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RAPD markers of mitochondrial origin exhibit lower population diversity and higher differentiation than RAPDs of nuclear origin in Douglas fir

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Abstract

We developed a method of screening RAPD markers for the presence of organelle DNA products using enriched organelle DNA probes, then used these markers to compare the structure of nuclear and mitochondria) RAPD diversity in Douglas fir. Of 237 screened RAPD fragments from 25 primers, 16% were identified as originating in the mitochondria) genome and 3% in the chloroplast genome. The mitochondria) DNA probe correctly distinguished fragments with known maternal inheritance (which is exclusive for the mitochondria) genome in the Pinaceae), and neither of the organelle probes hybridized to biparentally inherited fragments. Mitochondria) RAPD markers exhibited low diversity within populations compared to nuclear RAPD diversity (Hs = 0.03 and 0.22, respectively), but were much more highly differentiated than were fragments of nuclear origin at both the population (GsT = 0.18 and 0.05, respectively) and racial levels (GsT = 0.72 and 0.25, respectively). Both nuclear and mitochondria) DNA based phylogenetic analyses identified the varieties as monophyletic groups; the nuclear RAPD markers further separated the north and south interior races.

Keywords: Douglas fir, genetic variation, mitochondria) DNA, molecular markers, RAPD

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Introduction

Studies that utilize both cytoplasmic and nuclear DNA markers are common in phylogeographic analyses of animals (Avise 1994). Despite extensive literature on allozyme studies in plants (Hamrick & Godt 1989), however, there have been few studies that have compared patterns of diversity in nuclear and cytoplasmic markers (for reviews, see Arnold 1993; El Mousadik & Petit 1996). This is a consequence of the slow rates of nucleotide substitution in both the chloroplast and mitochondria) genomes of plants, and the complex structural dynamics of the plant mitochondria) genome (reviewed by Hipkins *et al.* 1994). However, the increasing abundance of gene sequence information, and the advent of PCR techniques, now allow efficient detection of cytoplasmic polymorphisms in plants (Taberlet *et al.* 1991; Demesure *et al.* 1995; Powell *et al.* 1995). This has prompted recent studies comparing these markers to nuclear markers (e.g. Vicario *et al.* 1995; McCauley *et al.* 1996), and has led to new insights on gene flow and the structuring of genetic diversity.

Results from comparisons between cytoplasmic and nuclear markers have generally conformed well to expectations from population genetic theory. The small effective population size and limited dispersal of most cytoplasmic markers should enhance differentiation among populations relative to nuclear markers (Moritz 1994). In most plant species where both organelle genomes are maternally inherited, chloroplast and mitochondria) DNA (mtDNA) markers exhibit this population level structuring (e.g. McCauley et *al.* 1996). However, in plant species such as conifers where the mitochondria) genome is maternally inherited while the chloroplast genome is predominantly paternally inherited (reviewed by Wagner 1992), the structure of nuclear and cytoplasmic diversity may be more complex (e.g. Strauss et *al.* 1993).

Douglas fir *[Pseudotsuga menziesii* (Mirb.) Franco] is a long-lived, monoecious species and is the dominant forest

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tree throughout most of its range in western North America. As with other Pinaceae conifers, the mitochondria) genome is maternally inherited (Marshall & Neale 1992), whereas the chloroplast genome is paternally inherited (Neale *et al.* 1986). Although they readily hybridize (Rehfeldt 1977), the two recognized varieties of Douglas fir (coastal and interior) are distinct based on needle morphology and colour, terpene chemistry (von Rudloff 1972), growth characteristics (Rehfeldt 1977), and allozymes (Li & Adams 1989). In addition, allozymes distinguish two racial groups within the interior variety (north and south interior; Li & Adams 1989). At the end of the Wisconsin glaciation (= 10 000 years ago), refugial populations of the interior races were likely scattered along the eastern edge of the Great Basin and in high-elevation populations of the southwestern USA (Critchfield 1984). Patterns of allozyme diversity suggest a single large refugium for the coastal variety near the Willamette Valley of western Oregon (Li & Adams 1989). Based on pollen distribution, the coastal and north interior races reached their present distribution only 7000 years ago (Tsukada 1982).

In a previous study of Douglas fir (Aagaard *et al.* 1995), we found that mtDNA sequences were commonly amplified using the randomly amplified polymorphic DNA (RAPD) technique (Williams *et al.* 1990). RAPDs of cytoplasmic origin have also been found by others (e.g. Thormann & Osborne 1992; Lorenz *et al.* 1994), and their common occurrence in Douglas fir RAPD profiles, as well as the high degree of racial differentiation, suggested that they might be a source of markers for population studies. Our goals in the current study were: (i) to develop a technique to allow identification of all cytoplasmic DNA markers in RAPD profiles; (ii) to quantify the frequency of organelle DNAs in RAPD profiles more intensively than was possible in our earlier study (Aagaard *et al.* 1995); (iii) to compare diversity and differentiation of organelle RAPD markers with nuclear RAPD markers at both population and racial levels; and (iv) to provide new data into the evolutionary history and racial phylogeny of Douglas fir.

Materials and methods

Sequential screening was used to identify organelle DNAs among RAPD profiles (Fig. 1). Application of this technique required the development of a mtDNA extraction protocol for Douglas fir, quantification of mitochondria) and chloroplast DNA enrichment, and demonstration of the ability of organelle DNA probes to identify genome-specific sequences and RAPD fragments of known genomic origin.

Mitochondria) DNA extraction

Mitochondria) DNA was extracted from embryogenic suspension cultures of Douglas fir (Gupta *et al.* 1988) using a modified version of a protocol developed for spruce by Dr P du Jardin, University of Gembloux, Belgium (personal communication). Packed embryogenic suspension cultures less than 2-weeks old were provided by Dr P K. Gupta of Weyerhauser Co. (Tacoma, Washington, USA). Cultures were precipitated in a tabletop centrifuge, the supernatant discarded, and 2 vol. of extraction buffer added (30 mm MOPS, pH 7.4, 0.35 M mannitol, 2.5 MM MgC12, 1.25 mm EGTA, 0.3% w/v PVP25, 2 mm sodium metabisulphite, 0.2% w/v BSA, 8 mm cysteine). Resuspended cells (= 15 mL) were ground on ice for 3 min with a mortar and pestle, 1 vol. of ice-cold extraction buffer was added, and the homogenate was filtered on one layer of miracloth and four layers of

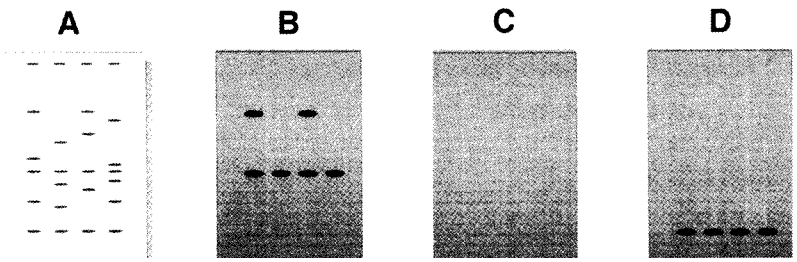

Fig. 1 Sequential screening via Southern hybridization to identify organelle DNA markers among RAPD profiles. A. RAPD fragments were separated on agarose gels and blotted onto nylon membranes for Southern analysis. B. Enriched mtDNA is isotopically labelled and hybridized to blots using standard Southern procedures. Mitochondria) DNA markers are those RAPD fragments showing a strong hybridization signal using phosphorimaging. C. Blots are then stripped and residual activity recorded. D. Enriched chloroplast DNA is isotopically labelled and hybridized to blots. Chloroplast DNA markers are those RAPD fragments showing a strong hybridization signal after discounting residual activity from the mtDNA probe.

cheesecloth. Filtrate was centrifuged twice at 1000 x g for 10 min, discarding the pellet each time; all centrifugation was carried out at 4 °C. Supernatant from the second centrifugation was transferred to a clean tube and centrifuged at 16 000 x g for 15 min and the pellet resuspended in 10 mL of ice-cold wash buffer (10 mm MOPS, pH 7.2, 0.35 M mannitol, 1 mm EGTA, 2 mm sodium metabisulfite, 0.1% w/v BSA); pellets from up to three samples (15 mL starting volumes) were combined and resuspended in the same 10 mL of wash buffer.

Resuspended pellets were centrifuged at 1000 x g for 10 min after which supernatant was transferred to a clean tube and centrifuged again at 16 000 x g for 15 min. The pellet was resuspended in 10 mL of freshly made DNase I solution and incubated at 15 °C for 30 min [10 mm MOPS, pH 7.2, 0.35 M mannitol, 2 mm sodium metabisulphite, 0.1% w/v BSA, 25 MM MgC12, 200 pg/mL DNase I (SIGMA)]. After incubation, 1 mL of 0.5 Nt EGTA was added and the solution centrifuged at 16 000 x g for 15 min. The pellet was resuspended in 10 mL of ice-cold wash buffer, transferred to a clean tube, and centrifuged at 16 000 x g for 15 min; resuspension of the pellet in wash buffer and centrifugation was repeated twice. After the final centrifugation, the pellet was resuspended in 2 mL of lysis buffer [40 mm Tris-HCI, pH 7.9,150 mm NaC1,15 mm EDTA, pH 8.0, 2% w/v sarkosyl,100 ug/mL self-digested proteinase K (2 h at 37 °C)] and incubated at 37 °C for 30 min, then at 55 °C for 60 min. DNA was extracted with 1 vol. phenol:chloroform:isoamyl alcohol (25:24:1), the aqueous phase treated with RNase (100 ug/mL RNase incubated at 37 °C for 2 h), and residual RNAse was removed with 1 vol. of phenol:chloroform:isoamyl alcohol. DNA was precipitated from the aqueous phase (150 mm NaCI) with 2 vol. of 95% ethanol.

Quantification of organelle DNA enrichment

The degree of enrichment of mtDNA, and of chloroplast DNA extracted previously (Hipkins 1993), using published chloroplast DNA-extraction protocols (Palmer 1992a), was quantified using Southern analysis and genome-specific probes. We loaded 0.2 jig of each organelle DNA, and 1 pg each of total genomic DNA from embryogenic suspension cultures, foliage, seed embryos, and seed megagametophytes onto 0.8% TAE agarose gels and electrophoresed them for 30 min at 0.8 V/cm. Total genomic DNA was isolated from embryogenic suspension cultures using the same protocol as for mtDNA extraction, eliminating the differential centrifugation and DNase treatment steps. Total genomic DNA was extracted from foliage using the modified CTAB protocol described in Strauss *et al.* (1993). Total genomic DNAs from seed embryos and megagametophytes were extracted using a modified CTAB protocol described previously (Aagaard *et al.* 1995). DNA was blotted and probed using standard Southern blotting techniques.

In order to determine mtDNA enrichment, a 0.7 kb coxIII fragment (labelled with [α^{32}P]-dCTP and [α32P]dATP) was amplified from Douglas fir and used as a probe. Primers used to amplify the coxIII fragment from Douglas fir were designed from the consensus sequence based on alignment of the coxIII sequences from different species listed in Hiesel *et al.* (1994; 5'-GTAGATCCAACTCCATGGCCT-3' and 5'-GCAGCTGCTTCAAAGCC-3'). Chloroplast DNA enrichment was determined using a 1.6 kb chloroplast DNA clone in pUC19 (Hipkins 1993). In the Douglas fir chloroplast genome, this fragment is nested between the 23S ribosomal RNA and the genes for rps12 and rps7 (Strauss *et al.* 1988). Phosphorimaging (Molecular Dynamics Phosphorimager SI, Sunnyvale, California, USA) was used to quantify the signal from organelle and total genomic DNAs, and the resulting signal ratios were used to determine approximate organelle DNA enrichment.

Enriched organelle DNAs as probes

The ability of the enriched organelle DNAs to correctly identify nuclear and organelle genes was tested using mitochondrial and chloroplast DNA fragments from Douglas fir, conserved nuclear genes, and RAPDs of known inheritance. We loaded 0.15 pg each of the 0.7 kb coxIII fragment, the 1.6 kb chloroplast DNA clone in pUC19, two nuclear sequences (3.0 kb actin gene cloned from soybean, Shah *et al.* 1982; 7.8 kb ribosomal DNA gene cloned from soybean, Varsanyi-Breiner *et al.* 1979), and RAPD amplification products that include fragments of known mitochondrial and nuclear origin from five RAPD primers studied previously (Aagaard *et al.* 1995) onto 2% TBE agarose gels that were electrophoresed at 30 V/cm for 3.5 h. Gels were blotted and probed with either enriched mitochondrial DNA or chloroplast DNA that was labelled using random primed labelling with 0.2 pg of the respective organelle DNA and both [a3ZP]dCTP and [a32P]-dATP (Boehringer Mannheim). Phosphorimaging plates were exposed to hybridized blots for = 48 h before scanning to enable weak signals to be detected on blots.

Douglas fir populations

Seeds representing two populations from each of the three races of Douglas fir were selected from existing seed collections. Coastal and north interior populations (Fig. 2) were selected from single tree seed collections maintained by the USDA Forest Service Pacific Northwest Research Station (Corvallis, Oregon, USA). Coastal seed collections were originally made from

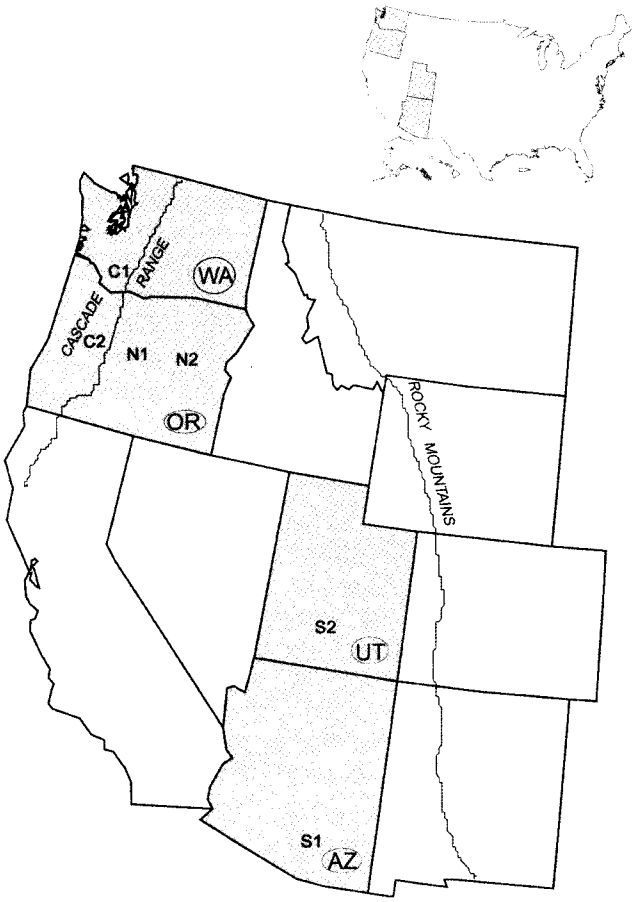

Fig. 2 The six populations of Douglas fir studied (Cl, Smith Creek; C2, Lacomb; Nl, Wildcat Mountain; N2, Baldy Mountain; SL Coronado; S2, Dixie) represent three recognized races (C = coastal, N = north interior, and S = south interior). All populations are located within the states of Arizona (AZ), Oregon (OR), Utah (UT), and Washington (WA).

natural populations in western Washington (Smith Creek; 34 maternal trees) and western Oregon (Lacomb; 22 maternal trees). North interior seed collections were originally made from natural populations in central (Wildcat Mountain; 36 maternal trees) and east-central (Baldy Mountain; 30 maternal trees) Oregon. Maternal trees were separated by 50-100 m and were the dominant or codominant trees within stands. South interior populations were selected from bulked seed collections stored at the USDA Forest Service Lucky Peak Nursery (Boise, Idaho). The southern Arizona bulked seed collection (Coronado) was made from a single stand of 30 maternal trees in a natural population, and the southern Utah bulked seed collection (Dixie) was made from two contiguous stands of 22 maternal trees each in a natural population; 30 and 29 seeds were analysed from the Coronado and Dixie collections, respectively.

Screening RAPD primers

RAPDs were amplified from DNA of two seed embryos from each of the three races of Douglas fir using 25 RAPD primers. These primers had been previously shown to give clear amplification profiles in Douglas fir. RAPDs were blotted, probed with enriched mtDNA, and exposed to phosphorimaging plates for 48 h and scanned as described above. The probes were then stripped from the blots by treating twice with 0.4 M NaOH at 42 °C, and residual activity recorded by again scanning the stripped blots. The blots were then reprobed with an enriched chloroplast DNA probe and exposed to phosphorimaging plates for 48 h before a final scanning.

Scoring of RAPD fragments

Only those RAPD fragments which were strong and reproducible were scored. Mitochondrial and chloroplast RAPD markers were identified based on strong hybridization signals relative to background; residual activity recorded from stripped blots was discounted when scoring chloroplast RAPD markers. Nuclear RAPD markers were considered to be those RAPDs which showed no evidence of hybridization to either organelle DNA probe. The total number of RAPD fragments amplified by each primer, including weak bands, and the number of mitochondrial and chloroplast RAPD bands, were quantified for each primer.

Scoring of RAPD markers in population surveys

Those RAPD primers that amplified reliably (UBC 234, UBC 328, UBC 330, UBC 336, UBC 411, UBC 428, UBC 460, UBC 504, and UBC 570) were used to study all individuals within each of the six populations of Douglas fir. Chloroplast markers were not included because we were unable to verify their pattern of inheritance (see Results). Nuclear RAPDs were treated as dominant Mendelian markers under Hardy-Weinberg equilibrium. Allele frequencies were calculated from null homozygote frequencies and corrected for dominance according to Lynch & Milligan (1994). Similar to most conifers, Douglas fir exhibits high outcrossing rates and genotype frequencies of seed embryos closely approximate Hardy-Weinberg proportions (Neale & Adams 1985). Even substantial deviations from Hardy-Weinberg frequencies (e.g. F_{IS} = 0.20) would have very little effect on estimates of diversity parameters for our nuclear RAPD data set (Aagaard 1997). Mitochondrial RAPDs identified solely on the basis of hybridization results (no inheritance information) were treated as haploid markers from which both 'allele' frequencies (i.e. frequency of individual mitochondrial RAPD fragments) and haplotype frequencies (i.e.

frequency of whole mitochondria) RAPD phenotypes) were calculated within populations.

Data analysis

Nei's (1973) gene diversity statistics, corrected for small sample size (Nei 1978) and small number of populations (Nei 1986), were calculated separately for nuclear and mitochondria) RAPD markers. A hierarchical analysis of differentiation due to population and race subdivision employed only the small sample size correction to allow for the decomposition of gene diversities (Nei 1986). All calculations were made using the computer program GENESTAT-PC 3.3 (Lewis 1994). Most calculations were made with completely monomorphic loci excluded (99% criterion), although population diversity parameters were calculated both with and without monomorphic loci. Standard errors for GsT values were calculated by jackknifing over loci according to the method of Weir & Cockherham (1984).

RAPD differentiation was also analysed with the analysis of molecular variance (AMOVA) approach of Excoffier *et al.* (1992) using simple dissimilarity [1-(the fraction of matches)] as a measure of genetic distance between individuals, as has been performed previously (e.g. Apostol *et al.* 1993). We used Black's (1996) computer program RAPDPLOT 2.4 to calculate the fraction of matches *(M)* using the formula $M = N_{AB}/N_T$, where N_{AB} is the total number of matches in individuals A and B (i.e. both fragments absent or present) and N_T is the total number of loci scored in the overall study. The computer program winAMOVA (Excoffier 1993) was used for all AMOVA calculations.

Allele frequencies from nuclear and mitochondria) RAPDs were used to construct separate nuclear and cytoplasmic phylogenetic trees. Using the computer package PHYLIP (Felsenstein 1995), we generated a genetic distance matrix using Nei's (1972) standard genetic distance from which an unrooted neighbour-joining tree was constructed (Saitou & Nei 1987). In order to establish statistical support for branch nodes, PHYLIP was used to generate 100 data sets from the original allele frequencies by bootstrapping over loci. These multiple data sets were used to create new distance matrices, neighbour-joining trees, and a consensus tree (Margush & McMorris 1981). Because haplotypes can differ by highly variable numbers of RAPD fragments, we used the RAPD mitochondria) 'allele' frequencies, rather than haplotype frequencies, to better represent genome similarity in phylogenetic analysis.

Results

Mitochondria) DNA enrichment

The mtDNA extraction protocol we used yielded =1 pg of enriched mtDNA from 10 g of packed suspension cultures. Mitochondria) DNA enrichment was only twofold greater than total genomic DNA isolated from embryogenic suspension cultures, but was more than 30-fold greater than enriched chloroplast DNA and total genomic DNA extracted from needle tissue. Chloroplast DNA enrichment was estimated to be only fourfold greater than for total genomic DNA from embryogenic suspension cultures and fivefold greater than total genomic DNA from seed megagametophytes, but was more than 25-fold greater than enriched mtDNA and total genomic DNA from needles and seed embryos. Enrichment ratios greater than 30-fold were difficult to quantify accurately due to low signal intensities in nonenriched tissues.

Ability of enriched mtDNA and cpDNA probes to identify genome-specific markers

The mtDNA probe hybridized to five RAPD fragments known to be maternally inherited, but showed no evidence of hybridization to three biparentally inherited RAPD markers (Aagaard *et al.* 1995). It did not detectably hybridize to the 7.8 kb ribosomal DNA sequence, hybridized weakly to the 3.0 kb actin gene sequence, and hybridized strongly to both the 1.6 kb chloroplast DNA clone and the coxIII mitochondria) sequence. The enriched chloroplast DNA probe hybridized strongly to the cpDNA clone, but showed no evidence of hybridization to the coxIII mitochondria) sequence or either of the nuclear gene sequences. It did not hybridize to either maternally inherited or biparentally inherited RAPD fragments.

Organelle DNAs among RAPD profiles

Sequential screening of RAPD markers unambiguously identified organelle fragments in most cases (Fig. 3). In our survey of 25 RAPD primers, we amplified 237 distinct RAPD fragments. Of these, 39 fragments (16%) hybridized to the enriched mtDNA probe and eight (3%) hybridized to the enriched chloroplast DNA probe (Table 1). For 16 of the 25 primers, at least a single fragment hybridized to the mtDNA probe. Of the eight RAPD markers identified with the chloroplast DNA probe, two also hybridized to the mtDNA probe. Most of the remaining RAPDs identified with the chloroplast DNA probe were difficult to reproduce in later RAPD amplifications, whereas RAPDs identified with the mtDNA probe were all reproducible.

Differentiation and diversity of nuclear and mitochondria) RAPD markers

Several fragments observed during screening of RAPD primers were too weak to score in all samples, and were

Primer	Sequence	No. of RAPD fragments		
		Total	mtDNA (%)	cpDNA (%)
OP-J1	CCC GGC ATA A	13	3 (23)	0
UBC 111	AGT AGA CGG G	6	0	0
UBC 114	TGA CCG AGA C	6	3 (50)	0
UBC 197	TCC CCG TTC C	14	2 (14)	0
UBC 234†	TCC ACG GAC G	9	2 (22)	0
UBC 264	TCC ACC GAG C	6	1 (17)	0
UBC 285	GGG CGC CTA G	6	2 (33)	0
UBC 304	AGT CCT CGC C	9	0	0
UBC 323	GAC ATC TCG C	13	1 (8)	1 (8)
UBC 327	ATA CGG CGT C	5	0	0
UBC 328†	ATG GCC TTA C	5	0	1* (20)
UBC 330†	GGT GGT TTC C	5	0	1* (20)
UBC 336†	GCC ACG GAG A	11	0	2 (18)
UBC 337	TCC CGA ACC G	10	3 (30)	2 (20)
UBC 341	CTG GGG CCG T	15	2 (13)	0
UBC 372	CCC ACT GAC G	13	0	0
UBC 387	CGC TGT CGC C	12	2 (17)	1 (8)
UBC 411†	GAG GCC CGT T	10	0	0
UBC 428†	GGC TGC GGT A	8	4 (50)	0
UBC 460†	ACT GAC CGG C	14	7 (50)	0
UBC 467	AGC ACG GGC A	12	3 (25)	0
UBC 497	GCA TAG TGC G	10	0	0
UBC 504†	ACC GTG CGT C	13	1 (8)	0
UBC 530	AAT AAC CGC C	3	2 (67)	0
UBC 570†	GGC CGC TAA T	9	1 (11)	0
Total		237	39 (16)	8 (3)

Table 1 RAPD primers, their sequences, the number of fragments identified from hybridization as putative mitochondrial (mtDNA) and chloroplast (cpDNA) markers, and the percentages of the total number of fragments amplified with each primer from two trees within each of the three races of Douglas fir (coastal, north interior, and south interior)

*RAPD fragments which hybridized to both the cpDNA and the mtDNA probes.
†RAPD primers used to amplify markers scored in the population study.

thus excluded from further analysis. In our population study, we selected 36 nuclear and 11 mitochondria) RAPD markers that were consistently amplified by nine primers. Five of the nuclear and two of the mitochondria) RAPD markers were monomorphic. We calculated diversity statistics based on the remaining 31 nuclear and nine mitochondria) RAPD markers.

Mean within-population diversity *(Hs)* was higher for nuclear than for mitochondria) RAPD markers (Table 2; 0.22 and 0.03, respectively), whereas total diversity (HT) was similar (0.32 and 0.28, respectively). Including monomorphic loci in the analysis reduced HS to 0.19 and 0.02 for nuclear and mitochondria) RAPD markers, respectively, and reduced HT to 0.27 and 0.23, respectively. Calculations of diversity parameters based on mitochondria) RAPD haplotypes resulted in *levels* of HS similar to that of nuclear RAPD markers *(Hs* = 0.24) and HT values that were more than threefold higher (HT = 0.86; Table 2). Haplotype diversity was highest in the Baldy Mountain population (HE = 0.58) where we found five mitochondria) haplotypes, and lowest in Wildcat Mountain, which was fixed for a single haplotype.

Differentiation among coastal populations of Douglas fir was low based on both nuclear and mitochondria) RAPD markers (GsT = 0.05 and 0.04, respectively; Table 3). Although nuclear RAPD differentiation among north interior populations was also low (GsT = 0.07), mitochondria) RAPD differentiation was high (GsT = 0.75). A similar trend was found among south interior populations (GsT = 0.25 and 0.84 for nuclear and mitochondria) RAPD markers, respectively). Overall, genetic differentiation was nearly threefold greater for mitochondria) than for nuclear RAPD markers (GsT = 0.91 and 0.34, respectively), and in both cases differentiation among races accounted for a majority of differentiation. These patterns were nearly identical when the AMOVA approach was used (over populations and races, (DST = 0.93, P = 0.002, and SST = 0.37, P = 0.002, for mitochondria) and nuclear RAPD markers, respectively).

Differentiation based on mitochondria) haplotypes was in most cases similar to calculations based on mitochondria) allele frequencies (Table 3). Haplotype differentiation and differentiation of mitochondria) allele frequencies were similar among coastal populations (GsT = 0.00 and 0.04, respectively), among north interior populations

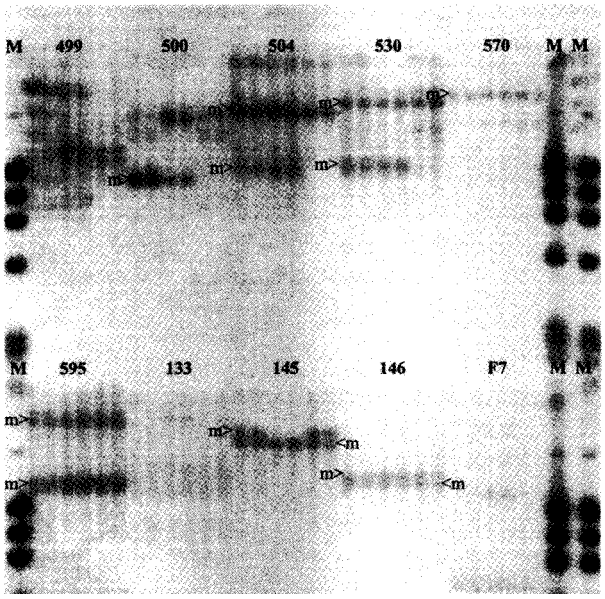

Fig. 3 Example of screening RAPDs for mitochondria) markers. The blot was probed using an enriched mtDNA preparation and standard Southern procedures in the second step of sequential screening. Nine UBC primers (499, 500, 504, 530, 570, 595, 133, 145, and 746) and one OPERON primer (F7) were used to amplify RAPDs from two trees from each of the three races of Douglas fir (coastal, north interior, and south interior, respectively; six trees per primer). RAPD markers scored as mitochondria) (m >) are based on signal intensity on the Southern, lack of background signal, and lack of hybridization when probed with the enriched chloroplast DNA probe. M is a DNA size marker with standard fragment lengths of 1353, 1078, 872, 603, and 310 by (0174 DNA/HaeIII digest).

(GsT = 0.71 and 0.75, respectively), and among south interior populations (GsT = 0.71 and 0.84, respectively). Overall, haplotype differentiation was lower than for mitochondria) allele frequencies (GsT = 0.74 vs. 0.91, respectively), due to the lower proportion of differentiation among races for mitochondria) haplotypes (GsT = 0.42 vs. 0.72 for mitochondria) allele frequencies).

Phylogeny of populations and races of Douglas fir

The pairs of Douglas fir populations from the three racial groups clustered together within races based on both nuclear and mitochondria) RAPD markers (Fig. 4). However, bootstrapping indicated that there is strong support only for the coastal race (both nuclear and mitochondria)) and the south interior race based on nuclear RAPD markers. The coastal and interior varieties (i.e. coastal vs. north and south interior together) were significant for both markers. Branch lengths appeared more heterogeneous for the mitochondria) markers than for the nuclear markers. Using populations from either the north or south interior race as an outgroup did not affect either the groupings identified or their degree of support for bootstrapping (data not shown).

Discussion

Evidence for the mitochondria) origin of RAPD markers

Our hybridizations to genes of known origin show that there was some nuclear and chloroplast DNA contamination present in our mtDNA preparations although it does

Table 2 Within-population nuclear and mitochondria) gene diversities (HF (± SE) and Hs), total diversity (HT) and their standard errors (SE) calculated in six populations from the three races of Douglas fir based only on polymorphic (31 and nine loci, respectively) and all (including monomorphic) RAPD markers (36 and 11 loci, respectively). Haplotype diversity was calculated from 14 mitochondria) haplotypes

Population (sample size)	Nuclear RAPD markers		Mitochondrial RAPD markers		
	Polymorphic	All	Polymorphic	All	Haplotypes
Coastal race					
Smith Creek (34)	0.27 ± 0.03	0.23 ± 0.03	0.01 ± 0.01	0.01 ± 0.01	0.12
Lacomb (22)	0.25 ± 0.04	0.21 ± 0.04	0.02 ± 0.02	0.02 ± 0.02	0.17
North interior race					
Wildcat Mountain (36)	0.27 ± 0.04	0.23 ± 0.04	0.00	0.00	0.00
Baldy Mountain (30)	0.24 ± 0.04	0.20 ± 0.04	0.08 ± 0.04	0.07 ± 0.04	0.58
South interior race					
Coronado (30)	0.15 ± 0.04	0.13 ± 0.03	0.02 ± 0.02	0.02 ± 0.01	0.19
Dixie (29)	0.15 ± 0.03	0.13 ± 0.03	0.04 ± 0.04	0.04 ± 0.03	0.39
Means H_S	0.22 ± 0.03	0.19 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.24
H_T	0.32 ± 0.03	0.27 ± 0.03	0.28 ± 0.07	0.23 ± 0.06	0.86

Table 3 Genetic differentiation (Nei's G_{ST} and standard genetic distance D) at different hierarchical levels in Douglas fir based on nuclear (31 loci) and mitochondrial (11 loci) RAPD markers, and mitochondrial RAPD haplotypes (14)

	Nuclear RAPD markers		Mitochondrial RAPD markers		
Hierarchical level	$G_{ST} \pm$ SE	D	$G_{ST} \pm$ SE	D	G_{ST} (haplotypes)
Within races*					
Coastal	0.05 ± 0.02	0.02	0.04 ± 0.03	0.00	0.00
North interior	0.07 ± 0.03	0.03	0.75 ± 0.52	0.13	0.71
South interior	0.25 ± 0.08	0.06	0.84 ± 0.15	0.21	0.71
Without regard to races†	0.34 ± 0.07	0.16	0.91 ± 0.03	0.08	0.74
Among populations within races†	0.05 ± 0.01		0.18 ± 0.09		0.29
Among races†	0.25 ± 0.07		0.72 ± 0.10		0.42

*Calculations of genetic differentiation among populations within races (G_{ST}) employed corrections for both small sample size (Nei 1978) and small number of populations (Nei 1986).
†Hierarchical analyses of the proportion of differentiation due to differences among races and populations employed only the correction for small sample size, as specified in Nei (1986).

not appear to have prevented us from identifying fragments of mitochondrial origin. The mtDNA probe hybridized weakly to a nuclear high-copy-number sequence (actin) and strongly to the 1.6 kb chloroplast DNA sequence. However, the low proportion of chloroplastDNA present in the enriched mtDNA probe relative to the enriched chloroplast DNA probe (= 3%), and the sequential use of both enriched organelle DNA probes in screening of RAPD blots, allowed us to exclude ambiguous RAPD fragments. The RAPD fragments which crosshybridized to both probes may have either resulted from chloroplast DNA contamination in the enriched mtDNA probe, or from chloroplast DNA sequences transferred to the mitochondrial genome (Blanchard & Schmidt 1995).

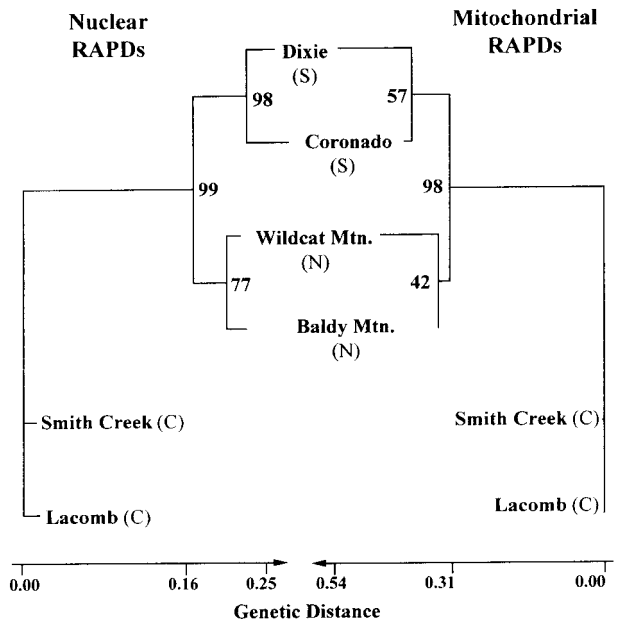

Fig. 4 Nuclear and mitochondrial RAPD phenograms. Nei's (1972) genetic distance was used to construct unrooted neighbour-joining trees using nitvL in (Felsenstein 1995) for six populations from the three races of Douglas fir (C = coastal; N = north interior; S = south interior) based on nuclear, and mitochondrial RAPD markers. Bootstrap values generated from bootstrapping over loci 100 times are listed at the branch nodes, indicating support for each Glade.

Two lines of evidence support our contention that the RAPD fragments we identified with the enriched mtDNA probe were amplified from the mitochondrial genome. First, hybridization results showed that our enriched mtDNA probe hybridized to all five RAPD fragments known to be maternally inherited (Aagaard *et al.* 1995), and only the mitochondrial genome is maternally inherited in Douglas fir (Marshall & Neale 1992). In contrast, three biparentally inherited RAPDs did not hybridize to the enriched mtDNA probe, and the enriched chloroplast DNA probe did not hybridize to either the maternally or biparentally inherited fragments.

Second, the selected mitochondrial RAPD fragments exhibited the population diversity patterns expected for mtDNA markers. Mitochondrial markers tend to show less diversity within populations than nuclear markers, and much greater differentiation among populations, than either nuclear or chloroplast DNA markers (Mitton 1994). For example, Strauss *et al.* (1993) found low levels of mitochondrial haplotype diversity within populations of the California closed-cone pine species based on RFLP haplotype diversity revealed by a coxI probe (mean HS = 0.07), but strong differentiation among populations (GsT varied between 0.75 and 0.96). Similarly Dong & Wagner (1993) found high differentiation among populations of subspecies of lodgepole pine based on RFLP haplotypes revealed by coxI and coxII RFLPs (FsT = 0.66).

In Douglas fir, maternally inherited RAPDs studied previously (Aagaard *et* al. 1995) showed that mitochondria) differentiation among races was high (GsT = 0.62 based on haplotype frequencies), similar to our findings in the present study (GsT = 0.74 for mitochondria) haplotypes). Although chloroplast DNA can also show strong differentiation among disjunct populations (Hong *et* al. 1993), this does not appear to be the case in Douglas fir (Ponoy *et* al. 1994). In addition, the small size and limited complexity of the Douglas fir chloroplast genome (Strauss *et* al. 1988; Hipkins *et* al. 1994), compared to that expected for the mitochondria) genome (see below), make it very unlikely that the many polymorphic mitochondria) RAPD markers we identified could be of chloroplast origin.

Unlike mitochondria) RAPDs, there is little evidence that many RAPD fragments originate in the chloroplast genome. Of the three reproducible markers that hybridized to the enriched chloroplast DNA probe, only one (UBC primer 328) showed polymorphism (interior races fixed for the dominant allele, coastal populations fixed for the null allele). Unfortunately, we were unable to verify its expected paternal inheritance (Neale *et* al. 1986) because the available pedigrees derived from racial hybrids were constructed from matriarchal lineages.

Abundance of mitochondria) RAPD markers in Douglas fir profiles

We found that 16% of all RAPD fragments amplified among races of Douglas fir hybridized strongly to the mtDNA probe, and are therefore likely to be of mitochondria) origin. A few other studies have similarly found that mtDNA sequences are amplified by RAPD primers. Probing with cloned organelle DNA sequences, Thormann & Osborne (1992) found that 5% of RAPD fragments amplified in Brassica were of mitochondria) origin, and less than 0.5% were of chloroplast origin. Similarly Lorenz *et* al. (1994) found mitochondria) RAPD fragments in greater proportion than chloroplast RAPD fragments based on inheritance studies in sugar beet. In most reports of strictly Mendelian inheritance for RAPD markers, such as Jermstad *et* al. (1994) in Douglas fir and Lu *et* al. (1995) in Scots pine, segregation of RAPD markers was studied in haploid megagametophytes of progeny from open pollinated crosses, or crosses between parents from close geographical proximity. Due to the expected low intrapopulation polymorphism of cytoplasmic markers, RAPDs of uniparental inheritance would be monomorphic among such crosses and thus their genomic origin remains cryptic.

There are two probable explanations for the higher proportion of mitochondria) RAPD fragments that we found in an earlier study of Douglas fir (Aagaard *et* al. 1995) compared with our present results. First, in the earlier study, where 45% of RAPDs exhibited maternal inheritance, we had primarily scored RAPD markers showing strong interracial differences. MtDNA characteristically exhibits interracial polymorphism (e.g. Strauss *et* al.1993), thus focusing on well-differentiated markers is likely to have preselected for RAPDs of mitochondria) origin. Second, in our earlier study we principally scored the most intense and strongly amplified RAPD fragments. Mitochondria) RAPDs often appeared to be the most intense bands on ethidium bromide-stained agarose gels. Lorenz *et* al. (1994) have also reported strong amplification of mitochondria) RAPDs relative to other RAPD fragments.

The size and complexity of the Douglas fir mitochondria) genome, as well as the expected prevalence of mtDNA in seeds, may contribute to the large number of mitochondria) RAPDs found. Mitochondria) genome size varies greatly in plants, and is often several hundred kb or larger (reviewed by Sederoff 1987). Although the size of the mitochondria) genome in Douglas fir is unknown, recent studies have indicated that it is in excess of 1 Mbp in the very closely related (Price *et* al. 1987) genus Larix (Kumar *et* al. 1995). This would provide a large number of sites for annealing of RAPD primers, especially as inverted repeats that promote RAPD amplification are common elements of mitochondria) sequences (Sederoff 1987). In addition, mtDNA is expected to be highly enriched in tissues such as seed embryos due to the need for rapid transcriptional capacity (Bendich 1987) and the formation of intergenomic mitochondria) molecules during intensive replication (Oldenburg & Bendich 1996). Seed embryo genomic DNA may therefore provide a template for RAPD amplification which is highly enriched in mtDNA.

Contrasting diversity and differentiation of nuclear and mitochondria) RAPD markers

We found that diversity within populations was sevenfold higher for nuclear than for mitochondria) RAPD markers despite comparable levels of total diversity (Table 2). Limited mitochondria) RAPD diversity within populations is expected based on limited dispersal and small effective population size of a maternally inherited haploid genome (reviewed by Birky et al. 1989). Periodic selection for advantageous mitochondria) mutations could also rapidly reduce mitochondria) diversity within populations due to the complete linkage of mitochondria) genes (Maruyama & Birky 1991).

The degree of population differentiation for mitochondria) vs. nuclear RAPD markers varied widely among regions. *GsT* was more than 10-fold higher than for nuclear RAPD markers among the north interior populations and more than threefold higher among south

interior populations (Table 3). In contrast, mitochondrial and nuclear differentiation were very similar among the coastal populations (GsT = 0.04 and 0.05, respectively). Although the geographical distance between populations of the north interior race is less than that between coastal populations, north interior populations are restricted to isolated mountain tops of the Ochoco (Wildcat) and Blue (Baldy) ranges in arid central and east-central Oregon (Fig. 2). Similarly, south interior populations are located in separate ranges of the Rocky Mountains of southern Utah and southern Arizona. Population fragmentation may therefore have predisposed populations of the interior variety to more rapid mitochondrial differentiation. The relative homogeneity of the coastal race may also be a consequence of a recent expansion from a single glacial refugium (located near the Willamette Valley of western Oregon during the Wisconsin glaciation; Tsukada 1982; Li & Adams 1989, although see Worona & Whitlock 1995). The higher susceptibility of the mitochondrial genome to the vagaries of genetic drift is reflected in its phylogenetic patterns, which show substantially higher variability in branch lengths for mitochondrial compared to nuclear RAPD markers among the interior populations (Fig. 4).

Mitochondrial diversity within populations calculated from allele frequencies was much lower than for mitochondrial haplotype diversity (Table 2). This is not surprising because diversity calculated from allele frequencies measures the average gene diversity within sections of the genome, whereas haplotype diversity is an integrated measure of variation over all mitochondrial genome regions, thus effectively treating the entire mitochondrial genome as a single locus. However, both measures give a consonant picture of the distribution of variation among vs. within populations. The lower proportion of racial haplotype differentiation in the present study compared to an earlier study (GsT = 0.42 and 0.73, respectively; Aagaard *et al.* 1995) probably results from our earlier scoring of mainly race-specific RAPD fragments based on maternal inheritance in interracial crosses. In contrast, sequential screening of RAPD fragments using enriched organelle DNAs should give a lessbiased picture of organelle diversity.

Utility of cytoplasmic markers in population and phylogenetic studies

Due to the high degree of mitochondrial differentiation among populations, mitochondrial-based phylogenetic trees have been proposed for use in identifying phylogenetically distinct populations for breeding and conservation (evolutionary significant units, ESUs; Avise *et al.* 1987; Moritz 1994). Our mitochondrial phylogenetic tree was concordant with the nuclear phylogenetic tree, suggesting that mtDNA can also be a useful phylogenetic indicator in Douglas fir. However, mitochondrial RFLP studies in pines (Strauss *et al.* 1993), including recent intensive RFLP analyses with a number of mtDNA probes (J. Wu, personal communication), have found that mtDNA markers can give very misleading phylogenies. This is not surprising given the low rates of point mutations and the high rate of structural rearrangements in mitochondrial genomes; the latter can give rise to convergent evolution of RFLP polymorphisms (Palmer 1992b). However, similar to our results, J. Wu found strong mtDNA differentiation among populations and also that mtDNA haplotypes were effective population-specific markers. Thus, although the phylogenetic value of mtDNA markers seems to vary widely, their strong differentiation among populations compared to nuclear markers will make them useful tools for identifying ESUs.

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References

Aagaard JE (1997) Genetic diversity and differentiation in Douglas-fir from RAPD markers of nuclear and mitochondrial origin. MSc Thesis, Oregon State University, Corvallis, USA.

Aagaard JE, Vollmer SS, Sorensen FC, Strauss SH (1995) Mitochondrial DNA products among RAPD profiles are frequent and strongly differentiated between races of Douglas fir. *Molecular Ecology, 4, 441-447.*

Apostol BL, Black IVWC, Miller BR, Reiter P, Beaty JB *(1993)* Estimation of family numbers at an oviposition site using RAPD-PCR markers: applications to the mosquito *Aedes aegypti. Theoretical and Applied Genetics, 86, 991-1000.*

Arnold J *(1993)* Cytonuclear disequilibria in hybrid zones. *Annual Review of Ecology and Systematics, 24, 521-554.*

Avise JC *(1994) Molecular Markers, Natural History, and Evolution.* Chapman and Hall, New York.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Niegel JE, Reeb CA, Saunders NC *(1987)* Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics, 18, 489-522.*

Bendich *Al (1987)* Why do chloroplasts and mitochondrial contain so many copies of their genome? *BioEssays, 6, 279-282.*

Birky CW, Fuerst P, Maruyama *T (1989)* Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics, 121, 613-627.*

Black IV WC (1996) *RAPDPLOT 2.4.* Department of Microbiology Colorado State University Fort Collins, Colorado.

Blanchard JL, Schmidt GW (1995) Pervasive migration of organellar DNA to the nucleus in plants. *Journal of Molecular Evolution,* 41, 397-406.

Critchfield WB (1984) Impact of the Pleistocene on the genetic structure of North American conifers. In: *Proceedings of the 8th North American Forest Biology Workshop* (ed. Lanner RM), pp. 70-118. Utah State University Logan, Utah.

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic noncoding regions of mitochondria) and chloroplast DNA in plants. *Molecular Ecology,* 4, 129-131.

Dong J, Wagner DB (1993) Taxonomic and population differentiation of mitochondria) diversity in *Pinus banksiana* and *Pinus contorta. Theoretical and Applied Genetics,* 86, 573-578.

El Mousadik A, Petit RI (1996) Chloroplast DNA phylogeography of the argan tree of Morocco. *Molecular Ecology,* 5, 547-555.

Excoffier L (1993) mNAtvtovn. Genetics and Biometry Laboratory, University of Geneva, Carouge, Switzerland.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondria) DNA restriction data. *Genetics,* 131, 479191.

Felsenstein J (1995) PHYLIP *(Phylageny Inference Package) version* 3.57c. Department of Genetics, University of Washington, Seattle, Washington.

Gupta PK, Dandekar AM, Durzan DJ (1988) Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts. *Plant Science,* 58, 85-92.

Hamrick JL, Godt MJ (1989) Allozyme diversity in plant species. In: *Plant Population Genetics, Breeding, and Genetic Resources* (eds Brown AHD, Clegg MT, Kahler AL, Weir BS), pp. 43-63. Sinauer Associates, Sunderland, Massachusetts.

Hiesel R, Cambettes B, Brennicke A (1994) Evidence for RNA editing in mitochondria of all major groups of land plants except the Bryophyta. *Proceedings of the National Academy of Science, USA,* 91, 629-633.

Hipkins VD (1993) Repeated sequences associated with inversions and length mutations in the chloroplast genomes of *Pinus* and *Pseudotsuga.* PhD Thesis, Oregon State University Corvallis, USA.

Hipkins VD, Krutovskii KV, Strauss SH (1994) Organelle genomes in conifers: structure, evolution, and diversity. *Forest Genetics,* 1,179-189.

Hong Y, Hipkins VD, Strauss SH (1993) Chloroplast DNA diversity among trees, populations and species in the California Closed-Cone Pines *(Pinus radiata, Pinus muricata* and *Pinus attenuata). Genetics,* 135,1187-1196.

Jermstad KD, Reem AM, Henifin JR, Wheeler NC, Neale DB (1994) Inheritance of restriction fragment length polymorphisms and random amplified polymorphic DNAs in coastal Douglas fir. *Theoretical and Applied Genetics,* 89, 758-766.

Kumar R, Lelu M, Small I (1995) Purification of mitochondria and mitochondria) nucleic acids from embryogenic suspension cultures of a gymnosperm, *Larix x leptoeuropaea. Plant Cell Reports,* 14, 534-538.

Lewis PO (1994) *GeneStat-PC* 3.3. Department of Statistics, North Carolina State University, Raleigh, North Carolina.

Li P, Adams WT (1989) Range-wide patterns of allozyme variation in Douglas fir *(Pseudotsuga menziesii). Canadian Journal of Forest Research,* 19,149-161.

Lorenz M, Weihe A, Borner T (1994) DNA fragments of organellar origin in random amplified polymorphic DNA (RAPD) patterns of sugar beet *(Beta vulgaris* L.). *Theoretical and Applied Genetics,* 88, 775-779.

Lu MZ, Szmidt AE, Wang XR (1995) Inheritance of RAPD fragments in haploid and diploid tissues of *Pinus sylvestris* (L.). *Heredity,* 74, 582-589.

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology,* 3, 91-99.

Margush T, McMorris FC (1981) Consensus n-trees. *Bulletin of Mathematical Biology,* 43, 239-244.

Marshall KA, Neale DB (1992) The inheritance of mitochondria) DNA in Douglas fir *(Pseudotsuga menziesii). Canadian Journal of Forest Research,* 22, 73-75.

Maruyama T, Birky CW (1991) Effects of periodic selection on gene diversity in organelle genomes and other systems without recombination. *Genetics,* 127, 449-451.

McCauley DE, Stevens JE, Peroni PA, Raveill JA (1996) The spatial distribution of chloroplast DNA and allozyme polymorphisms within a population of *Silene alba* (Caryophyllaceae). *American Journal of Botany,* 83, 727-731.

Mitton JB (1994) Molecular approaches to population biology. *Annual Review of Ecology and Systematics,* 25, 45-69.

Moritz C (1994) Applications of mitochondria) DNA analysis in conservation: a critical review. *Molecular Ecology,* 3, 401-411.

Neale DB, Adams WT (1985) The mating system in natural and shelterwood stands of Douglas fir. *Theoretical and Applied Genetics,* 71, 201-207.

Neale DB, Wheeler NC, Allard RW (1986) Paternal inheritance of chloroplast DNA in Douglas fir. *Canadian Journal of Forest Research,* 16,1152-1154.

Nei M (1972) Genetic distance between populations. *The **American Naturalist,*** 106, 283-292.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science, USA,* 70, 3321-3323.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics,* 89, 583-590.

Nei M (1986) Definition and estimation of fixation indices. *Evolution,* 40, 643-645.

Oldenburg DJ, Bendich AJ (1996) Size and structure of replicating mitochondria) DNA in cultured tobacco cells. *The Plant Cell,* 8, 447-461.

Palmer JD (1992a) Organelle DNA isolation and RFLP analysis. In: *Plant Genomes: Methods for Genetic and Physical Mapping* (eds Beckman J, Osborn TC), pp. 35-53. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Palmer JD (1992b) Mitochondria) DNA in plant systematics: applications and limitations. In: *Molecular Systematics of Plants* (eds Soltis PS, Solos DE, Doyle JJ), pp. 36-49. Chapman and Hall, New York, London.

Ponoy B, Hong YP, Woods J, Jaquish B, Carlson JE (1994) Chloroplast DNA diversity of Douglas fir in British Columbia. *Canadian Journal of Forest Research,* 24, 1824-1834.

Powell JR, Morgante M, McDevitt R, Vendramin GG, Rafalski JA (1995) Polymorphic simple sequence repeat regions in chloroplast genomes: applications to the population genetics of pines. *Proceedings of the National Academy of Science, USA,* 92, 7759-7763.

Price RA, Olsen-Stoikovich J, Lowenstein JM (1987) Relationships among the genera of Pinaceae: an immunologieal comparison. *Systematic Botany,* 12, 91-97.

Rehfeldt GE (1977) Growth and cold hardiness of intervarietal hybrids of Douglas fir. *Theoretical and Applied Genetics, 50,* 3-15.

von Rudloff E (1972) Chemosystematic studies in the genus *Pseudotsuga. I.* Leaf oil analysis of the coastal and Rocky Mountain varieties of the Douglas fir. *Canadian Journal of Botany,* 50,1025-1040.

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and* Evolution, 4, 406-425.

Sederoff RR (1987) Molecular mechanisms of mitochondria) genome evolution in higher plants. *The American Naturalist,* 130, S30-S45.

Shah DM, Hightower RC, Meagher RB (1982) Complete nucleotide sequence of a soybean actin gene. *Proceedings of the* National *Academy of Science, USA,* 79,1022-1026.

Strauss SH, Palmer JD, Howe G, Doerksen A (1988) Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. *Proceedings of the National Academy of Science, USA,* 85, 3898-3902.

Strauss SH, Hong YP, Hipkins VD (1993) High levels of population differentiation for mitochondria) DNA haplotypes in *Pinus radiata,* muricata, and *attenuata. Theoretical and Applied Genetics,* 86, 60511.

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology,* 17,1105-1109.

Thormann CE, Osborn TC (1992) Use of RAPD and RFLP markers for germplasm evaluation. In: *Proceedings of the* Symposium: *Applications of RAPD Technology to Plant Breeding,* pp. 9-11. Joint Plant Breeding Symposia Series, Minneapolis, Minnesota.

Tsukada M (1982) *Pseudotsuga menziesii* (Mirb.) Franco: its pollen dispersal and later quaternary history in the Pacific Northwest. *Japanese Journal of* Ecology, 32, 159-187.

Varsanyi-Breiner A, Gusella JF, Keys C, Housman D, Sullivan D, Brisson N, Verma DS (1979) The organization of a nuclear DNA sequence from a higher plant: molecular cloning and characterization of soybean ribosomal DNA. *Gene,* 7, 317-334.

Vicario F, Vendramin GG, Rossi P, Lio P, Gianini R (1995) Allozyme, chloroplast DNA and RAPD markers for determining genetic relationships between *Abies alba* and the relic population of *Abies nebrodensis. Theoretical and Applied Genetics,* 90, 1012-1018.

Wagner DB (1992) Nuclear, chloroplast, and mitochondria) DNA polymorphisms as biochemical markers in population genetic analyses of forest trees. New Forests, 6, 373-390.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution, 38,1358-1370.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic *Acids Research, 18,* 6531-6535.

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