Diversity and genetic structure in populations of *Pseudotsuga menziesii* (Pinaceae) at chloroplast microsatellite loci

F. Viard, Y.A. El-Kassaby, and K. Ritland

Abstract: Genetic variation was compared between uniparentally-inherited (chloroplast simple sequence repeats, cpSSRs) vs. biparentally-inherited (isozyme and random amplified polymorphic DNA, RAPD) genetic markers in Douglas-fir (*Pseudotsuga mensiezii*) from British Columbia. Three-hundred twenty-three individuals from 11 populations were assayed. In Douglas-fir, the cpSSR primer sites were well-conserved relative to *Pinus thunbergii* (11 of 17 loci clearly amplified), but only 3 loci were appreciably polymorphic. At these cpSSR loci, we found an unexpectedly low level of polymorphism within populations, and no genetic differentiation among populations. By contrast, the nuclear markers showed variation typical of conifers, with significant among-population differentiation. This difference is likely the outcome of both historical factors and high pollen dispersal.

Key words: chloroplast microsatellites, isozymes, RAPDs, gene flow, pollen flow.

Résumé : Les profils de diversité et structure génétique obtenus, en Colombie-Britannique chez l'espèce *Pseudotsuga mensiezii*, avec des marqueurs à hérédité uniparentale (microsatellites chloroplastiques) ont été comparés aux profils obtenus avec des marqueurs à hérédité bi-parentale (enzymes et ADN polymorphe amplifié au hasard, RAPD). Trois-cent vingt-trois individus issus de 11 populations ont été analysés. Un niveau élevé de conservation des amorces microsatellites chloroplastiques, originellement définies sur l'espèce *Pinus thunbergii*, a été observé chez *P. mensiezii* (11 loci sur 17 testés sont amplifiés), mais seulement 3 loci se sont avérés polymorphes. De façon inattendue, ces loci microsatellites chloroplastiques ont révélé un faible niveau de polymorphisme au sein des populations ainsi qu'une absence de différences génétiques entre populations. En revanche, les marqueurs nucléaires ont montré des profils de diversité et structure génétique typiques de ceux généralement observés chez les conifères. Ces résultats suggèrent l'influence conjointe d'évènements historiques et d'importantes dispersions polliniques.

Mots clés : microsatellites chloroplastiques, marqueurs enzymatiques, RAPD, flux de gènes, dispersion pollinique.

[Traduit par la Rédaction]

Introduction

In plant species, gene migration occurs first through pollen, then through seeds. These components of migration often differ in scale, and consequently, differ in their impact on genetic population structure and variation. The distribution of such genetic variation within and among populations is of importance to the adaptation and adaptability of a species (Slatkin 1985; Slatkin 1987; Levin 1988) and for gene conservation efforts (El-Kassaby and Ritland 1996*a*, 1996*b*).

Received August 6, 2000. Accepted January 24, 2001. Published on the NRC Research Press Web site April 20, 2001.

Corresponding Editor: R.S. Singh.

F. Viard.[1] Laboratoire Génétique et Evolution des Populations Végétales, Unité de Recherche Associée – Centre National de la Recherche Scientifique (UPRESA–CNRS) 8016, Fédération de Recherche – CNRS (FR–CNRS) 1818, Bat. SN2, Univ. Lille 1, 59655 Villeneuve d'Ascq, France.
Y.A. El-Kassaby and K. Ritland. Department of Forest Sciences, University of British Columbia, Vancouver, BC V6T 1Z4, Canada.

[1]Corresponding author at Station Biologique de Roscoff – Place Georges-Teissier, BP 74, 29682 Roscoff CEDEX, France (e-mail: viard@sb-roscoff.fr).

In forest management, knowledge of dispersal is of practical importance because pollen migration can reduce the genetic gain in seed orchards and breeding programs, and also alter the genetic composition of open-pollinated families used in progeny tests (Adams 1992; Ellstrand 1992).

Isozymes have been traditionally used to assess population genetic structure and gene dispersal in plant species (see reviews by Loveless and Hamrick 1984; Hamrick and Godt 1990; Hamrick et al. 1992). Hamrick et al. (1992) showed that forest trees have high genetic diversity within populations (0.151 on average for gymnosperms) and low genetic differentiation (G_{st} value equal to 0.073 on average for gymnosperms), compared to other plant species. More recently, RAPD (random amplified polymorphic DNA) markers have been used for population genetic analyses (Rafalski and Tingey 1993; Haymer 1994; Lynch and Milligan 1994; Powell et al. 1996*b*). Most recently, the advent of uniparentally inherited markers has opened a new way to assess the relative magnitudes of seed and pollen dispersal. Comparisons of patterns of variation between paternally-inherited and nuclear-inherited markers allow inferences about pollen movement and paternity (McCauley 1995; Powell et al. 1996*a*; Latta and Mitton 1997).

Because chloroplasts evolve slowly and exhibit little variation below the species level (Clegg et al. 1994), cpDNA

 DOI: 10.1139/gen-44-3-336

markers have been largely used for phylogenetic inferences (Olmstead and Palmer 1994) and to some extent, for within-species genetic studies (Soltis et al. 1992; Ennos et al. 1999). However, certain noncoding regions contain variable microsatellites; using surrounding conserved regions, "universal primers" have been designed to amplify these microsatellite loci (Powell et al. 1995*a*, 1995*b*; Vendramin et al. 1996; see references in Provan et al. 1999*a*). Microsatellite markers are a well-known opportunity to find polymorphism at fine scales due to their highly polymorphic nature (Jarne and Lagoda 1996; Powell et al. 1996*a*).

In the present study, we surveyed Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) populations from British Columbia for their pattern of genetic variation at both nuclear and chloroplast marker loci. In British Columbia and northwestern U.S.A., Douglas-fir is among the most valuable softwood species, and the object of several breeding programs in both countries. Pollen in Douglas-fir, as in other conifers, is dispersed by wind, but since Douglas-fir pollen is larger than most other conifers and lacks wings or bladders, a relatively short dispersal distance is expected. However, direct estimates of pollen flow are difficult to make due to high background pollen loads (Allen and Owens 1972).

We assayed 323 individuals sampled from 11 populations of Douglas-fir in British Columbia for chloroplast microsatellite (cpSSR) and nuclear (RAPD) markers, and also drew upon the isozyme data of El-Kassaby and Ritland (1996*a*). We (*i*) check for the conservation of cpSSR markers derived from pine species (Vendramin et al. 1996) to *Pseudotsuga mensiezii*, (*ii*) evaluate their usefulness to study genetic variation within and among natural populations in Douglas-fir, and (*iii*) compare the estimates derived from the paternally inherited chloroplast genetic markers to those derived from two types of biparentally inherited markers, isozymes, and RAPDs.

Materials and methods

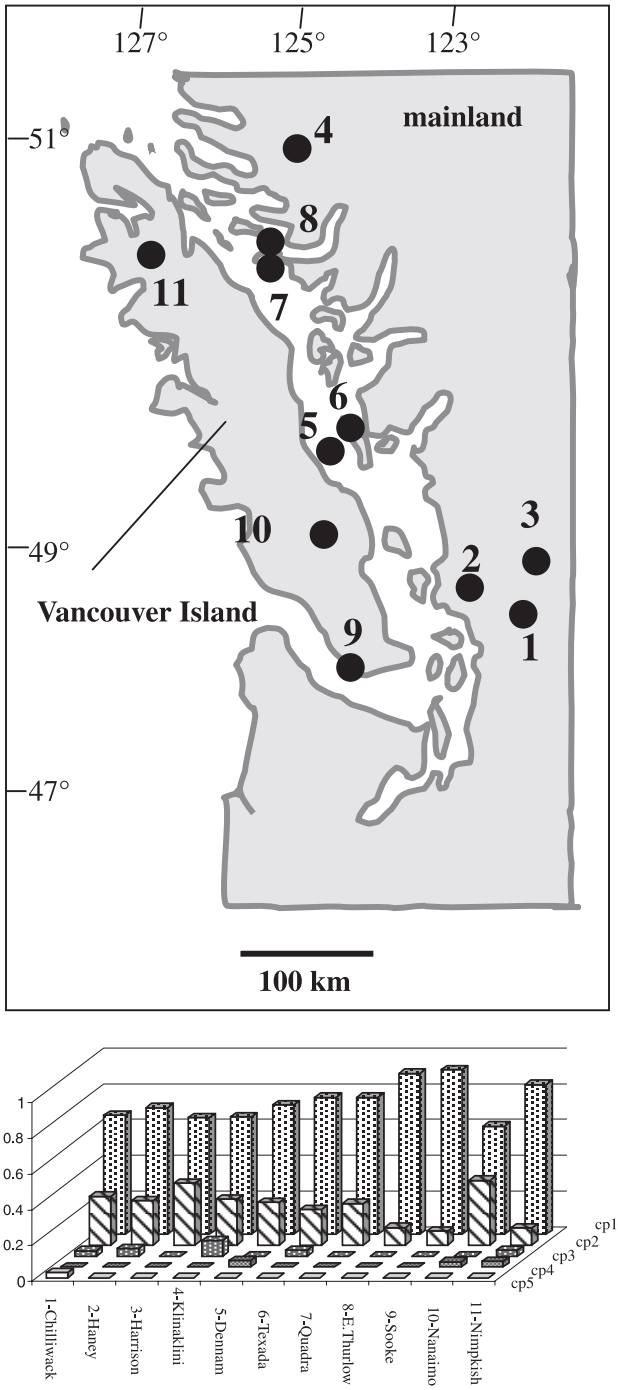
Sampling design

We sampled 323 individuals from 11 natural populations in British Columbia (B.C.); these 11 are a subset of the 49 populations previously analyzed for isozymes (El-Kassaby and Ritland 1996*a*). The location of these populations is given in Fig. 1. These populations represent different geographical areas in B.C.: four populations from the mainland, three on Vancouver Island, and four on small islands between Vancouver Island and the mainland. DNA was extracted from about 30 vegetative buds (sampled from 30 different trees) for each of the 11 populations.

RAPD and cpSSR assay

Standard methods for RAPD assay were followed (Hadrys et al. 1992). The PCR amplifications, performed using a Perkin-Elmer DNA thermocycler, included a preliminary 7 min denaturation at 94°C, followed by a total of 45 cycles of 1 min denaturing at 95°C, 1 min annealing at 35°C and 2 min extension at 72°C, with a final extension step of 72°C for 10 min. The reaction mix (25 μL) consisted of 200 ng of template DNA, 1.9 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.2 μM primer (10-base oligonucleotide), 2 U of Taq polymerase (Amplitaq, Perkin-Elmer). After screening more than 100 RAPD primers, four RAPD primers where chosen for assays (OA11, OB08, 268, and 269), as they produced clean, consistent banding patterns. Over these four primers, 48 different zones of

Fig. 1. Samples localization and histogram of haplotype frequencies. Five haplotypes were defined by the combination of the alleles (hereafter named by size in base pairs) occurring at the three polymorphic loci (*Pt26081*, *Pt63718*, and *Pt71936*), respectively: cp1, 126/110/166; cp2, 125/110/166; cp3, 125/110/167; cp4, 126/110/167; cp5, 125/111/166.



banding activity, each representing a putative marker locus, were scored. To facilitate comparisons among populations, 3 to 4 DNA samples from each population were run on a gel. RAPDs are sometimes not inherited in a Mendelian fashion (Ritland and Ritland 2000), as (1) co-migrating bands may be due to variation at two or more loci; (2) amplification can be unreliable or dependent on the

Table 1. Primer conservation of 20 cpSSR primer pairs in pine species.

Locus	*PT1254*	*PT9383*	*PT15169*	*PT26081*	*PT30204*	*PT36480*	*PT41093*	*PT45002*	*PT48210*
T_m (°C)	58	58	60	60	58	58		64	60
Pseudotsuga mensiezii	0	–	+	++	0	+		0	0
Pinus thunbergii	+	+	+	+	+	+	+	+	+
P. leucodermis	+	+	+	+	+	+	+	+	+
P. halepensis complex	+	+	+	+	+	+	+		
P. resinosa		+	+	+	+	+	+		
P. sylvestris	+	+	+		+	+	+	+	+
P. torreyana	+	+	+		+	+	+	+	+
P. pinaster		+	+	+	+	+	+		
Abies alba		0	+	+	+	+	–		+
A. nordmanniana		0	+	+	+	+	–		+
A. cilica		0	+	+	+	+	–		+
A. numidica		0	+	+	+	+	–		+
A. pinsapo		0	+	+	+	+	–		+
A. cephalonica		0	+	+	+	+	–		+

Note: Locus refers to the locus name given in Vendramin et al. (1996). T_m is the annealing temperature used on the Douglas-fir samples. Blank, 0, – and + stand for not tested, no amplification, not scorable, and scorable amplification pattern, respectively. For Douglas-fir, + and ++ stand for scorable pattern monomorphic and polymorphic, respectively. Ref. refers to the study from which the results were retrieved: 1, Powell et al. (1995*b*); 2, Vendramin et al. (1996); 3, Bucci et al. (1998); 4, Echt et al. (1998); 5, Provan et al. (1998); 6, Provan et al. (1999*b*); 7, Vendramin et al. (1998); 8, Vendramin et al. (1999); 9, Vendramin and Ziegenhagen (1997); 10, present study.

Table 2. Allele frequencies at four cpSSR loci in 11 populations (323 individuals) of Douglas-fir. The names of the alleles refer to the size (in base pairs) of the amplified fragment.

		Pt26081		*Pt63718*		*Pt71936*		*Pt110048*
		Alleles (bp)						
Population	*N*	125	126	110	111	166	167	86
Chilliwack	31	0.36	0.64	0.97	0.03	0.97	0.03	1
Haney	24	0.29	0.71	1	0	0.96	0.04	1
Harrison	23	0.35	0.65	1	0	1	0	1
Klinaklini	29	0.31	0.69	1	0	0.93	0.07	1
Denman	28	0.25	0.75	1	0	0.96	0.04	1
Texada	28	0.25	0.75	1	0	0.96	0.04	1
Quadra	29	0.21	0.79	1	0	1	0	1
E. Thurlow	27	0.11	0.89	1	0	1	0	1
Sooke	25	0.08	0.92	1	0	1	0	1
Nanaimo	31	0.39	0.61	1	0	0.97	0.03	1
Nimpkish	27	0.11	0.89	1	0	0.96	0.04	1

genetic background; (3) bands of differing size may be caused by length variation at a single locus; and (4) products may arise from chloroplast and mitochondrial DNA. Instances (1) and (2) were minimized by choosing relatively few, distinct zones of activity on each gel. Instance (3) occurs in 3–10% of RAPD loci, but finding these loci is difficult and their net effect is to duplicate an observation. Instance (4) is expected to be rare based on the relative sizes of nuclear vs. organelle genomes. The tradeoff of these limitations is the large number of putative loci revealed by the RAPD technique.

Chloroplast microsatellite markers (cpSSRs) were designed from the complete *Pinus thunbergii* chloroplast sequence by Vendramin et al. (1996). An aliquot of 17 primer pairs was kindly provided for preliminary tests by G. Vendramin. The PCR amplifications were performed using a MJ-PTC100 thermocycler, with the following method: 3 min denaturing at 95°C, followed by 20 cycles of 45 s denaturing at 94°C, 45 s annealing at T_m and 45 s extension at 72°C, with a final extension step of 72°C for 10 min. The reaction solution (20 μL) contained 3 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.75 pmol of forward and reverse primers, 0.5 pmol of M13-labelled primer, 1 U of Taq polymerase (Amplitaq, Perkin-Elmer), and 20 ng of DNA. An M13-tail was added to the forward primer (Gibco BRL) to use an M13-labelled primer on a Licor automated sequencer (Oetting et al. 1995). Fifteen microlitres of stop solution (fuchsin-based) was added prior to the loading of 1 μL onto the sequencing gel. For two primer pairs (namely Pt63718 and Pt110048), co-amplification was possible as both primer pairs shared the same annealing temperature and allele sizes were non-overlapping.

Data analyses

From the isozyme data set of El-Kassaby and Ritland (1996*a*), the 323 individuals, for which RAPDs and cpSSRs were assayed, were retrieved. Specific analyses detailed below were conducted for each class of markers.

At the within population level, for each sample and each isozyme and cpSSR locus, the allele frequencies, the mean number of alleles (N_{all}), and the gene diversity (H_e) were estimated by using Fstat 2.3 (Goudet 1995). For the isozyme loci, the observed heterozygosity (H_o) and the *f*-values (i.e., the F_{is} estimates according to Weir and Cockerham (1984)) were also estimated using this

PT51873	*PT63718*	*PT71936*	*PT79951*	*PT87268*	*PT100783*	*PT102584*	*PT107148*	*PT107517*	*PT109567*	*PT11048*	Ref.
	60	60	58	58		58	58	58	60	60	
	++	++	+	+		0	+	+	+	+	10
+	+	+	+	+	+	+	+	+	+	+	1, 2
+	+	+	+	+	+	+	+	+	+	+	1, 2
		+		+						+	1, 3
		+		+						+	4
+	+	+	+	+	+	+	+				1, 5
+	+	+	+	+	+	+	+				6
		+		+							7
		+	+	+						+	8, 9
		+	+	+						+	9
		+	+	+						+	9
		+	+	+						+	9
		+	+	+						+	9
		+	+	+						+	9

Table 3. Number of individuals (N) and gene/haplotype diversity (H_e) per population estimated for each marker.

Population	N	$H_{e(cp)}$	$H_{e(rapd)}$	$H_{e(iso)}$
Chilliwack	33	0.494	0.224	0.154
Haney	24	0.453	0.221	0.132
Harrison	23	0.474	0.268	0.148
Klinaklini	35	0.509	0.224	0.147
Denman	29	0.431	0.228	0.175
Texada	30	0.384	0.262	0.165
Quadra	30	0.370	0.244	0.167
E. Thurlow	30	0.186	0.258	0.177
Sooke	25	0.153	0.239	0.145
Nanaimo	33	0.515	0.236	0.160
Nimpkish	31	0.295	0.243	0.164
Overall	323	0.388	0.241	0.158
Standard deviation		0.127	0.016	0.014

Note: *cp*, Chloroplastic SSRs; *iso*, isozyme.

Table 4. Genetic differentiation among populations ($\hat{F}_{st}$) and hierarchical analyses of genetic differentiation.

Parameter	cpSSR	RAPD	Isozyme
$\hat{F}_{st}$	0.019	0.072	0.018
	$P = 0.098$	***	***
Hierarchical analyses			
$\hat{F}_g$	0.02	0.074	0.02
	$P = 0.12$	***	***
$\hat{F}_p$	0.006	0.008	0.004
	$P_1 = 0.90$; $P_2 = 0.44$; $P_3 = 0.01$	***	***

Note: For the hierarchical analyses, the populations were clustered into three groups: (*i*) group 1: mainland populations (1, 2, 3, 4), (*ii*) group 2: small islands populations (5, 6, 7, 8), (*iii*) group 3: Vancouver Island populations (9, 10, 11). $\hat{F}_g$ and $\hat{F}_p$ are estimates of the genetic differentiation among groups and among populations within groups, respectively (see Materials and methods for details). Probability values of the exact tests associated to these estimations are indicated by stars or by exact values. ***$P < 10^{-5}$.

software. Tests for deviations from Hardy–Weinberg expectations and for genotypic linkage disequilibria among loci at each allozymic locus were computed within each population with Genepop 3.1d (Raymond and Rousset 1995*b*). For the RAPD loci (which exhibit dominance), allele frequencies were estimated according to Lynch and Milligan (1994), and gene diversity values (H_e) were estimated by using TFPGA (Miller 1997), which has algorithms designed for RAPDs. Tests for genotypic linkage disequilibria among RAPD loci were computed within each population with Genepop 3.1d. Finally, because of the nonrecombining nature of the chloroplast genome, cpDNA haplotypes were treated as alleles at a single locus. Diversity values based on haplotype frequencies were calculated using Arlequin v. 2.000 (Schneider et al. 2000).

For both isozymes and RAPDs, exact tests for allelic differentiation were performed with Genepop 3.1d, and F_{st} was estimated according to the Weir and Cockerham (1984) method with TFPGA. For a hierarchical analysis, three groups were defined: mainland populations (populations 1, 2, 3, 4), small island populations (5, 6, 7, 8) and Vancouver Island (9, 10, 11; see also Fig. 1). In this, $\hat{F}_{st}$ was partitioned into $\hat{F}_p$ and $\hat{F}_g$, denoting differentiation of populations within groups and among groups, respectively. For haplotypic differentiation of cpSSRs, an analysis of molecular variance (AMOVA, Excoffier et al. 1992; Excoffier and Smouse 1994), which distributes the genetic variance within and among populations (or within and among groups for hierarchical analyses) was conducted using Arlequin v. 2.000.

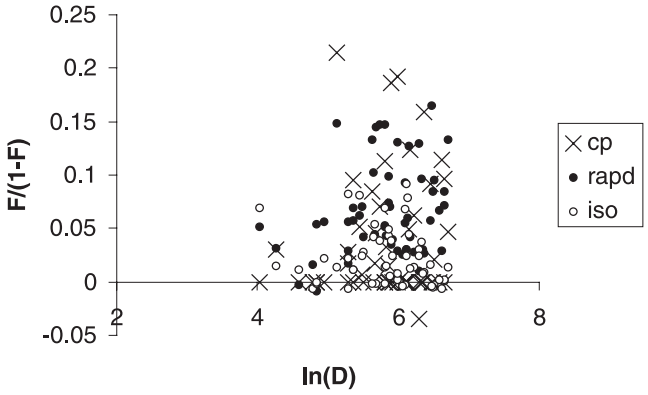
Isolation by distance (Slatkin 1993) was assessed by testing for an association between geographic and genetic distances, estimated as a Spearman's rank correlation coefficient, at various geographical levels. The null hypothesis of no association was tested with Mantel-like permutations of the genetic ($\hat{F}_{st}/(1 - \hat{F}_{st})$) and geographic (ln(geographic distance)) matrices with Genepop 3.1d.

Results

Transferability and polymorphism of cpSSRs

To ascertain the transferability across genera of the SSR

Fig. 2. Isolation by distance. Estimates of pairwise genetic differentiation between populations are plotted against pairwise distance (in km). Mantel tests are nonsignificant ($P > 0.10$).



primer pairs of Vendramin et al. (1996), 17 primer pairs were tested on a sample of 12 individuals from three natural populations (Texada, E. Thurlow, and Sooke). Six out of the 17 primer pairs gave no amplification or ambiguous results (presence of several bands, lack of reproductibility; Table 1). Of the 11 that produced unambiguous amplifications, only three (*Pt26081*, *Pt63718*, and *Pt71936*) showed more than one allele. These three polymorphic primer pairs were assayed in all 323 sampled individuals (Table 2). In addition, *Pt110048* was co-amplified with *Pt63718*; but this fourth locus had only one variant (86 base pairs) across all 323 individuals. Each of the three polymorphic loci exhibited two alleles, separated by one base pair only. For two loci (*Pt63718* and *Pt71936*), one out of the two alleles was present only at a very low frequency. Except for the population of Klinaklini, the frequency of the rarer allele was always below five percent at the within-population level. *Pt26081* is thus the only polymorphic locus at the 95% level criterion.

Altogether, the combination of the alleles at each of the three polymorphic loci constituted five haplotypes. The distribution of these haplotypes is given in Fig. 1. Two out of the five haplotypes (cp1 and cp2) were present in all populations; these differ by one change at locus *Pt26081*. The other three haplotypes occurred at low frequency and were scattered among populations. Haplotype diversities ranged from 0.153 to 0.509 among the populations with a mean of 0.388 (Table 3). The mainland populations generally exhibited higher diversity.

Gene diversity of biparentally inherited markers

Over the 11 populations surveyed, 17 of 20 isozyme loci and 46 of 48 RAPD loci were polymorphic (allelic frequencies are available upon request to the corresponding author). Genotypic associations between pairs of isozyme loci were present in 2 of 135 cases (*6PG1* and *SKD1*, *Fes2* and *PGM1)*, but these occured in just one and three populations, respectively. Within populations, the mean number of alleles ranged from 1.9 to 2.3, and averaged 3.05. F_{is} estimates were near zero ($\hat{F}_{is} = 0.0332$, 95% CI = –0.056; 0.1042). We could thus assume Hardy–Weinberg equilibrium to estimate allele frequencies of RAPDs. Genotypic associations between pairs of RAPD loci were observed between a single pair of loci (in nine out of 11 populations; Fisher's test $P < 10^{-5}$); thus one of these loci was removed from further analysis. Gene diversities at RAPD loci were very similar to those for isozymes (Table 3).

Genetic structure at chloroplast and nuclear markers

Analyses of molecular variance for the cpSSR markers found no differentiation among populations (exact test, $P = 0.098$ over all populations), and only 1.85% of the variance was explained by the variation among populations. By contrast, a significant ($P < 10^{-5}$), but slight differentiation was observed for both isozyme and RAPD markers (Table 4).

The sampled populations were distributed over three distinct geographic areas (mainland, Vancouver Island, and small islands), reflecting an east–west longitudinal cline for climate, primarily precipitation. A hierarchical analysis of genetic variability was carried out using this classification, with $\hat{F}_g$ representing the variation among these groups and $\hat{F}_p$ the variation among populations within groups. For the cpSSR markers, a low and nonsignificant $\hat{F}_g$ value was obtained, but $\hat{F}_p$ was significant ($P = 0.01$) for group 3 (Vancouver Island). With both RAPDs and isozymes, $\hat{F}_g$ and $\hat{F}_p$ were small but significantly different from zero (Table 4). $\hat{F}_g$ was also higher than $\hat{F}_p$.

Because the partitioning of the populations into these groups is somewhat arbitrary, an isolation by distance analysis was then carried out. For all three sets of markers, the Mantel tests for independence between geographic and genetic distances were nonsignificant. The plot of geographical distance against genetic distance is given in Fig. 2.

Discussion

Transferability, polymorphism, and mutation rates of the cpSSR loci

The primers used in the present study were first designed using the complete chloroplast sequence of *Pinus thunbergii* by Powell et al. (1995*b*) and Vendramin et al. (1996). Vendramin et al. (1996) showed 100% success in cross-amplification for 20 primer pairs among seven different *Pinus* species. They also demonstrated high level of conservation in other genera (65%, 75%, and 75% in *Abies alba*, *Cedrus atlantica*, and *Picea abies*, respectively). Here, we found that 65% of 17 primer pairs tested were amplified and scorable in *Pseudotsuga menziesii*. Table 1 summarizes our results and compares the success of these primers in other studies of the Pinaceae family. As for angiosperms (Weising and Gardner 1999), a high level of primer conservation is observed. However, as commented by Provan et al. (1999*a*), it should be noted that by increasing the phylogenetic distances between the source species and the focus species, the level of conservation (i.e., scorable amplification pattern) is decreased (Table 1).

In our sample of 323 individuals from 11 populations, five haplotypes were identified. Two were predominant and occurred in all populations (cp1 and cp2), whereas three were rare. This is due to the low level of polymorphism of two of the three loci used. At the geographical scale of our study, these two loci did not fit the 95% level criterion for polymorphism. The overall level of haplotypic variation here observed is considerably lower than that reported in other

Table 5. Genetic diversity revealed by cpSSRs in the Pinaceae.

Species	Geographical area	N_{pop}	N_{ind}	N_p/N_l	N_{all}	N_{haplo}	H_e	F_{st}	Ref.
Pinus brutia	Mediterranean	8	72	8/9	–	40	0.30	0.29	1
P. eldarica	Middle-East	2	48	8/9	–	13	0.27	0.07	1
P. halepensis	Mediterranean	10	127	8/9	38	28	0.22	0.31	1
P. resinosa	Canada	7	159	9/9	25	23	0.62	0.12	2
P. sylvestris	Europe	7	330	13/17	–	174	0.98	0.32	3
P. pinaster	Mediterranean basin	10	240	7/9	24	34	0.81	0.23	4
P. leucodermis	Mediterranean basin	7	–	10/10	29	48	–	–	5
P. torreyana	California	1	64	0/17	1	1	0.00	–	6
Abies alba	Europe	17	714	2/2	26	90	0.85	0.13	7
Pseudotsuga menziesii	Canada (B.C.)	11	323	3/11	6	5	0.38	0.02	8

Note: N_{pop}, N_{ind}, N_p/N_l, N_{all}, and N_{haplo} refer to numbers of populations, individuals, polymorphic loci (compared to the total number of loci tested), alleles over all loci, and haplotypes, respectively; H_e and F_{st} refer to estimates of haplotype diversity and of F_{st}, respectively. References: 1, Bucci et al. 1998, 2, Echt et al. 1998, 3, Provan et al. 1998, 4, Vendramin et al. 1998, 5, Powell et al. 1995*b*, 6, Provan et al. 1999*b*, 7, Vendramin et al. 1999, 8, present study.

studies of cpSSRs in Pinaceae (see Table 5). In some of these species, variation was so high that unique haplotypes (specific to a given individual) and population-specific haplotypes were found. For example, by using 13 polymorphic loci among seven Scottish populations, Provan et al. (1998) found that 38% of the trees could be identified by a unique haplotype. The reason for our low polymorphism is unknown. There is not a clear relationship between the phylogenetic distance, the transferability of primers, and the level of polymorphism (in Table 1 and Table 5, see for example, *Abies alba*, *P. torreyana*, and *P. pinaster*). Thus, low mutation rates and (or) peculiar biological features of Douglas-fir populations in B.C. may explain the low polymorphism observed.

Compared to nuclear microsatellites, chloroplast microsatellites have low mutation rates. Provan et al. (1999*b*) estimated mutation rates ranging from 3.2×10^{-5} to 7.9×10^{-5} for 17 cpSSR assayed in a population of *Pinus torreyana*. Those estimates are at the lower bound of the mutation rates estimated with nuclear microsatellites (10^{-2} to 10^{-5} according to Jarne and Lagoda 1996). Thus, the genetic distribution of neutral markers should be affected mainly by migration and not by mutation (Ennos et al. 1999).

Low mutation rates at cpSSR loci cannot alone explain the results observed in the Douglas-fir populations, for the following reasons. First, this is not a general feature of the loci we used. For example, three primers used in the present study (namely *Pt26081*, *Pt71936*, and *Pt110048*) were also analyzed by Bucci et al. (1998) to assess the genetic relationships among three species of the *Pinus halepensis* complex. Eight, eight and four alleles were reported on *Pt26081*, *Pt71936*, and *Pt110048*, respectively, over the whole study (247 individuals from 20 populations distributed in the Mediterranean basin and the Middle East). They observed up to 10 haplotypes within a single population. Second, the levels of polymorphism and diversity observed across populations with both RAPDs and isozymes are of the same order of magnitude as those observed with cpSSRs (Table 3). Each class of markers also showed a high proportion of loci with skewed distribution of allele frequencies; a property of long-lived woody species (Conkle 1992; Hamrick et al. 1992; Aguirre-Planter et al. 2000). Thus, the small number of haplotypes and the moderate level of haplotype diversity observed in the Douglas-fir populations may highlight peculiar biological and historical features of Douglas-fir populations from B.C.

Organelle vs. nuclear comparisons: Insights about the pollen flow

Chloroplasts are paternally inherited in Douglas-fir (Neale et al. 1986) and thus migrate through both seeds and pollen. Under either the island model or the isolation by distance model, a greater differentiation for paternally inherited (chloroplast) genes is expected compared to biparentally inherited (nuclear) genes (Ennos 1994; Hu and Ennos 1997). Unexpectedly, among our Douglas-fir populations, cpSSR loci showed a lack of genetic differentiation, whereas isozymes and RAPDs did show significant differentiation. As discussed above, it is unlikely that mutations at cpSSR loci have significantly decreased genetic differentiation, as mutation rates are probably low. A more parsimonious hypothesis is that the pollen pool is highly homogeneous, and that the significant genetic population differentiation observed with biparentally inherited markers is mainly due to limited seed dispersal, relative to pollen dispersal.

Whether our hypothesis is correct is an open question. It is well known that pollen disperses further than seeds, and that conifers are highly outbreeding (Hamrick et al. 1992), and previous studies based on chloroplast markers demonstrated a low level of population differentiation, indicative of high pollen migration rates (Latta and Mitton 1997; Provan et al. 1998). However, by using both paternity analyses and direct measures, Campbell (1991) demonstrated a discrepancy between estimates of pollen movement and realized gene flow, which could be due to post-pollination effects or difficulty of sampling. The viability of pollen as well as the density also decrease over long distance (Latta et al. 1998). In Douglas-fir, despite wind dispersal of pollen, the lack of wing and the heavy weight probably limit pollen dispersal (Allen and Owens 1972). Also, Burczyk and Prat (1997) demonstrated a significant effect of male–female mating distance in Douglas-fir, with 43% of matings resulting from

outcrossing with surrounding males. The characterization of maternally inherited markers (in mtDNA) could be very helpful to ascertain this hypothesis.

Historical factors

Aside from high pollen flow, historical factors may have had major effects on the overall low diversity, and lack of genetic structure, of cpSSRs in Douglas-fir. The presence of only two common haplotypes in all populations is suggestive of an ancient major bottleneck. It is well known that several glaciations during the Pleistocene have caused major range fluctuations in most temperate and boreal species (Ferris et al. 1999). With migration from refugia during the interglacials, strong genetic differentiations can occur via founding events. However, in previous Douglas-fir studies (Yeh and O'Malley 1980; El-Kassaby and Ritland 1996*a*; Aagaard et al. 1998*a*) and in studies of other temperate long-lived woody species (Hamrick et al. 1992; Austerlitz et al. 2000; Vendramin et al. 2000), populations do not show the dramatic structure expected from such founding events. Under different models of colonization, Austerlitz et al. (2000) demonstrated that in forest trees, founder effects are reduced by delayed reproduction. Such effects are evident in the small genetic differentiation among populations before glaciation and colonization of refugia (Aguirre-Planter et al. 2000; Austerlitz et al. 2000).

In addition to these biological (time to sexual reproduction) and historical (glaciation) effects, more contemporary migration effects are suggested by our data on cpSSRs. Out of the five haplotypes identified over the 11 populations, two (cp1 and cp2) were found in all the populations whereas three were scarcely found (cp3 to cp5). Given the low frequencies of the cp3 and cp5 haplotypes, the test for isolation by distance as well as hierarchical analyses did not reveal any group structure or geographical distance effects. However, interestingly the cp3 and cp4 haplotypes were not randomly distributed: cp3 is located in three out of the four mainland populations as well as in the population sampled in Texada Island (one of the small islands closed to the mainland) and cp4 is distributed within only two populations of Vancouver Island and within Denman Island, which is also very close to that major island. This indicates that a slight isolation by distance effect may occur in the Douglas-fir populations of B.C. These new variants (i.e., alleles 110 at *Pt63718* and 167 at *Pt71936*) at very low frequencies may be recent mutations that occurred in delimited area and which spread through step-by-step migration.

In conclusion, our assay of cpSSRs in Douglas-fir produced an unexpectedly low level of polymorphism within populations, and no genetic differentiation among populations. This result is likely the outcome of both historical factors and high pollen dispersal. The slight although significant genetic structure observed at nuclear loci is probably a consequence of limited seed dispersal. Mitochondrial, maternally inherited markers (Aagaard et al. 1998*b*) would be of value to clarify the relative importance of pollen vs. seed dispersal in genetically constructing the population.

Acknowledgements

This research was supported by grants from the Natural Science and Engineering Research Council of Canada (NSERC) to K.R. The authors would especially like to thank G.G. Vendramin for sending us aliquots of the cpSSR primers, and Carol Ritland for her great help in the laboratory.

References

Aagaard, J.E., Frutovskii, K.V., and Strauss, S.H. 1998*a*. RAPDs and allozymes exhibit similar levels of diversity and differentiation among populations and races of Douglas-fir. Heredity, **81**: 69–78.

Aagaard, J.E., Frutovskii, K.V., and Strauss, S.H. 1998*b*. RAPD markers of mitochondrial origin exhibit lower population diversity and higher differentiation than RAPDs of nuclear origin in Douglas fir. Mol. Ecol. **7**: 801–812.

Adams, W.T. 1992. Gene dispersal within forest tree populations. New For. **6**: 217–240.

Aguirre-Planter, E., Furnier, G.R., and Eguiarte, L.E. 2000. Low levels of genetic variation within and high levels of genetic differentiation among populations of species of *Abies* from southern Mexico and Guatemala. Am. J. Bot. **87**: 362–371.

Allen, G.S., and Owens, J.N. 1972. The life history of Douglas-fir. Information Canada, Ottawa.

Austerlitz, F., Mariette, S., Machon, N., Gouyon, P.-H., and Godelle, B. 2000. Effects of colonization processes on genetic diversity: differences between annual plants and tree species. Genetics, **154**: 1309–1321.

Bucci, G., Anzidei, M., Madghiele, A., and Vendramin, G.G. 1998. Detection of haplotypic variation and natural hybridization in *halepensis*-complex pine species using chloroplast simple sequence repeat (SSR) markers. Mol. Ecol. **7**: 1633–1643.

Burczyk, J., and Prat, D. 1997. Male reproductive success in *Pseudotsuga menziesii* (Mirb.) Franco: the effects of spatial structure and flowering characteristics. Heredity, **79**: 638–647.

Campbell, D.R. 1991. Comparing pollen dispersal and gene flow in a natural population. Evolution, **45**: 1964–1968.

Clegg, M.T., Clegg, M.T., Gaut, B.S., Learn, G.H., and Morton, B.R. 1994. Rates and pattern of chloroplast DNA evolution. Proc. Natl. Acad. Sci. U.S.A. **91**: 6795–6801.

Conkle, M.T. 1992. Genetic diversity—seeing the forest through the trees. New For. **6**: 5–22.

Echt, C.S., DeVerno, L.L., Anzidei, M., and Vendramin, G. G. 1998. Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. Mol. Ecol. **7**: 307–316.

El-Kassaby, Y., and Ritland., K. 1996*a*. Genetic variation in low elevation Douglas-fir of British Columbia and its relevance to gene conservation. Biodivers. Conserv. **5**: 779–794.

El-Kassaby, Y., and Ritland, K. 1996*b*. Impact of selection and breeding on the genetic diversity in Douglas-fir. Biodivers. Conserv. **5**: 795–813.

Ellstrand, N.C. 1992. Gene flow among seed plant populations. New For. **6**: 241–256.

Ennos, R.A. 1994. Estimating the relative rates of pollen and seed migration among plant populations. Heredity, **72**: 250–259.

Ennos, R.A., Sinclair, W.T., Hu, X.-S., and Langdon, A. 1999. Using organelle markers to elucidate the history, ecology and evolution of plant populations. *In* Molecular Systematics and Plant Evolution. *Edited by* P.M. Hollingsworth, R.M. Bateman, and R.J. Gornall. Taylor & Francis Ltd., London. pp. 1–19.

Excoffier, L., and Smouse, P.E. 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. Genetics, **136**: 343–359.

Excoffier, L., Smouse, P., and Quattro, J. 1992. Analysis of molecular variance inferred from metric distances among DNA

haplotypes: application to human mitochondrial DNA restriction data. Genetics, **131**: 479–491.

Ferris, C., King, R.A., and Hewitt, G.M. 1999. Isolation within species and the history of glacial refugia. *In* Molecular Systematics and Plant Evolution. *Edited by* P.M. Hollingsworth, R.M. Bateman, and R.J. Gornall. Taylor & Francis Ltd., London. pp. 20–34.

Goudet, J. 1995. Fstat (version 1.2): a computer program to calculate F-statistics. J. Hered. **86**: 485–486.

Hadrys, H., Balcik, M., and Schierwater, B. 1992. Application of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. **1**: 55–63.

Hamrick, J.L., and Godt, J.W. 1990. Allozyme diversity in plant species. *In* Plant population genetics, breeding, and genetic resources. *Edited by* A.H.D. Brown, M.T. Clegg, A.L. Kalher, and B.S. Weir. Sinauer Associates Inc., Sunderland, Mass. pp. 43–63.

Hamrick, J.L., Godt, M.J.W., and Sherman-Broyles, S.L. 1992. Factors influencing levels of genetic diversity in woody plant species. New For. **6**: 95–124.

Haymer, D.S. 1994. Random amplified polymorphic DNAs and microsatellites: what are they, and can they tell us anything we don't already know? Ann. Entomol. Soc. Am. **87**: 717–722.

Hu, X.-S., and Ennos, R.A. 1997. On estimation of the ratio of pollen to seed flow among plant poplations. Heredity, **79**: 541–552.

Jarne, P., and Lagoda, J.-L. 1996. Microsatellites, from molecules to populations and back. Trends Ecol. Evol. **11**: 424–429.

Latta, R.G., and Mitton, J.B. 1997. A comparison of population differentiation across four classes of gene marker in limber pine (*Pinus flexilis* James). Genetics, **146**: 1153–1163.

Latta, R.G., Linhart, Y.B., Fleck, D., and Elliot, M. 1998. Direct and indirect estimates of seed versus pollen movement within a population of ponderosa pine. Evolution, **52**: 61–67.

Levin, D.A. 1988. Consequences of stochastic elements in plant migration. Am. Nat. **132**: 643–651.

Loveless, M.D., and Hamrick, J.L. 1984. Ecological determinants of genetic structure in plant populations. Annu. Rev. Ecol. Syst. **15**: 65–95.

Lynch, M., and Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. Mol. Ecol. **3**: 91–99.

McCauley, D.E. 1995. The use of chloroplast DNA polymorphism in studies of gene flow in plants. Trends Ecol. Evol. **10**: 198–202.

Miller, M.P. 1997. Tools for Population Genetic Analyses (1.3): A windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by the author. Department of Biological Sciences, Northern Arizona University, Flagstaff, Ariz. (e-mail: mpm2@jan.ucc.nau.edu).

Neale, D.B., Wheeler, N.C., and Allard, R.W. 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. Can. J. For. Res. **16**: 1152–1154.

Oetting, W.S., Lee, H.K., Flanders, D.J., Wiesner, G.L., Sellers, T.A., and King, R.A. 1995. Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics, **30**: 450–458.

Olmstead, R.G., and Palmer, J.D. 1994. Chloroplast DNA systematics: a review of methods and data analysis. Am. J. Bot. **81**: 1205–1224.

Powell, W., Morgante, M., Andre, C., McNicol, J.W., Machray, G.C., Doyle, J.J., Tingey, S.V., and Rafalski, J.A. 1995*a*. Hypervariable microsatellites markers provide a general source of polymorphic DNA markers for the chloroplast genome. Curr. Biol. **5**: 1023–1029.

Powell, W., Morgante, M., McDevitt, R., Vendramin, G.G., and Rafalski, J.A. 1995*b*. Polymorphic simple sequence repeat regions in chloroplast genomes: applications to the population genetics of pines. Proc. Natl. Acad. Sci. U.S.A. **92**: 7759–7763.

Powell, W., Machray, G.C., and Provan, J. 1996*a*. Polymorphism revealed by simple sequence repeats. Trends Plant Sci. **1**: 215–222.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., and Rafalski, A. 1996*b*. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. **2**: 225–238.

Provan, J., Soranzo, N., Wilson, N.J., McNicol, J.W., Forrest, G.I., Cottrell, J., and Powell, W. 1998. Gene-pool variation in caledonian and european scots pine (*Pinus sylvestris* L.) revealed by chloroplast simple-sequence repeats. Proc. R. Soc. Lond. B Biol. Sci. **265**: 1697–1705.

Provan, J., Russell, J.R., Booth, A., and Powell, W. 1999*a*. Polymorphic chloroplast simple sequence repeat primers for systematic and population studies in the genus *Hordeum*. Mol. Ecol. **8**: 505–511.

Provan, J., Soranzo, N., Wilson, N.J., Goldstein, D.B., and Powell, W. 1999*b*. A low mutation rate for chloroplast microsatellites. Genetics, **153**: 943–947.

Rafalski, J.A., and Tingey, S.V. 1993. Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends Genet. **9**: 275–280.

Raymond, M., and Rousset, F. 1995*a*. An exact test for population differentiation. Evolution, **49**: 1280–1283.

Raymond, M., and Rousset, F. 1995*b*. Genepop (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. **86**: 248–249.

Ritland, C., and Ritland, K. 2000. DNA fragment markers in plants. *In* Molecular Methods in Ecology. *Edited by* A.J. Baker, Blackwell Scientific, Oxford, U.K. pp. 208–234.

Schneider, S., Roessli, D., and Excoffier, L. 2000. Arlequin: a software for population genetics data analysis. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Slatkin, M. 1985. Gene flow in natural populations. Annu. Rev. Ecol. Syst. **16**: 393–430.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. Science, **236**: 787–792.

Slatkin, M. 1993. Isolation by distance in equilibrium and nonequilibrium populations. Evolution, **47**: 264–279.

Soltis, D.E., Soltis, P.S., and Milligan, B.G. 1992. Intraspecific chloroplast DNA variation: systematics and phylogentic implications. *In* Molecular Systematics of Plants. *Edited by* P.S. Soltis, D.E. Soltis, and J.J. Doyle. Chapman and Hall, New York. pp. 117–150.

Vendramin, G.G., and Ziegenhagen, B. 1997. Characterization and inheritance of polymorphic plastid microsatellites in *Abies*. Genome, **40**: 857–864.

Vendramin, G.G., Lelli, L., Rossi, P., and Morgante, M. 1996. A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. Mol. Ecol. **5**: 595–598.

Vendramin, G.G., Anzidei, M., Madaghiele, A., and Bucci, G. 1998. Distribution of genetic diversity in *Pinus pinaster* Ait. as revealed by chloroplast microsatellites. Theor. Appl. Genet. **97**: 456–463.

Vendramin, G.G., Degen, B., Petit, R.J., Anzidei, M., Madaghiele, A., and Ziegenhagen, B. 1999. High level of variation at *Albies alba* chloroplast microsatellite loci in Europe. Mol. Ecol. **8**: 1117–1126.

Vendramin, G.G., Anzidei, M., Madaghiele, A., Sperisen, C., and Bucci, G. 2000. Chloroplast microsatellite analysis reveals the presence of population subdivision in Norway spruce (*Picea abies* K.). Genome, **43**: 68–78.

Weir, B.S., and Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. Evolution, **38**: 1358–1370.

Weising, K., and Gardner, C. 1999. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome, **42**: 9–19.

Yeh, F.C.-H., and O'Malley, D.O. 1980. Enzyme variations in natural populations of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, from British Columbia. 1. Genetic variation patterns in coastal populations. Silvae Genet. **29**: 3–4.