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Chloroplast DNA in *Pinus monticola*

2. Survey of within-species variability and detection of heteroplasmic individuals

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Summary. Within-species variability of a restriction site in the chloroplast (cp) DNA in *Pinus monticola* has been surveyed. Frequencies of two variants of the cp genome are significantly different in interior versus coastal populations. Paternal inheritance of the cp genome predominates, though some individuals have both variants of the genome. The presence of heteroplasmic individuals indicates occasional biparental inheritance.

Key words: Species variability – Western white pine – *Pinus monticola* – Heteroplasmic trees – Chloroplast DNA

Introduction

Western white pine, *Pinus monticola* Dougl. ex D. Don, has a high growth rate and excellent wood qualities. However, its use in reforestation in western North America is prevented by its susceptibility to white pine blister rust, *Cronartium ribicola* J. C. Fisch, a fungal pathogen introduced to western Canada at the turn of this century. A program to breed white pine with increased resistance to blister rust has been in existence in the U.S. for some time (Bingham 1983) and a similar program has been established recently in western Canada (Hunt et al. 1987).

Information on the population structure of western white pine would be useful in determining the number and size of breeding zones required for this program. Some western Canadian species, e.g. Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] and lodgepole pine (*Pinus contorta*), show marked biological and physiological adaptation, which limits the usefulness of coastal seed inland and vice versa. Western white pine in British Columbia is separated into coastal and interior wet-belt populations with about 200 km of drier area between them (Fig. 1). Data from provenance trials to determine whether this geographic separation is accompanied by adaptive differentiation are limited. Indirect evidence provided by studies of isozyme (Steinhoff et al. 1983) and terpene (Hunt and von Rudloff 1977) variation indicates a high degree of within-population variation without strong between-population differentiation, though the terpene data indicate some clustering of Vancouver Island populations.

Variability in DNA sequences detected as variability in the size of fragments produced by restriction endonucleases provides another measure of species variation (Beckmann and Soller 1983; Helentjaris et al. 1985). The degree of diversity of cpDNA in angiosperm species is less than that of animal mitochondrial DNA or plant nuclear DNA, which may limit the utility of cpDNA analysis in studies of angiosperm population structure (Palmer 1987). However, conifer chloroplast genomes for which data are available differ from those of angiosperms; they lack a large inverted repeat region and probably contain dispersed repeats (Lidholm et al. 1988; Strauss et al. 1988; Tsai and Strauss 1988; White 1989). Both of these characteristics have been associated with increased cp genome variability due to structural rearrangements (Palmer and Thompson 1981). Previous work has shown that *P. monticola* also lacks the inverted repeat and may contain dispersed repeats (White 1989).

This study was undertaken to assess the usefulness of cpDNA variation at a restriction site in analyzing population structure in western white pine. For this purpose, the distribution of variant genomes in individual trees from eight populations in each of the coastal and interior white pine regions in British Columbia were examined.

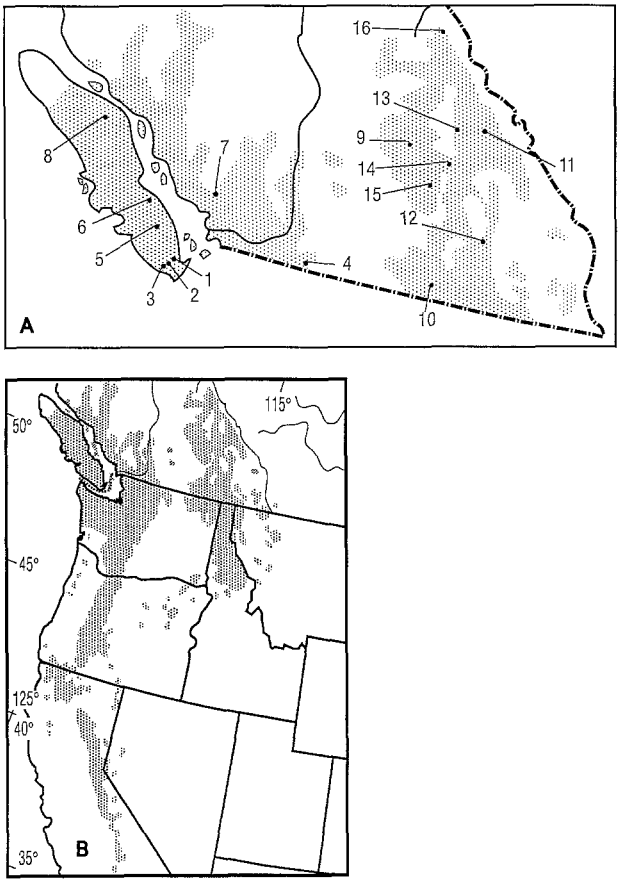

Fig. 1. **A** Distribution of *Pinus monticola* in British Columbia. Locations of the populations listed in Table 1 are indicated by *numbers*. **B** Distribution of *P. monticola* in North America (Source: Little 1971)

Methods and materials

Provenance survey

Seeds were collected from trees of eight coastal and eight interior populations. Collections were made from trees a minimum of 20 m apart in native stands with white pine distributed throughout the stand (M. Meagher, personal communication). Location of collections is shown in Fig. 1. Seeds were grown for two seasons, and crude total DNA was extracted individually from two offspring of four to five parent trees per population (total of eight or ten trees per population). In some cases, extractions were performed with seedlings which had completed only one season's growth.

Crude total DNA was obtained from cell homogenates in buffered osmoticum (White 1986) by pelleting the homogenate of 5–10 g of needles at 3,000 × g for 5 min, suspending the pellet in PTE containing 1 mg/ml protease, (approximately 4 ml/pellet from 10 g tissue), adding 10 µl/ml of 10% Triton-X-100, and digesting at room temperature for 2–3 h. CsCl was added at 1 g/ml, 100 µl/ml of 10 mg/ml ethidium bromide was added, the refractive index was adjusted to 1.3870–1.3910 and samples were centrifuged at 270,000 × g overnight in a vertical rotor. The DNA band was removed and dialyzed against three changes of TE buffer in the cold over a period of 20 h.

Crude DNA was digested with SalI plus BamHI and SalI plus PstI, and Southern blots were prepared and probed with a cloned 4.3-kb HindIII fragment of white pine cpDNA as described previously (White 1989). Frequency of trees with major hybridizations to normal or variant fragments were scored. Frequencies in coastal versus interior populations were subjected to the Wilcoxon-Mann-Whitney two-sample test of location (Steel and Torrie 1980).

Heteroplasmy

To determine whether the presence of both normal and variant hybridizations was due to incomplete digestion in trees which showed both, or whether these trees were truly heteroplasmic, the time course of digestion was examined. Approximately 12 µg crude total DNA was digested in a total reaction volume of 140 µl, with 1 µl each of 10 units/µl PstI and SalI or with the same amount of BamHI and SalI. After 10 min, 0.5, 1, 2, 3, 4 and 5 h, 20-µl aliquots were removed and 5 µl of gel loading buffer was added. More enzyme (10 units) was added to the digestions at 1-h intervals. Stopped aliquots were held at 5 °C until they were loaded on gels and separated electrophoretically as described previously.

Results

Provenance survey

Description of variable restriction site. In the previous study, a 4.3-kb HindIII fragment of white pine cpDNA was cloned and located near one end of the 21.1-kb Sal fragment (White 1989). This fragment, designated 1-1E, was found to show variable hybridization patterns in some trees. In most trees it hybridized to a 6.3-kb Pst/Sal fragment and a 6.9-kb Bam/Sal fragment ("normal" hybridizations). However in some trees it hybridized to 3.7-kb and 2.6-kb Pst/Sal fragments and 6.2-kb and 0.7-kb Bam/Sal fragments ("variant" hybridizations). Figure 2 shows a physical map consistent with these results in which some trees have an additional Sal site in the 21.1-kb Sal fragment, 2.6 kb from the site separating this fragment from the 3.9-kb Sal fragment. The cloned fragment 1-1E provided a convenient probe for identifying which trees had cp genomes containing the variable site.

Frequency of variant cp genome in populations. The frequency of trees with the variant cp genome, i.e. trees in which the major hybridization of 1-1E was to variant fragments, is given in Table 1.

Heteroplasmy

In extracts of some trees, 1-1E hybridized both to "normal" (6.3-kb and 6.9-kb) and "variant" (3.7- and 2.6-kb and 6.2- and 0.7-kb) fragments. These hybridization signals were consistent and did not change after prolonged digestion. The rate of increase of hybridization to clone 1-1E of fragments from an extract showing strong variant hybridization and weak normal hybridization is illustrated in Fig. 3. The normal and variant hybridizations have parallel kinetics, increasing to a maximum level

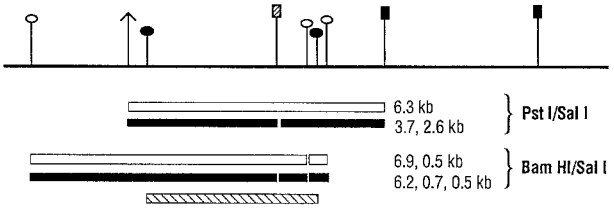

Fig. 2. Detail of restriction map near variable SalI site. *Open bars* indicate "normal" fragments hybridizing to probe, *solid bars* indicate "variant" fragments. *Hatched bar* shows position of probe (4.3-kb Hind fragment). ■ = SalI sites; ▨ = variable SalI site; ↑ = PstI sites; ○ = BamHI sites; ● = HindIII sites

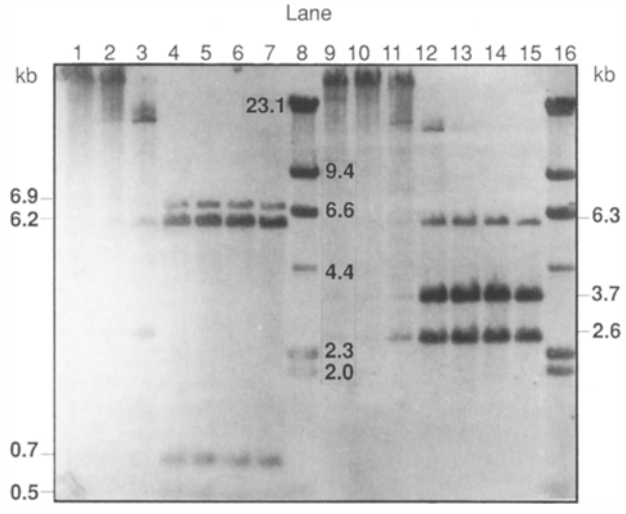

Fig. 3. Time course of digestion on DNA from a single seedling with both chloroplast genomes. *Lanes 1–8:* digested with BamHI plus SalI. *Lanes 9–15*: digested with PstI plus SalI. *Lanes 8 and 16* are size standards. Sample lanes, l–r, after 10 min, 0.5 h, 1, 2, 3 and 4 h digestion

after 2 or 3 h of digestion. Hybridizations attributable to incomplete digestion are strong for the first hour and then disappear.

Hybridization intensities were not equal when the normal and variant fragments occurred in the same tree; either the variant or normal genome predominated. Considerably more trees with the variant genome predominating were heteroplasmic than trees with the normal genome. Of the 20 trees in which the intensity of the variant bands was strongest, 10 had weak normal fragment signals. Of 124 trees in which the intensity of the normal fragments was strongest, only 10 had weak variant fragment signals.

Discussion

Provenance survey

Variable restriction site. The 4.3-kb cloned HindIII fragment was homologous to a region of the 21.1-kb SalI fragment, which in some trees contained an extra SalI site, presumably due to a single base substitution generating the new recognition site. In *Pisum, Lycopersicon-Solanum* and *Brassica,* the rate of parallel or back mutation at a restriction site has been shown to be low in comparisons of cpDNA variation (Palmer et al. 1985). Hence, shared restriction site variants are likely to indicate shared ancestry in trees containing them.

Frequency of variable cp genome in populations. The occurrence of cp genomes with the variant SalI site was significantly higher in interior populations of white pine than in coastal populations. The data in Table 1 show all

Table 1. Frequency of trees with variant cp genomes. Location numbers correspond to those on Fig. 1A

Coastal			Interior		
Location	Population	Frequency	Location	Population	Frequency
1	Bamberton	0	9	Barriere	0
2	Butchart Lake	0	10	Christina Lake	0.3
3	Butler Main	0	11	Heather	0.3
4	Manning Park	0	12	Lyall Creek	0.25
5	McKay	0	13	Mount Revelstoke	0.125
6	North West Bay	0.1	14	Perry River	0.4
7	Whistler	0.4	15	Salmon Arm	0.125
8	Woss/Davies	0	16	Valemont	0.1

Analysis	Rank sum	Populations	Cases
Interior	87	8	76
Coastal	49	8	68
	Z = 2.00		P(Z) = 0.0456

but one of the interior populations had trees with the variant, while most coastal populations lacked it entirely. Of the remaining coastal populations, most had a low frequency of trees with the variant. The exception was the Whistler population, from the upper end of a fjord valley leading to the interior plateau of British Columbia. Differentiation of subpopulations would result in the abnormally distributed frequencies of the variant evident within the coastal area populations. Further sampling and survey of the presence of this and other cp genome variants may give evidence about the degree of differentiation of coastal and interior white pine, whether and what subpopulation patterns occur and how they are affected by pollen dispersal. The present data indicate there are differences in the pollen cloud of coastal and interior western white pine.

Heteroplasmy

In the majority of conifers for which data are available, cpDNA is predominantly paternally inherited (Neale et al. 1986, 1988; Szmidt et al. 1987; Wagner et al. 1987). This also appears to be the case in *P. monticola,* since offspring of the same mother tree frequently had different variants of the cp genome (22% of pairs of open-pollinated siblings).

Some seedlings had both variants. Prolonged digestion did not eliminate hybridization of the probe to the larger fragment nor increase the intensity of hybridization to the two smaller fragments, indicating the signals were not due to incomplete digestion but to the presence of both genomes in those seedlings. The occurrence of heteroplasmic trees could be the result of biparental inheritance from trees with different genomes, heteroplasmic gametes, or somatic mutation. This last possibility is less likely, given a low incidence of parallel or back mutation in cpDNA (Palmer et al. 1985), since the variants observed were always the same. The trees were all sampled in their first or second growing season. The possibility of biparental cpDNA inheritance has been raised in *P. contorta* × *P. banksiana* putative hybrids (Govindaraju et al. 1988). Biparental inheritance in the trees examined here is consistent with the observation that mixed genomes were detected in proportion to the frequency of the alternate genome. Thus, in the trees with the normal genome predominating, only 8% were heteroplasmic, while in the trees with the variant genome predominating, 50% also had the normal genome, in proportion to the much higher frequency of the normal genome.

Hybridization intensities indicated that one genome always predominated in heteroplasmic trees. Whether somatic segregation would reduce the degree of heteroplasmy in older trees could not be determined, since the trees were small and destroyed in sampling.

Concluding remarks

Restriction fragment length polymorphisms (RFLPs) in cpDNA have potential as genetic markers in studies of population variation, especially in species such as western white pine in which variation in other biochemical markers is high. RFLPs due to variation at a restriction site can be rapidly screened in Southern blots of crude DNA extracts from a small amount of tissue once a cloned probe covering the variable site is available. Information on the physical location of the site prevents confusion should the probe have non-chloroplast DNA homologies (Stern and Palmer 1984; Ayliffe et al. 1988). Significant maternal contribution to cpDNA inheritance in western white pine can be detected in trees with a low frequency variant of the chloroplast genome.

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