). Estimates of biparental inbreeding were low and ranged from -0.5 to 2.2%. The overall multilocus outcrossing rate estimated for the pooled stands was high ($n=104$ families; $t_m=93.8\%$) and the mean single locus outcrossing rate was slightly lower ($t_s=90.6\%$). Both t_m and t_s estimates were significantly different from unity (Table 2). The biparental inbreeding estimate ($t_m-t_s=3.2\%$) was significantly greater than zero.

When mating system parameters were estimated by SSC (small, medium or large), there were no significant differences among groups in multilocus outcrossing rates (t_m), single locus outcrossing rates (t_s) and biparental inbreeding rates (t_m-t_s) (Table 2). All SSCs showed

amounts of selfing and biparental inbreeding that were significantly greater than zero. Correlation of paternity estimates were small for all SSCs. However, small stands had a slightly, but significantly, higher estimate of correlation of paternity (1.6%) than large and medium-sized stands (both 0.7%).

Based on the r_p values, the estimated effective population size (N_{ep}) was 62.5 trees for small stands and 143 trees each for the medium-sized and large stands. The TwoGener analysis yielded negative Φ_{FT} values for small and large stands, and thus we could not calculate a N_{ep} estimate from these values. When values of Φ_{FT} are near zero, then N is very large. In medium-sized stands, there was low, but significant differentiation among pools from different families ($\Phi_{FT}=2.6\%$). The N_{ep} for medium-sized stands estimated as $(2\Phi_{FT})^{-1}=29$, was much lower than when estimated as $r_p^{-1}=142$.

Primary selfing rates

Trees in the medium SSC had a significantly higher proportion of empty seeds than the large SSC (Table 3). At the individual family-level, the allozyme-based estimates of selfing ranged between 6.8 and 13% (mean = $9.6 \pm 1.3\%$ SD), and the mean estimate of primary selfing over all trees was 39.5% ($\pm 10.7\%$ SD). Mean family level estimates of selfing did not differ among SSCs, but the primary selfing rates were significantly higher in medium stands than in large stands (Table 3). The proportion of empty seeds and selfing rates were both lowest in the large stands, but these two measures were not significantly correlated ($n=102$ trees, Pearson's $r=0.102$; $P=0.31$).

Genetic diversity

The six variable loci (*Fum1*, *Idh1*, *Lap1*, *Lap2*, *Pgm1*, and *Pgm2*) showed high levels of allelic diversity. In the filial subpopulation (ie seeds) we detected a total of 26 alleles,

Table 2 Mating system parameter estimates for all 23 stands pooled in one populations, and separately for small, medium and large stands of white spruce

Group	N	t_m (SD)	t_s (SD)	t_m-t_s (SD)	r_p (SD)	N_{ep}	Φ_{FT} (P-value)	N_{ep}
Pooled stands	104	93.8* (0.8)	90.6* (0.7)	3.2 [†] (0.5)	0.010 [†] (0.001)	100	-0.025 (1.00)	—
Small	32	94.5 ^{a,*} (1.4)	90.5 ^{a,*} (0.7)	4.0 ^{b,†} (0.8)	0.016 ^{a,†} (0.002)	62.5	-0.055 (1.00)	—
Medium	36	92.7 ^{a,*} (1.4)	90.5 ^{a,*} (0.7)	2.1 ^{a,†} (0.9)	0.007 ^{b,†} (0.001)	142.9	0.026 [†] (0.01)	19
Large	36	95.3 ^{a,*} (1.0)	91.2 ^{a,*} (0.6)	4.1 ^{a,†} (0.6)	0.007 ^{b,†} (0.001)	142.9	-0.017 (1.00)	—

Note: mating system estimates are not means but estimated simultaneously for all trees within a group.

N , number of families sampled. Multilocus estimate of the percentage of outcrossed seeds (t_m), mean single-locus estimate of the percentage of outcrossed seeds (t_s), percentage of biparental inbreeding (t_m-t_s), correlation of paternity (r_p), pollen pool differentiation among families (Φ_{FT}), and the effective number of pollen donors (N_{ep}) estimated both from r_p and Φ_{FT} .

*Significantly $<100\%$, [†]Significantly $>0\%$; Estimates in a column followed by the same superscript letters are not significantly different.

Table 3 The effect of stand size class (SSC) and Stand (nested within SSC) on mean percentage of empty seeds, percentage self-fertilized seeds and percentage of primary selfing in 11 small, 6 medium and 6 large stands of white spruce

No. families sampled	SSC			ANOVA-effect	
	Small 30	Medium 36	Large 36	SSC 2 df F-ratio (P-value)	Stand (SSC) 20 df F-ratio (P-value)
% empty seeds (SE)	44.4 ^{a,b} (2.5)	48.8 ^a (2.3)	34.6 ^b (2.3)	5.28 (0.01)	2.48 (0.002)
% selfing (SE)	9.7 ^a (2.4)	9.7 ^a (2.2)	9.4 ^a (2.2)	0.55 (0.59)	0.88 (0.61)
% primary selfing (SE)	40.9 ^{a,b} (1.8)	44.0 ^a (1.6)	33.9 ^b (1.6)	5.56 (0.012)	2.16 (0.0084)

Means in a row followed by the same superscript letters are not significantly different.

Table 4 Genetic diversity measures of filial populations from 23 stands of white spruce grouped into three stand size classes (SSC): small, medium and large stands

SSC	N	He	Ho	f	A	Ae	AR	H	F _{IS}	LGP	G _A (O)	G _A (E)
Small	11	0.329	0.331	-0.005	2.77	1.57	2.16	0.361	0.003	7.20	22	33.2
Medium	6	0.368	0.364	0.011	3.31	1.65	2.06	0.311	0.015	9.91	30	45
Large	6	0.345	0.339	0.016	3.22	1.59	2.19	0.343	-0.003	9.80	30	43.6
Mean over all populations	23	0.344	0.342	0.004	3.03	1.60	2.14	0.343	0.002	8.59	26.3	39
ANOVA (Among SSC)												
All stands F _[2,22]		1.49	0.92	0.77	5.80	1.11	2.67	2.67	0.19	5.13	11.70	6.12
P-value		0.25	0.41	0.77	0.01	0.35	0.09	0.10	0.83	0.02	0.0004	0.008
Large & med. F _[1,11]					0.29					0.02	0.04	0.73
P-value					0.60					0.90	0.80	0.70

N, number of stands; He, mean expected heterozygosity; Ho, mean observed heterozygosity; f, multilocus inbreeding coefficient; A, mean number of alleles per locus; Ae, number of effective alleles per locus; AR, mean allelic richness adjusted for sample size; H, mean He adjusted for sample size; F_{IS}, multilocus inbreeding coefficient adjusted for sample size; LGP, latent genetic potential; G_A(O), genotypic additivity observed; G_A(E), genotypic additivity expected.

Table 5 Estimates of pollen genetic diversity at the stand level at six allozyme loci in 23 white spruce stands

	SSC			P-value
	Small N = 11	Medium N = 6	Large N = 6	
Mean A/L _(pollen)	2.790	3.361	3.250	0.55
Mean AR _(pollen)	2.188	2.217	2.247	
Locus		H _(pollen)		0.68
Fum1	0.220	0.228	0.173	
Idh1	0.404	0.457	0.432	
Lap1	0.247	0.238	0.266	
Lap2	0.274	0.263	0.269	
Pgm1	0.518	0.504	0.510	
Pgm2	0.454	0.501	0.500	
Mean H _(pollen)	0.353	0.365	0.358	

Two-sided P-values of differentiation among stand size classes (SSC) were obtained from 100 permutations.

N, number of stands; A/L_(pollen), mean number of alleles per locus; AR_(pollen), mean allelic richness corrected for sample size; H_(pollen), mean genetic diversity corrected for sample size at each locus.

and the number of alleles per locus ranged between three and five with a mean of 4.3 alleles per locus. The measures of genetic diversity of the filial populations from 23 white spruce stands are in Table 4 (details provided in Supplementary Table 2). Stand level genetic diversity in the filial generation measured by He, Ho, and Ae did not differ significantly among the SSCs. There was also no significant difference in the inbreeding coefficient, f, among SSCs. Measures of genetic diversity that are sensitive to the sample size, such as A, LGP, G_A(O), G_A(E), were all slightly higher in medium-sized stands, but were not significantly different from those in larger stands (Table 4). All four measures, however, were significantly lower in small stands due to the smaller number of seeds sampled per stand, and to the lower number of families present in these stands.

Outcross pollen pool genetic diversity

We found no significant difference in pollen diversity among the three SSCs. Although we detected fewer alleles in the pollen pool of small stands, there was no significant difference in either gene diversity, H_(pollen) or allelic richness, AR_(pollen) among the SSCs (Table 5). The

significant difference in the number of alleles among SSC is likely due to the smaller sample sizes in the small stands, where as few as 96 embryos were sampled in some small stands (Table 1). Gene diversity and allelic richness measures were both corrected for differences in sample sizes.

Discussion

Pollen pool differentiation

Overall, there were no genetic differences in the pollen pool among stands or families of white spruce in the studied fragmented landscape, despite the significant difference in ovule allele frequencies among stands. This result is particularly striking in stands where every reproductive tree was sampled and, therefore, every genotype is known. The pollen pool allele frequency in these stands does not reflect the locally available pollen pool, suggesting that a large proportion of the pollen comes from the surrounding trees outside the stands. The lack of pollen pool differentiation can explain the lack of differentiation in mating system parameters among SSCs and levels of genetic diversity among the filial populations from different SSCs.

Outcrossing and inbreeding rates

Despite the large range of stand sizes sampled (single trees to contiguous forest with >500 white spruce trees) and the large inter-stand distances (up to 3 km), no effect of stand size on the outcrossing rates of white spruce was detected from the molecular marker data alone. The high outcrossing rates (overall $t_m = 93.8\%$ and mean $t_s = 90.6\%$) observed in our study were similar to previously reported rates for white spruce by Denti and Schoen (1988; mean $t_s = 93.1\%$) and Cheliak *et al.* (1985; mean $t_s = 98\%$). Although self-fertilization (6.2%) and biparental inbreeding (3.2%) were significantly greater than zero, these values were not significantly different among SSCs. There were also no significant differences in inbreeding coefficients of the filial populations from the small, medium-sized, and large stands.

Results from previous studies in tropical trees (eg Fuchs *et al.*, 2003) do show a trend towards higher inbreeding in fragmented stands. However, these results are not statistically significant. Other, studies of tropical forest trees mating systems have also failed to find a

significant effect of forest fragmentation on levels of inbreeding in forest tree populations based on molecular marker data alone (Hall *et al*, 1996; Young *et al*, 1996). In contrast to previous mating system studies in fragmented populations, we sampled a large number of embryos ($\sim 10\,000$) and obtained small standard errors ($< 2\%$) for the mating system estimates; thus providing more precise mating system estimates for white spruce. Given the large sample sizes of both seeds and populations in this study, the failure to detect an effect of fragmentation is unlikely to be due to low power and represent type II statistical errors. Rather our study provides strong evidence that there is no genetic effect of fragmentation in this species.

Nevertheless, failing to detect inbreeding differences in trees among different size stands using molecular data does not mean that fragmentation does not increase inbreeding. As many tropical trees are self-incompatible, and trees in general, including temperate conifers, have high levels of early inbreeding depression, most inbred embryos are eliminated before they can be sampled with molecular markers. Thus, viable assayed seeds may show high levels of outcrossing even when high rates of self-pollination or self-fertilization have occurred. This seems to be case in our study where small and medium-sized stands had a higher proportion of empty seeds (aborted embryos) than large stands, and the estimated primary selfing rates in the medium-sized stands were significantly greater than in large stands. Selfing estimates based on the empty seed data followed the same pattern as allozyme-based selfing estimates, where the lowest estimates of selfing were found in large stands. However, there was no correlation between the two measures at the individual tree level. The lack of correlation between the seed viability and allozyme-based selfing estimates is due to individual tree differences for inbreeding depression and possibly also micro environmental conditions.

Effective number of pollen parents (N_{ep})

In our study, we observed high estimates for the effective number of pollen parents in white spruce. These results are consistent with the general trend observed in other conifer species. Based on N_{ep} estimates from 19 different studies of forest trees, Smouse and Sork (2004) outlined three main trends for this parameter: (1) wind pollinated species show higher N_{ep} -values than animal pollinated species; (2) trees with a higher conspecific density have higher N_{ep} (eg El-Kassaby and Jaquish, 1996); (3) The density of other species impedes pollen dispersal yielding higher N_{ep} estimates in stands with more open canopies (eg Dyer and Sork, 2001).

Our overall estimate of $N_{ep} = 100$, is similar to estimates from other conifer species. In *Pinus sylvestris*, $N_{ep} > 70$ (Robledo-Arnuncio *et al*, 2004), in *Larix occidentalis*, N_{ep} ranges from 10 to 1000 (El-Kassaby and Jaquish, 1996) and in *Thuja plicata*, $N_{ep} = 40$ (O'Connell *et al*, 2001). In contrast, N_{ep} between 1 and 2 was estimated from a paternity analysis in a seed orchard of white spruce (Schoen and Stewart, 1986; Smouse and Sork, 2004). In the seed orchard study, however, pollen from outside the orchard was not considered, even though it was only isolated by 1 km from natural white spruce stands. As the results of our study show, a considerable amount of

pollen can travel over this distance in white spruce and the actual N_{ep} in the seed orchard study is probably much larger. Unlike conifers, wind-pollinated oaks and animal-pollinated species tend to show much lower estimates of N_{ep} (Smouse and Sork, 2004). In general, $N_{ep} \leq 12$ has been reported for these nonconiferous species. One exception is *Acacia melaxylon*, where estimates of N_{ep} range from 16 to 33 (Muona *et al*, 1991).

Although there was no significant difference in conspecific tree density among SSCs in our study, we found that N_{ep} was smaller ($< 50\%$) in small stands, than in medium-sized and large stands. This result is consistent with pollen-limitation that we observed in these small stands (O'Connell *et al*, 2006). If a proportion of the seeds are sired by local trees, and there are a few dominant males in a stand, we would also expect some correlation of paternity, especially in small stands with only a few trees. In fact, we have found a slightly higher correlation of paternity, and thus a lower estimate of N_{ep} in small stands than in medium-sized and large stands. Nevertheless, correlation of paternity in small stands was still very low ($r_p = 1.6\%$) and N_{ep} was high for all SSCs.

While previous studies suggest that a reduction in the surrounding canopy can increase pollen flow (Young and Merriam, 1994; Dyer and Sork, 2001) we did not find any evidence of this in our more fragmented (ie small) stands. All stands regardless of size were relatively open and probably experienced high levels of gene flow. The reduction in the total amount of pollen received in small stands could have increased the proportion of the pollen cloud made up by pollen contributed by local trees. However, we failed to detect any differentiation in the pollen pool among small stands.

Estimates of N_{ep} calculated from Φ_{FT} did not correlate well with estimates of N_{ep} calculated from r_p . We found significant differentiation among pollen pools in trees from medium-sized stands, but no differentiation among pollen pools in trees from the small or large stands. Possible reasons for this discrepancy between the two estimates are: (1) the medium SSC showed the lowest outcrossing rate (Table 2), which could inflate the estimate of Φ_{FT} . However, the observed differences in outcrossing rates among the groups are probably not large enough to account for the pollen pool differentiation (Burczyk and Koralewski, 2005). (2) The data set we used for MLTR to estimate r_p also included megagametophyte genotype data for approximately 10% of the seeds, leading to a more accurate estimate of mating system parameters. Nevertheless, the overall estimate of Φ_{FT} was negative, and like r_p , suggests there was very little pollen pool structure in these fragmented white spruce stands.

Genetic diversity of filial population

The results of our study demonstrate that the SSC and fragmentation did not adversely affect the genetic diversity of the filial seed population in white spruce. There was remarkably little difference in filial genetic diversity among SSCs, considering the more than 100-fold difference in the number of trees per stand. This lack of differentiation is especially marked between the medium-sized and large stands where equal sample sizes make it possible to directly compare the levels of genetic diversity among filial populations. Although

primary selfing rates were lower in large stands, genetic diversity of the resultant seed did not differ significantly from seeds from medium-sized stands. Trees in the medium-sized stands showed the highest levels of primary selfing, yet also had the filial populations with the highest levels of genetic diversity. As the primary selfing rate is partially estimated from the empty seed data (inbred embryos that have been aborted), the viable, and thus assayed seeds will represent the more heterozygous individuals. The elimination of inbred and less genetically heterozygous embryos is therefore one of the primary mechanisms that maintains genetic diversity in conifers.

Genotypic diversity measures [$G_A(O)$ and $G_A(E)$] and *LGP* did not differ significantly between the medium-sized and large stands. However, these measures were all lower in the small SSC. This was mainly due to smaller number of maternal genotypes in these stands. As all families were sampled in these small stands, these are accurate measures of the genotypic diversity of these stands that show the limited number of genotypes possible in small stands. Limited seed dispersal may lower genotypic diversity in isolated stands in the short term. Nevertheless, because of the high gene flow, as suggested by the outcross pollen pool allele frequencies, the loss of genotypes in these small stands may potentially be recovered in subsequent generations.

The lack of differentiation in genetic diversity among filial populations can be directly attributed to the genetic diversity of the pollen pool that arises from panmixia. Measures of genetic diversity of the pollen pool were similar in all SSCs. The low number of alleles detected in the filial population and outcross pollen pool of small stands is probably due to a smaller sample size in this SSC. Gene diversity and allelic richness were both corrected for differences in sample size and did not differ among SSCs (Tables 4 and 5).

Conclusions

In this study, we detected a low impact of fragmentation on the mating system of the parental population and the genetic diversity of the filial population of white spruce. We found a reduction in the number of effective pollen parents (N_{ep}) in small stands, but the resultant seed maintained high levels of diversity. Genetic data from seed populations alone may overstate outcrossing rates if inbreeding depression has lethal effects that occur very early in the development of seeds. Inbreeding depression could, therefore, mask the negative genetic effects of fragmentation in seed populations. Molecular markers also do not detect other impacts of habitat disturbance on reproductive fitness of natural populations. We have previously found that small and isolated stands are likely to be pollen-limited, and have a significantly lower overall fecundity (ie number of viable seeds per cone) than larger stands and contiguous forests (O'Connell *et al*, 2006).

Nevertheless, the resultant seeds in fragmented and geographically isolated stands maintain high levels of heterozygosity and allelic richness. These results are significant for conifers, and trees in general, that are well-known for their high genetic diversity (Hamrick and Godt, 1996). The selection against inbred seeds and seedlings, may partly explain how trees maintain such a

high heterozygosity. Our results also show that high gene flow between stands also contributes to the high genetic diversity of seeds produced in small, geographically isolated stands. In this study, we provide strong empirical evidence of extensive gene flow over a landscape capable of maintaining high genetic diversity and connectivity between populations of a common temperate conifer. White spruce is representative of most temperate conifers, which usually show high outcrossing rates and high genetic diversity. The source of pollen and the quantification of pollen-mediated gene flow into these small isolated stands of white spruce are addressed in another manuscript (O'Connell, Mosseler and Rajora, unpublished).

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