HIGH LEVELS OF CHLOROPLAST GENETIC VARIATION DIFFERENTIATE COASTAL AND INTERIOR DOUGLAS-FIR (*PSEUDOTSUGA MENZIESII*) LINEAGES IN SOUTHERN BRITISH COLUMBIA

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ABSTRACT

We surveyed genetic variation in chloroplast DNA (cpDNA) of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) from the coastal and interior regions of British Columbia. Restriction digestion of a 1765 base pair (bp) region of the cpDNA molecule revealed 31 distinct haplotypes in a total of 58 trees. A total of 15 haplotypes were observed in coastal trees while 16 haplotypes were detected in trees from the interior of British Columbia. Gene diversity was high overall and slightly higher in the coastal sample 0.95 (SD 0.02) than in the interior sample 0.88 (SD 0.05). The coastal and the interior populations were significantly differentiated from each other as none of the cpDNA haplotypes were shared between the regions. This suggests that gene flow from pollen dispersal is restricted between these two regions, and that possibly the two regions were colonized by different sources following the end of the Fraser glaciation approximately 10,000 years before the present time. The simple method developed for assaying cpDNA heterogeneity in Douglas-fir will be useful for further study of biogeography and population structure of the species and for monitoring pollen flow in seed orchards.

Key words: Douglas-fir, *Pseudotsuga menziesii*, chloroplast DNA, genetic variation.

INTRODUCTION

An important aspect of forest conservation genetics is understanding population structure and mating dynamics. Surveys of molecular genetic variation can reveal isolation of populations and whether populations have arisen from distinct ancestral lineages. Conservation of adaptive genetic variation and ancestral phylogenetic lineages is important because this genetic legacy allows for adaptation to environmental change and provides the raw materials for artificial selection programs.

In forest tree genetics, population structure has been revealed by quantitative genetic approaches (CHRISTOPHE & BIROT 1979; CHING & HINZ 1978; REHFELDT 1989) as well as a variety of molecular genetic techniques. Using allozyme analysis, SCHNABEL *et al.* (1993) detected population differentiation among Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) populations of the Great Basin while MERKLE and ADAMS (1987) examined population structure among breeding zones of coastal Douglas-fir in southwest Oregon and found little evidence of population differentiation. Also with allozyme analysis, LI and ADAMS (1989) surveyed genetic heterogeneity across the entire range of Douglas-fir and found evidence for a genetic split between interior and coastal populations.

The potential for population structuring among Douglas-fir populations is clearly indicated in the above studies. Notable with regard to the distribution of Douglas-fir in British Columbia is the genetic split between coastal and interior Douglas-fir populations observed by LI and ADAMS (1989). These observations are concordant with the classification of Douglas-fir into two varieties, coastal (var. *menziesii*) and interior (var. *glauca*) (VON RUDLOFF 1972; for a review see HERMANN & LAVENDER 1990) based on differences in leaf secondary metabolites and cold tolerance. Range boundaries between the two varieties in British Columbia are the Fraser River and the Coast Mountains, although geographic separation between the two varieties is not absolute.

In Douglas-fir, dispersal of genetic material occurs through both seeds and pollen. In attempting to understand Douglas-fir population structure, it is important to determine if populations are exchanging genetic material, and whether exchange is through pollen or seed dispersal. Over small spatial scales both seed and pollen are likely to be vectors of genetic exchange. However, because of the higher dispersal potential of pollen, pollen flow is likely to be a more important determinant

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of genetic structure over large spatial scales.

Monitoring pollen flow is also important in seed orchard operations aimed at producing high quality seed. Lack of orchard panmixis and introgression of exogenous pollen from trees outside the orchard reduce the genetic worth (GW) of seed produced (WOODS *et al.* 1996).

Pollen flow has been studied using traps (GREENWOOD 1986; CARON & LEBLANC 1992) but this approach does not lead to estimates of fertilization events or provide information regarding historical pollen dispersal. Because Douglas-fir cpDNA is carried by pollen and the fertilized embryo incorporates only the male-derived cpDNA (NEALE *et al.* 1986; OWENS & MORRIS 1991) genetic analysis of cpDNA provides a convenient way to study pollen flow.

In order to make meaningful inferences regarding pollen flow and population structure utilizing genetic surveys, a modest amount of genetic variability must be detectable. There is good reason to believe that in the Douglas-fir cpDNA molecule there is sufficient genetic variability to make genetic surveys of this molecule worthwhile. PONOY *et al.* (1994) carried out restriction fragment length polymorphism (RFLP) analysis of cpDNA from coastal, interior and transition zone Douglas-fir in British Columbia using a DNA hybridization approach and detected an overall gene diversity of 0.78. It is likely that at a large portion of the variation observed by PONOY *et al.* (1994) is due to a hypervariable cpDNA region identified by HIPKINS *et al.* (1995) because survey of other potentially polymorphic regions of the cpDNA molecule (VIARD *et al.* 2001; Nelson, R.J. unpublished) have shown little or no variability. The hypervariable region identified by HIPKINS *et al.* (1995) contains a region of direct sequence repeats. Survey of variation in this hypervariable region was utilized by STOEHR *et al.* (1998) for analysis of pollen flow within a Douglas-fir seedlot. This study showed high levels of genetic variation, detecting 13 unique haplotypes in a sample of 20 trees and demonstrated that analysis of this cpDNA region could provide valuable insight into pollen flow. Analysis of this hypervariable region would likely be useful for population genetic studies. The laboratory method employed by STOEHR *et al.* (1998), however, is radionuclide-based and labour intensive making it unsuitable for screening large numbers of samples.

In support of further studies of Douglas-fir population structure and silviculture activities, we developed a simple non-radioactive method to survey cpDNA genetic variation. We then used this method to determine the amount of native genetic variation present in trees from the coast and interior of British Columbia. This data was evaluated to determine the degree of reproductive isolation of coastal and interior Douglas-fir populations and the utility of the technique developed for seed-lot evaluation.

MATERIALS AND METHODS

Vegetative samples (buds) of coastal British Columbia Douglas-fir were obtained from 25 seed orchard clones originating from Vancouver Island and the coastal mainland of British Columbia. Samples from the interior of British Columbia were obtained from open pollinated trees from selections of the Sushwap-Adams Lake breeding zone.

Total DNA was isolated from 1 to 4 fresh buds per individual tree according to GUILLEMAUT and MARECHAL-DROUARD (1992).

Polymerase Chain Reaction (PCR) primers PM12L (CAGGGCGGTACTCTAACCAA), and PM12R (AGATCACGTGCGTGTGAAAA) were designed to amplify a 1765 bp DNA fragment (positions 356 to 2120) of a highly polymorphic region of the chloroplast genome (GENBANK acc. # L20416) identified by HIPKINS *et al.* (1995). Polymerase chain reaction was carried out in Qiagen PCR buffer with 0.25 units of Qiagen Hot Star *Taq* DNA polymerase (Qiagen Corp. Mississauga, Ontario). Each 50 µl PCR required 4 µl of DNA extract and contained 24 pmol (0.48 µM) of each primer, and 80 µM of each nucleotide. An MJ PTC-100 thermal cycler (MJ Research, Watertown, Mass.) was used to carry out PCR in 96-well microtiter plates. Temperature cycling was initiated with incubation at 95 °C for 15 minutes followed by 38 cycles of: 94 °C for 30 seconds, 62 °C for 30 seconds, 72 °C for 2 minutes. Following amplification, the PCR product was concentrated to 15 µl by ethanol precipitation. All of the PCR product from each 50 µl PCR was subjected to restriction digestion with enzymes *HincII*, *BslI*, and *Tsp45I* (New England Biolabs, Beverly, Mass.). Restriction digestion proceeded for 3.75 hours under conditions suggested by the manufacturer. Digestion was terminated by adding 4.0 µl of loading dye (50 mM EDTA pH 8.0, 30 % glycerol, 0.25 % bromphenol blue) and 10 µl of this solution was loaded per gel electrophoresis lane.

The DNA fragments from restriction digestions were size-fractionated on non-denaturing polyacrylamide gels that were 17 cm wide by 14.5 cm long and contained 10 % acrylamide in a bis-acrylamide ratio of 19:1. Gels contained 2X TAE buffer (SAMBROOK *et al.* 1989), and were run for 12 hours at 70 volts. Each gel included three 20 base-pair marker (GenSura Labs Inc., Del Mar, California) lanes to create a molecular size grid and 24 individual tree samples. Gels were stained with 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide in water and bands

were visualised with ultraviolet light. Digital images of gels were obtained with an Eagle-eye system (Stratagene Corp., San Diego, Calif.). Gels were scored and band size determined with INTELLIGENT QUANTIFIER version 2.1.2A (Millipore Corp., Bedford, Mass.).

Testing of genetic homogeneity of the coastal and interior samples was accomplished with a *G*-based exact test based on haplotype frequencies (GOUDET *et al.* 1996); this was carried out with GENEPOP version 3.1 (RAYMOND & ROUSSET 1997).

RESULTS

Restriction digestion of the 1765 bp chloroplast DNA fragment with *BslI, HincII,* and *Tsp45I,* produced 19, 21 and 17 different patterns respectively, revealing a high degree of polymorphism in the 58 trees that were analysed. Each of these unique banding patterns contained between one and seven bands. Individual enzyme patterns for each tree were combined to create 31 different composite haplotypes (Table 1). In the 24 coastal samples 15 different haplotypes were observed. In the 34 Interior samples 16 haplotypes were observed (Table 1). Two coastal trees that were progeny of the same male parent shared haplotype 7 suggesting haplotypes are stable through reproductive transmission.

Genetic diversity in both the coastal and interior samples was high at 0.95 (SD 0.02) and 0.88 (SD 0.05), respectively (Table 2). No haplotypes were found in common between the coastal and interior trees. A *G*-based exact test of genetic homogeneity resulted in a rejection of the null hypothesis (H_o = homogeneity of samples) with $P < 0.00001$.

Table 1. Haplotypes observed using three restriction enzymes and composite haplotype frequencies for coastal and interior Douglas-fir populations.

BsII	HincII	Tsp45I	Haplotype #	Coastal	Interior
A	A	A	1	2	0
B	B	B	2	4	0
B	B	C	3	2	0
B	I	B	4	2	0
B	P	Q	5	0	2
C	B	B	6	1	0
C	B	C	7	3	0
C	I	E	8	1	0
D	C	B	9	1	0
E	D	D	10	1	0
F	E	B	11	1	0
G	F	E	12	1	0
H	F	F	13	2	0
I	G	G	14	1	0
I	G	H	15	1	0
J	H	G	16	1	0
K	J	I	17	0	1
L	L	J	18	0	1
L	R	J	19	0	11
L	R	P	20	0	1
M	M	K	21	0	1
N	N	L	22	0	1
N	N	N	23	0	2
N	T	J	24	0	2
O	O	M	25	0	1
P	N	N	26	0	3
Q	S	K	27	0	1
R	K	Q	28	0	1
R	Q	Q	29	0	4
R	R	K	30	0	1
S	U	O	31	0	1
			Total:	24	34

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Table 2. Summary statistics of Douglas-fir cpDNA analysis. Number of samples analyzed (*N*), number of haplotypes observed, and gene diversity (s.d. in parentheses) is shown for each sample.

	Coastal	Interior
N	24	34
Number of haplotypes	15	16
Gene diversity	0.95 (0.02)	0.88 (0.05)

DISCUSSION

We detected 31 different chloroplast haplotypes in a total of 58 trees. Since high variability was observed in two different locales this observation is not likely to be a local effect but rather is due to a general high level of genetic variability of the 1765 bp region of cpDNA examined. The 1765 bp region consists of two complete and one partial tRNA genes and a large intergenic region comprised of multiple tandem repeats that bear similarity to the tRNA gene, *trnY*. Repeated sequences found in tandem are subject to recombination events that generate sequence length variants (PALMER 1991). Variability in this region has been shown to be largely due to differences in the number of repeats (HIPKINS *et al.* 1995). Because this region is intergenic, new sequence variants confer no selective advantage or disadvantage and thus are neither rapidly fixed nor eliminated from the population. Taken together, high mutation rate and lack of selective pressure result in a large amount of genetic variation in this region maintained within populations.

The genetic diversity we detect (0.88 to 0.95) is substantially higher than detected in Douglas-fir with allozymes. YEH and O'MALLEY (1980) detected gene diversity of 0.16 in coastal Douglas-fir while SCHNABEL *et al.* (1993) detected gene diversity of 0.14 in Rocky Mountain Douglas-fir. Because the number of allelic states at allozyme loci is at least partly constrained by the fact that they code for a functional enzyme, it is likely that the higher levels of genetic variation detected in our study is due to the lack of strict functional constraints on the specific cpDNA region studied rather than a general higher level of variation in cpDNA.

Taking a RFLP-DNA hybridisation approach to survey genetic variation of almost the entire cpDNA molecule, PONOY *et al.* (1994) observed gene diversities of 0.72, 0.85, and 0.59 in coastal, transition and interior populations of Douglas-fir respectively. In this study, 16 different haplotypes were observed in a total of 72 trees from three biogeoclimatic zones. Here we detect higher gene diversity with 31 different haplotypes in 54 trees from two biogeoclimatic zones. This is most likely due to the fact that PONOY *et al.* (1994) used restriction enzymes with a six base pair recognition sequence and did not specifically target a known hypervariable region of the cpDNA molecule. Analysis of cpDNA specific microsatellite loci in Douglas-fir from coastal British Columbia revealed little variability (VIARD *et al.* 2001). In addition, in our laboratory exploration of variability in cpDNA sequences that are orthologous and polymorphic between Douglas-fir and other tree species, suggests that there is not a great deal of sequence variation outside the region studied here (NELSON, R.J. unpublished).

In our study we detect high levels of variability because we target a highly variable region and employ restriction enzymes that have four or five base pair recognition sequences. With our approach we survey for variation at from six to eight restriction enzyme recognition sites per enzyme per 1765 bp. Also, because the variable region is split into multiple fragments, small indels that may not change the sequence at a restriction enzyme recognition site, can be easily detected. The levels of variation we observe with the simple non-radionucleide based method developed are similar to levels of variation seen by Stoehr *et al.* (1998) in their study of this hypervariable region.

In contrast to the study of PONOY *et al.* (1994) which found many of the same chloroplast haplotypes in coastal and interior Douglas-fir, we found that none of the chloroplast haplotypes detected with our method were in common between trees of these two regions. Our results suggest a very low level of contemporary pollen flow between Douglas-fir of the coast and interior of British Columbia. The striking difference in haplotype distribution may further reflect a split between lineages dating back 10,000 years, the time of the retreat of the Cordilleran ice sheet, when coastal and interior regions may have been colonized by separate populations of Douglas-fir. This hypothesis is supported by the genetic study of LI and ADAMS (1989) who found that Douglas-fir from coastal British Columbia grouped with coastal Douglas-fir from Washington, Oregon and California while Douglas-fir from the interior of British Columbia grouped with populations from the non-coastal states of Montana and Idaho and the interior of Oregon and Washington. In order to explore this hypothesis further more samples from the interior and coastal populations of Douglas-fir in British Columbia and areas south of the Cordilleran ice sheet margin should be analysed and compared with the data presented here. The method described here also provides a convenient method by which the range and overlap zones of the coastal and interior Douglas-fir varieties could easily be mapped.

Chloroplast DNA markers have been employed to elucidate paternity in Douglas-fir (STOEHR *et al.* 1998).

These authors identified 13 different haplotypes in 20 seed orchard clones. In the present study we identify 15 different haplotypes in 24 trees. Therefore, the power to discriminate cpDNA haplotypes is similar in both studies. STOEHR *et al.* (1998) found that the level of gamete discrimination achieved was useful in assessment of exogenous pollen levels and assessment of natural selfing within the seed orchard. Because the method we have developed is technically less demanding than that of STOEHR *et al.* (1998) yet shows high discrimination power, it will facilitate assessment of chloroplast variation in silvaculture operations.

Even so, it is likely that we have underestimated the amount of genetic variability in the cpDNA region studied because the gel electrophoresis method used in our analysis resolved fragments only in the 60 to 500 base pair range. Simulated restriction digestion analysis of the hypervariable region shows fragments outside of the size range of resolution of the gels used. These bands, which may be variable in size, could be scored by further optimising gel electrophoresis conditions.

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