We examined fine-scale genetic structure of mountain hemlock (Tsuga mertensiana) in an old-growth stand and an adjacent seedling population, with the goal of detecting the effects of fragmentation. Three hundred and six old-growth trees and 195 naturally regenerating seedlings were genotyped at 5 microsatellite loci. Genetic diversity was similar across old-growth life stages and within the clear-cut seedlings. Significant inbreeding was found in the adult class (30+ cm diameter at breast height) of old-growth seedlings and in the adjacent natural regeneration. Relatedness was significantly associated with physical distance for both the oldest age class and for regenerating seedlings in the adjacent clear-cut, whereas intermediate classes showed no such association. As intermediate classes show no isolation by distance, the associations that arise probably occur from single cohort regeneration that clearly has taken place in the clear-cut, and possibly when the oldest old-growth trees were established. Parentage analysis suggested that large-scale fragmentation, such as this clear-cut, allowed for increased long-distance seed dispersal. We conclude that long-lived tree populations can consist of a cohort mosaic, reflecting the effects of fragmentation, and resulting in a complex, age-dependent, local population structure with high levels of genetic diversity.

The effect of fragmentation on genetic structure has been well studied in neotropical and tropical forest species; yet, temperate rainforests have all but been ignored. This may be because compared with tropical forests, temperate forests are thought to be less biologically distinctive or less diverse. It may also be because of increasing public awareness at the profound deforestation of tropical rainforest. In North America, however, the Pacific coastal coniferous forest is considered to be an important habitat for several endemic species and many intact predator-prey systems (Moola et al. 2004). Although not as dramatic, in British Columbia alone, 9% of the total forested landbase has been clear-cut for commercial purposes and 70.1% of areas deemed feasible for logging are keystone ecosystems (Moola et al. 2004). In the 1800s, the arrival of the European settlers into the Lower Fraser Basin led to a decrease in coniferous deciduous forest cover by 46% with concomitant increase in agricultural and urban cover by 26% (Boyle 1997). Once protected from timber harvesting by virtue of the rugged landscape, untouched old-growth, montane forests of British Columbia are now vulnerable to new logging technologies (Moola et al. 2004).

In tropical and neotropical forests, human-induced landbase changes affect pollen and seed dispersal impacting mating systems and genetic structure of the colonizing trees in the fragmented terrain (Murawski and Hamrick 1992; Hall et al. 1994; White et al. 1999; Pither et al. 2003). Whether genetic consequences are muted in temperate forests may depend on preexisting genetic structure, mating system, dispersal ecology, time since deforestation, and the size of land-cover change relative to the distribution of the species (Aldrich et al. 1998). In temperate forests, trees occur in higher population densities with a more widespread distribution and are largely wind dispersed (Nason and Hamrick 1997). If the fragmentation event is small relative to how far seeds disperse, then recolonizing seedlings from an outcrossed, widespread tree species will largely come from many different adults. Thus, these seedlings will exhibit high levels of diversity and very little genetic structure. By contrast, if the fragmentation event is large relative to the variance in seed dispersal, then recolonizing seedlings will likely be recruited from the nearest adults resulting in lower levels
of genetic diversity with higher levels of relatedness (Hamilton 1999).

Here, we present a case study of the relationship between fragmentation, fine-scale genetic structure, and age structure in mountain hemlock, as inferred by variation at microsatellite loci. Microsatellite studies of remnant temperate populations suggest a loss in allelic richness and extensive fine-scale structure in regenerated seedling populations (Fore et al. 1992; Young et al. 1993; Aldrich and Hamrick 1998; Epperson and Chung 2001; Baucom et al. 2005). Unlike previous studies though, we consider not only the remnant forest but also the genetic diversity of recolonizing individuals. Natural history observations, coupled with the fine-scale resolving power of microsatellites, detail the impact of disturbance on genetic structure and dispersal. Although we examined only one population, it serves to validate our hypotheses and suggest further research. We hypothesize that seedling populations that have regenerated after fragmentation (clear-cutting) will show a loss of local genetic structure, relative to both adult and seedling cohorts (old growth), due to increased gene flow.

Materials and Methods

Study Species and Field Site

Mountain hemlock (Tsuga mertensiana, Pinaceae) is a predominantly outcrossing conifer (Ally et al. 2000). Both protogyyny (female cones are receptive before pollen release) and synchrony of female and male components are observed among individuals (Means 1990). Both pollen and seeds within this tree are well adapted for wind-mediated dispersal. Like many other Pinaceae, mountain hemlock pollen is sac-cate (Owens and Bladke 1983), whereas the mature seed has a seed wing 2.67 times the size of the seed itself (Owens and Molfrt 1975).

We chose a population of mountain hemlock on the south-facing slope of Mount Cheam, British Columbia, Canada, at an elevation of 1400 m. The Lower Fraser Valley old-growth forest is a stand of mixed-species conifers, dominated by mountain hemlock and pacific silver fir (Abies amabilis), with some yellow cedar (Chamaecyparis nootkatensis). Adjacent to the old-growth stand on 3 sides was a 27-year-old clear-cut, harvested in 1976 and allowed to naturally regenerate. In 1993, the clear-cut regeneration was determined to have 2496 stem/ha, of which 77% was pacific silver fir, 19% western hemlock, and 2% mountain hemlock and yellow cedar (Nelson E, personal communication).

In the old-growth population, we sampled trees within 4 plots covering an area of approximately 1.5 ha. The natural regeneration from the southern and eastern edges of the standing clear-cut was sampled along 2 length transects of 100 m each. Foliation was sampled where accessible; otherwise bark was collected. In the old-growth stand, trees were measured for diameter at breast height (DBH). A subset of these trees (n = 156) were cored to determine age. Only seedlings greater than 0.33 m in height were sampled. In all areas, trees were mapped into a Cartesian coordinate system.

Microsatellite Assay

DNA was isolated from 496 leaf and 5 bark samples after a modification of the CTAB protocol of Doyle JJ and Doyle JL (1990). Leaf tissue (0.15–0.20 g) and/or bark (0.20 g) was homogenized with liquid nitrogen and incubated in 1800 µL CTAB isolation buffer at 65–70 °C waterbath for 45 min followed by a chloroform–phenol wash. Samples were tested for several microsatellite loci initially designed for Tsuga hetero-phylla (Amarasinghe et al. 2002; Wellman et al. 2003). We chose to assay 5 SSR loci: EE10, EE12, EE06, HS26, and HS29. Polymerase chain reactions were performed with 10 µL reaction volumes. The reaction mixture contained 1.0 µL of 10× Erika Hagelberg buffer, 1.0 µL of 2.0 mM dNTP, 0.5 pmol of each forward and reverse primers, 0.5 pmol M13 IRD-labeled primer, 1 unit Taq DNA Polymerase, and 30–50 ng of DNA template. Samples were amplified with profiles specific for each primer pair, loaded on a LI-COR 4200 automated sequencer for assay. Microsatellite products were detected by M13 tailed or end-labeled primers. DNA fragments were sized using LI-COR size standards (cat. no. 4200–44), labeled with IRD dye.

Statistical Analysis

Two comparisons of local genetic structure were made: 1) between old-growth trees and clear-cut seedlings and 2) among 4 life history stages (seedlings, saplings, and 2 classes of adults) within the old-growth stand. A FORTRAN 95 program was written by K. Ritland to estimate expected heterozygosity (H_e) as one minus the sum of the squared observed gene frequencies, averaged over loci, and to estimate the inbreeding coefficient (F) as 1 – H_o/H_e, where H_o was the observed heterozygosity, averaged over loci. Standard errors (SEs) were found by bootstrapping. To determine whether the allelic distributions among old-growth classes were identical, a Markov chain estimation of Fisher’s exact probability test was performed using GENEPOP (Raymond and Roussset 1995). Default values were used for the Markov chain parameter. Using GENEPOP, we also performed Fisher’s exact test of allele differentiation and genotypic differentiation to determine if the old-growth and clear-cut seedling populations were from the same gene pool. We measured pairwise relatedness using Lynch and Ritland’s (1999) estimator between pairs of individuals at 5, 10, 15, 20, 25, and 30 m apart using the program SPAGeDi (Hardy and Vekemans 2002). Significance of the estimates was found by determining 95% confidence intervals (CIs) following Sokal and Rohlif (1995).

To quantify differences in effective seed dispersal between the clear-cut and old-growth populations, we employed a parentage analysis to assign parents to seedlings. The maximum likelihood estimation procedure of Marshall et al. (1998) was used because it assigns the most likely parent with a high level of confidence (95%) when the entire pool of candidate parents have not been sampled. Offspring in the old-growth stand were pooled to include both the seedling and the sapling classes, for a total of 125 individuals. This pooling is justifiable given that mountain hemlock has a
nonreproductive juvenile growth phase of 20–30 years (Fowells 1965). Candidate parents included both classes of adults for a total of 180 individuals. Assignment of parentage was for only one candidate parent, not for both. The delta criterion, the difference in the log-odds (LOD) scores between the most likely parent and the next most likely parent, was calculated.

Results

Old-Growth Life History Stages

The 306 old-growth individuals were divided into 4 classes based on DBH: 101 seedlings (0–9.9 cm), 24 saplings (10.0–14.9 cm), 69 mature adults (15.0–29.9 cm), and 112 older adults (30+ cm). We found that a regression of diameter on age was significant ($F = 453.3894, \text{degrees of freedom} = 155, P < 0.0001$) predicting 74.64% of the variation of tree age. Although older trees showed greater error variation for diameter class, trees were classified into life history stages using the convention of DBH, rather than age classes. As there is greater error variation at the tail end of the regression, by grouping cohorts by size class we are more likely to sample all individuals of a given age cohort.

Among the old-growth classes, there were no significant differences in expected heterozygosity (Table 1). Levels of inbreeding $F$ were significantly different from zero in 2 of 4 old-growth classes (seedlings: $0.069 \pm 0.018$ and old adults: $0.060 \pm 0.019$). Figure 1 gives a plot of pairwise genetic relatedness against physical distance. In the old growth, seedlings, saplings, and mature adult classes showed no significant association of relatedness with physical distance, even at the smallest distance interval of 5 m. Mean pairwise relatedness was found to be $0.026 \pm 0.0154$ for seedlings, $0.1009 \pm 0.1161$ for saplings, and $0.0034 \pm 0.0194$ for mature adults. For the 30+ cm class of adults, however, a significant positive autocorrelation ($P = 0.05$) was found in the 5-m distance interval ($0.0862 \pm 0.0161$ see Figure 1).

Old-Growth versus Clear-Cut Populations

The highest expected heterozygosity was found among seedlings in the clear-cut cohort class ($0.908 \pm 0.002$; Table 1). This value, although did not differ significantly from any of the old-growth diameter classes, was significantly higher than the average of all old-growth classes ($0.899 \pm 0.003$; Table 1), although this difference is a mere 1%. As well, levels of inbreeding did not differ between clear-cut seedlings and any individual old-growth life history class. Although the clear-cut seedlings showed lower inbreeding ($0.036 \pm 0.011$) than the mean of the old-growth classes ($0.046 \pm 0.010$), this difference was not significant. Fisher’s exact test, however, showed that the clear-cut and the old-growth seedlings were significantly different from each other, both genotypically and in allelic composition.

Although both types of seedlings showed positive levels of inbreeding, there was no indication of significant microspatial genetic structuring among the old-growth seedlings (Figure 1). In contrast, the clear-cut seedlings showed very strong genetic structure at the smaller distance class intervals, with a decline in relatedness and autocorrelation at the larger distance classes (Figure 1). Values of mean pairwise relatedness for all old-growth seedlings within the 0- to 1-m class were $0.0007 \pm 0.0122$ and $0.0771 \pm 0.0174$ for clear-cut seedlings. For old-growth seedlings within the 1- to 2-m distance interval, the estimate of mean pairwise relatedness was $0.0112 \pm 0.0204$, whereas for clear-cut seedlings it was $0.0309 \pm 0.0124$.

Among the clear-cut seedlings, parentage analysis of 180 candidate parents assigned 100 parents to 195 offspring. For the old-growth seedlings, of a possible 180 candidate parents, 73 were assigned parentage to 125 offspring (101 seedlings plus 24 saplings). The delta criterion, the difference in the LOD scores between the most likely parent and the next most likely parent, was 1.80 for a 95% CI. Using information on who were candidate parents, we calculated the physical distance from each candidate parent to the putative offspring. We then examined effective seedling dispersal distributions for both old-growth seedlings and clear-cut seedlings. The old-growth seedling dispersal distribution had a mean distance of 65.70 m [standard deviation (SD) = 35.92 m]. The smallest parent–offspring distance was 3.45 m and the largest distance between parent and offspring was 162.26 m. Unlike the old-growth seedlings, clear-cut offspring dispersed on average 90.51 m (SD = 38.16 m) with a range of 16.74–207.43 m. A Kolmogorov–Smirnov test found that the 2 distributions significantly differed, with a maximum difference between the cumulative distributions 0.03063 ($P < 0.0001$).

Discussion

Our results suggest that this fragmentation event did not change the level of genetic diversity between the old growth and clear-cut individuals but did affect the allelic composition. Long-distance dispersal of mountain hemlock seeds and pollen from unsampled areas into the adjacent clear-cut could facilitate admixture and change allelic composition. The 43.3-ha clear-cut in 1976 that surrounded the old-growth stand removed all trees, thus allowing for extensive long-distance seed and pollen dispersal. This is evident from the seedling dispersal distributions, where effective dispersal

<table>
<thead>
<tr>
<th>Table 1. Genetic diversity ($H_e$) and inbreeding ($F$) for old-growth versus Clear-Cut Populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life history class</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Clear-cut seedlings</td>
</tr>
<tr>
<td>Old growth</td>
</tr>
<tr>
<td>Seedlings</td>
</tr>
<tr>
<td>Saplings</td>
</tr>
<tr>
<td>Mature adults</td>
</tr>
<tr>
<td>Old adults</td>
</tr>
<tr>
<td>Mean for old growth</td>
</tr>
</tbody>
</table>
was more substantial in the higher distance classes for the clear-cut (range 16.7–207 m) than for the old growth (range 3.5–162 m). As well, Fisher’s exact test of differentiation found that the clear-cut seedlings are neither just a subset of the old-growth adult genetic pool nor from the same pool as the old-growth seedlings.

Contrary to expectations, though old-growth seedlings showed nearly twice the level of inbreeding as clear-cut seedlings, among the former group there was no significant level of relatedness. Two factors may have contributed to this result. Old-growth adults, within a 10-m distance interval, were related (at most 0.10, third cousin relationship). If the contribution of related alleles to the pollen pool was disproportionately high, this might facilitate consanguineous matings among near neighbors. A high level of mean pairwise relatedness in these adults likely determines the positive inbreeding coefficient in the 0- to 9.9-cm old-growth seedling class. Although minimal inbreeding was found in the clear-cut seedlings, we paradoxically found high levels of relatedness (approximately 0.10, cousins a third order relationship) among pairs of clear-cut seedlings at the shortest distance class. Fine-scale structure can develop when the variance in the seed dispersal is smaller than the variance in pollen dispersal (Kalisz et al. 2001). Without the effect of overlapping seed shadows, sibling and cousin clusters could form in the clear-cut explaining the higher mean genetic relatedness estimate in clear-cut seedlings.

Most plant population studies suggest that spatial genetic differentiation is greater than temporal genetic structure (Muona et al. 1987; Schnabel and Hamrick 1995; Caujape-Castells and Pedrola-Monfort 1997). This and other studies (Linhart et al. 1981; Hamrick et al. 1993) have shown that adult age classes can retain microspatial genetic structuring, as adults with a diameter of 30+ cm exhibited significant spatial structure at a 5-m distance class (Figure 1). Relatedness appears to decay exponentially with increasing pairwise distance, implying genetic structure in adults is a consequence primarily of localized seed dispersal.

Two factors, however, may bias estimates of inbreeding: null alleles and a temporal Wahlund effect (Hamrick et al. 1993; Aldrich et al. 1998). Although several other studies have found significant inbreeding levels associated with fragmented forests (Aldrich et al. 1998; Dayanandan et al. 1999; White et al. 1999), most were unable to distinguish inbreeding from the effect of null alleles. Null alleles occur when mutations or duplication/inversion events in the flanking region

![Figure 1](image-url). Lynch and Ritland’s (1999) mean pairwise relatedness ($r_{xy}$) calculated as a function of distance between individuals (5, 10, 15, 20, 25, 30 m) in the old-growth stand. Dotted lines represent upper and lower 95% CIs around zero relatedness. A distance interval refers to all pairs of observations within 0–5 m, 5–10 m, etc. (a) Seedlings (0–9.9 cm), (b) saplings (10–14.9 cm), (c) mature adults (15–29.9 cm), and (d) old adults (30.0+ cm).
of the sequence repeat prevent amplification of an allele (Pemberton et al. 1995). Null allele frequencies generally vary among loci, and loci with conspicuous nulls will show higher single locus inbreeding coefficients. On a per-locus basis, we found that one locus had a higher inbreeding coefficient but this did not substantially affect estimates. As well, null alleles should affect each class equally, so that relative comparisons are still valid. Secondly, a temporal Wahlund effect results from pooling of allele frequencies from different cohorts (Hartl and Clark 1997). The extent of this temporal effect was examined by subdividing the 30+ cm class into 4 diameter classes. Although overall, the 30+ cm class showed significant inbreeding, no significant inbreeding was found when this class was subdivided. This could be the result of small sample sizes and thus large standard errors or it may reflect the presence of a temporal Wahlund effect.

Long-lived organisms like mountain hemlock are ideal for studying temporal and spatial genetic differentiation because different age cohorts can be examined simultaneously. We inferred retrospectively the impact of clear-cutting on genetic structure and diversity, and also examine how levels of genetic diversity, inbreeding, and genetic structure change with life history stages within old-growth stands. With a growing worldwide concern for the impact of human disturbance on species persistence, understanding the interplay of demography and genetic structure is essential for developing effective conservation practices.

Acknowledgments

This study was possible because of the helping hands of Dr Carol Ritland in the laboratory and Dr Jamie Skidmore in the field. Thanks to anonymous reviewers for thoughtful comments that improved the quality of the manuscript. This study was funded by grants from the Natural Sciences and Engineering Research Council of Canada and Forest Renewal British Columbia to D.A. and K.R.

References


Received June 22, 2006
Accepted September 6, 2006

Corresponding Editor: David Wagner