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Classifying seedlots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA

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Summary. Chloroplast DNA (cpDNA) restriction analysis was used to classify five reforestation seedlots as to species. The material included two Sitka spruce (*Picea sitchensis* (Bong.) Carr.), one white spruce (*P. glauca* (Moench) Voss) from interior British Columbia, and two putative hybrid seedlots from the coast-interior introgression zone in British Columbia. The cpDNA patterns generated by Bam-HI and Bcl-I from individual trees of Sitka spruce, white spruce, western white spruce (*P. glauca* var. *albertiana* (S. Brown)), and Engelmann spruce (*P. engelmanni* (Parry)) were species-specific. They were used as reference patterns for comparisons. In addition, two controlled crosses between white and Sitka spruce were analyzed to demonstrate the paternal inheritance of cpDNA in spruces. The cpDNA restriction patterns for the five seedlots were obtained from composite samples of seedlings from each lot and compared to the typical cpDNA patterns of each species. Restriction patterns for the two Sitka spruce seedlots agreed with those from the Sitka spruce tree, while patterns for the white spruce seedlots from British Columbia agreed with those from the white spruce tree, lacking evidence of any Engelmann spruce component in the sample. On the other hand, one putative hybrid seedlot showed cpDNA patterns similar to white spruce while the other showed fragments unique to both Sitka and white spruce, indicating that this was a hybrid seedlot. The analysis of cpDNA restriction polymorphism has proven to be an effective tool for classifying seedlots in regions of introgression. To our knowledge, these results provide the first demonstration of the use of cpDNA analysis for solving practical forestry problems.

Key words: *Picea sitchensis* – *P. glauca* – *P. engelmanni* – Introgression – cpDNA restriction analysis

Introduction

Natural stand seed collections of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and white spruce (*Picea glauca* (Moench) Voss) provide the majority of seed sown in British Columbia nurseries for the production of seedlings used in artificial regeneration programmes of these two species. The natural ranges of Sitka spruce and white spruce overlap along river drainages such as the Skeena, Nass, and Bulkley (Roche 1969). The presence of the two species in sympatric populations and the apparent lack of reproductive barriers to hybridization have permitted the creation of several introgression zones in these regions (Daubenmire 1968; Roche 1969). There is no satisfactory method available at present to identify hybrid populations.

The chloroplast DNA (cpDNA) has been extensively studied in many angiosperms (for review, see Palmer 1985). It is well known that in angiosperms, the cpDNA genome is usually inherited from the female parent (Palmer 1985). Another typical feature of cpDNA is its evolutionary conservatism (Palmer 1985 and many others).

Recently, the cpDNA of gymnosperms has also become accessible to study owing to the extraction protocols of White (1986) and Szmidt et al. (1986). Analyses of cpDNA inheritance in some gymnosperms have revealed that, in contrast to angiosperms, it is contributed by the male parent (Neale et al. 1986; Szmidt et al. 1987). The use of restriction analysis of cpDNA in conifers for spe-

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cies indentification and phylogenetic studies has also been suggested (Neale et al. 1986; Szmidt et al. 1987; Wagner et al. 1987).

In this study, the restriction analysis of the chloroplast DNA has been used to classify composite seed samples as to Sitka spruce, white spruce or their hybrids. Furthermore, typical Sitka and white spruce composite samples were compared to cpDNA obtained from individual trees of Sitka spruce (*Picea sitchensis* (Bong.) Carr.), white spruce (*P. glauca* (Moench) Voss) western white spruce (*P. glauca* var. *albertiana* (S. Brown)) and Engelmann spruce (*P. engelmanni*, Parry). Controlled crosses between Sitka and white spruces were used to determine the mode of cpDNA inheritance.

Material and methods

Plant and seed material

Seeds used in this study were taken from bulk natural stand collections, collected by the Silviculture Branch of British Columbia Ministry of Forests and Lands. This included two Sitka spruce seedlots, one "interior spruce" seedlot and two seedlots from regions of introgression (putative hybrids). The term "interior spruce" is the operational name for both white and Engelmann spruce in British Columbia (B.C.), because of their similar nursery cultural regimes and planting sites. In addition, fresh needle samples of Sitka, western white, white, and Engelmann spruce and two Sitka × white spruce crosses (made by the late C. Syrach-Larsen) were collected from individual standing trees in the Hørsholm Arboretum in Denmark. The location, latitude, longitude and elevation for the five seedlots are listed in Table 1. Table 2 shows the origin of the spruce trees from Hørsholm.

Seedlots were individually sown in plastic trays (60 × 40 × 10 cm). The trays were filled with pure peat and placed in a greenhouse. Greenhouse day time/nighttime temperatures were maintained at 25 °C and 15 °C, respectively, with an 18 h photoperiod. Relative humidity was maintained at 70%–90%. The trays were irrigated daily and fertilized each week with Superba liquid fertilizer (Supra Ceres, Landskrona, Sweden) three weeks after germination.

Four months after gemination, 40–60 individual seedlings of each seedlot were harvested and bulked to provide a composite sample of fresh needles needed for the cpDNA extraction.

CpDNA isolation

Chloroplast DNA was extracted from needles according to the method described by Szmidt et al. (1986). This method represented a modified version of White's (1986) extraction protocol. In summary, needles (100 g, fresh weight) were frozen in liquid nitrogen and ground to powder. The powder was mixed with 350 ml of extraction solution, filtered and washed by centrifugation. The chloroplasts were separated by sucrose step-gradient centrifugation and lysed. CpDNA was further purified on CsCl gradient, dialyzed and used for restriction analysis.

Restriction endonuclease analysis

Four restriction endonucleases, Bam-HI, Bcl-I, Kpn-I and Sac-I (Boehringer, Mannheim, FRG), each of which recognizes a specific six base pair nucleotide sequence for cleavage, were used. Electrophoresis of restriction fragments was carried out for 16 h in 0.8% agarose gels (Maniatis et al. 1982).

Table 1. Location of the five British Columbia seedlots

Species	B.C. seed-lot no.	Location	Lat. (°N)	Long. (°W)	Alt. (m)
Sitka spruce	4115	Prince Rupert	54	130	20
Sitka spruce	4127	Queen Charlotte Islands	53	132	244
Putative hybrid	4189	Kitwanga	55	128	380
Putative hybrid	3964	Kispiox River	55	127	350
"Interior spruce"	3999	Dungate Creek	54	126	785

Table 2. Origin of the individual spruce trees from Hørsholm

Species	Origin
Sitka spruce	Danish plantation (seed source: Washington, USA)
Sitka spruce × white spruce	Controlled cross performed at Hørsholm *
(Sitka × white) × white spruce	Controlled cross performed at Hørsholm *
Western white spruce	Fairbanks/AK, USA
White spruce	Yukon, Canada
Engelmann spruce	Colorado, USA

* The crosses were made by the late C. Syrach-Larsen; the origin of the parental trees is not known

The gels were then photographed in UV light using 665/667 film (Polaroid). The size of individual cpDNA fragments was determined based on an algorithm described by Schaffer and Sederoff (1981).

Results and discussion

Differences among species

Identical cpDNA restriction patterns were observed among the different species when Kpn-I and Sac-I were used. Therefore, these two endonucleases were excluded from the discussion.

Restriction patterns and the size of restriction fragments (in kb) generated by Bam-HI for the spruce species and the five seedlots are presented in Fig. 1. Three distinct phenotypes were observed. The Sitka spruce was characterized by the presence of three unique fragments (6.79, 5.75 and 4.61 kb), while the Engelmann, white and western white spruce were characterized by two specific fragments (10.19 and 6.41 kb). It was not possible to differentiate between the Engelmann and white spruce due to their identical fragment patterns (Fig. 1). White

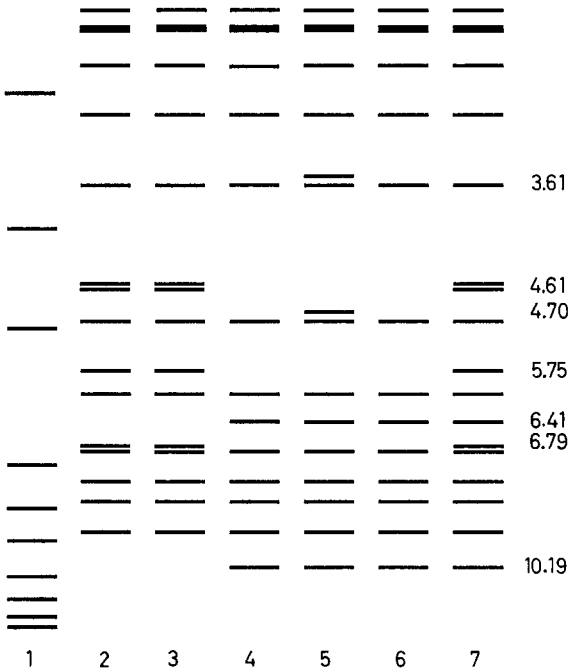

Fig. 1. Schematic drawing illustrating cpDNA restriction patterns from three spruce species and putative hybrid seedlot 4189 generated by Bam-HI. *Lane 1*: 1 kb ladder, (BRL); *lane 2*: Sitka spruce, 4115; *lane 3*: Sitka spruce, Hørsholm; *lane 4*: white spruce, Hørsholm; *lane 5*: western white spruce, Hørsholm; *lane 6*: Engelmann spruce, Hørsholm; *lane 7*: putative hybrid, 4189

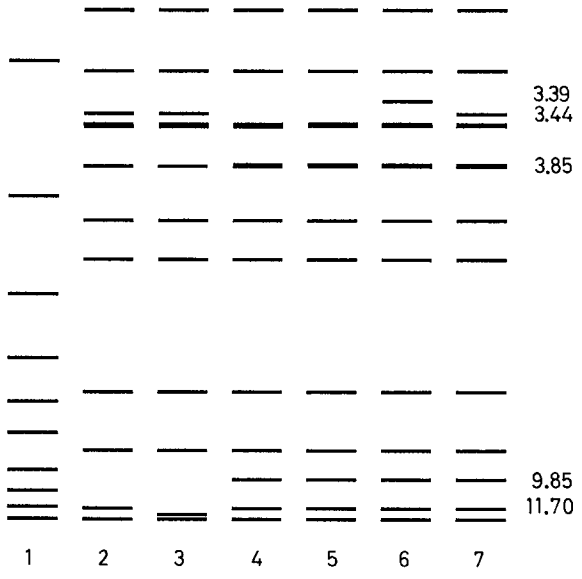

Fig. 2. Schematic drawing illustrating cpDNA restriction patterns from three spruce species and putative hybrid seedlot 4189 generated by Bcl-I. *Lane 1*: 1 kb ladder, (BRL); *lane 2*: Sitka spruce, 4115; *lane 3*: Sitka spruce, Hørsholm; *lane 4*: white spruce, Hørsholm; *lane 5*: western white spruce, Hørsholm; *lane 6*: Engelmann spruce, Hørsholm; *lane 7*: putative hybrid, 4189

and western white, of the other hand, were easily differentiated from each other based on two fragments (4.70 and 3.61 kb) that were unique to western white spruce.

The seedlots 4115 and 4127 were not different from one another and conformed to the restriction pattern observed for the single tree from Hørsholm (Fig. 1). The seedlot 3999 showed a restriction pattern different from the two B. C. Sitka samples and was identical to both white and Engelmann spruce samples from Hørsholm (Fig. 1).

Bcl-I restriction patterns and the size of restriction fragments are presented in Fig. 2. Three distinct phenotypes were observed. Sitka spruce was uniquely identified by the presence of one fragment (3.44 kb) that is not present in the white, western white or Engelmann spruces. Engelmann spruce was unique due to the presence of a specific fragment (3.39 kb) that was not present in other samples. Thus, Engelmann spruce was successfully separated from white spruce. White and western white spruce were not different from each other, but were easily identifiable from both Sitka and Engelmann spruce.

Differences were observed in restriction patterns between the two seedlots (4115, 4127) and the Sitka spruce individual when Bcl-I was used. The Sitka spruce individual had one unique fragment (11.70 kb) which was not present in any other sample. The 3999 seedlot was identical to the Hørsholm white spruce tree (Fig. 2).

The conformity of B.C. seedlot 3999 to the typical white spruce restriction pattern strongly suggests the absence of an Engelmann spruce component in that seedlot.

Mode of cpDNA inheritance

One Sitka × white spruce-controlled cross as well as a backcross ((Sitka × white) × white) were assayed for their restriction patterns. They gave phenotypes identical to the Hørsholm white spruce, indicating paternal inheritance of the cpDNA (data not shown).

Hybrid seedlot identification

The identification of the two spruce hybrid seedlots is based on three assumptions: (1) the mode of inheritance of cpDNA is paternal; (2) the reference seedlots (i.e. B.C. Sitka and white) are identified with certainty, and (3) hybrid seedlots should show a mixture of restriction fragments that are unique to each species concurrently, due to the presence of both species' pollen in the introgression zone.

The conformity of the reference seedlots to their typical cpDNA form is also evidence of absence of introgressive hybridization as well as low intra-populational variation, since they were extracted from composite samples of 40–60 individuals.

The restriction patterns for hybrid seedlots 3964 and 4189 that were generated by Bam-HI are presented in Figs. 1 and 3. Seedlot 3964 showed patterns identical to those present for seedlot 3999 (white spruce, B.C.), indicating that this seedlot is typical white spruce. Yeh and Arnott (1986), using electrophoretic and morphological data, classified this seedlot as white spruce (based on isozyme data) and as an intermediate (based on budset characteristics and needle serration). Seedlot 4189, on the other hand, showed fragments that were typical for both species, indicating that this seedlot was from a zone of introgression, conforming to our assumption of the concurrent appearance of both types in a composite sample. Yeh and Arnott (1986) classified this seedlot as being Sitka spruce (based on isozyme data and the degree of needle serration), but as an intermediate between Sitka and white spruce for terminal bud formation.

The restriction patterns generated by Bcl-I for these two hybrid seedlots are presented in Figs. 2 and 3. Similar to the fragment patterns obtained from the Bam-HI, the Bcl-I has confirmed our observations and conclusion regarding the classification of these two seedlots. Seedlot 3964 exhibits a banding pattern typical of white spruce, whereas seedlot 4189 shows a restriction pattern containing fragments unique to both Sitka and white spruce.

Although Yeh and Arnott's (1986) electrophoretic and morphological data were in close agreement, our results concur with their classification of seedlot 3964, but not 4189. It must be emphasized that the electrophoretic data are subject to the absence of unique allozyme markers (Copes and Beckwith 1977) and that the morphological data are subject to environmental influences (Falkenhagen and Nash 1978). Seedlot classification based on allozyme frequencies is also subject to the influence of sample size (El-Kassaby and Sziklai 1983) and the proportion of each species in the sample analyzed. Employing isozyme analyses of small samples from seedlots containing disproportionate amounts of one species over the other will lead to biased estimates of the actual proportions of each species in these seedlots. In cases where one species dominates the sample analyzed, the seedlot will be classified as to the dominant species. In cpDNA restriction analysis of composite samples, the only criterion is the presence or absence of fragments unique to each species.

The fact that introgression zones contain a mixture of F_1 hybrids, backcrosses to the two parent species and F_2 hybrids makes both isozyme markers and morphological traits unqualified for classifying seedlots. Furthermore, Yeh and Arnott (1986) employed isozyme analysis of the megagametophyte for their seedlot classification. This method, however, was only effective for identifying F_1 hybrids. Megagametophyte data alone would not allow the detection of pollen contribution in the samples analyzed if introgression was due to pollen flow from a stand

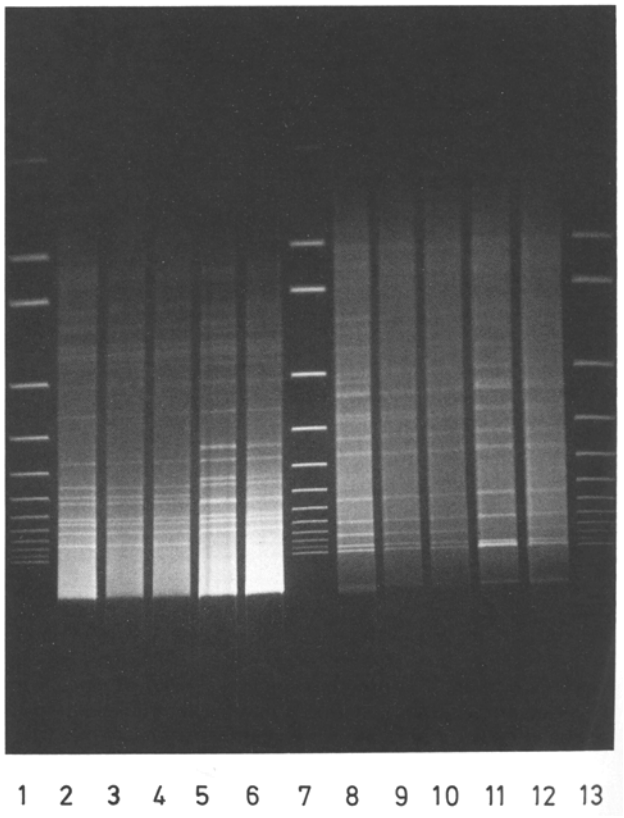

Fig. 3. Restriction fragments of cpDNA from three spruce species and composite seedlots separated by 0.8% agarose electrophoresis. *Lanes 2–6*: Bam-HI; *lanes 8–12*: Bcl-I. *Lane 1*: 1 kb ladder (BRL); *Lane 2*: Engelmann spruce; *lane 3*: white spruce; *lane 4*: putative hybrid seedlot 3964; *lane 5*: Sitka spruce 4115; *lane 6*: putative hybrid seedlot 4189; *lane 7*: 1 kb ladder (BRL); *lane 8*: Engelmann spruce; *lane 9*: with spruce; *lane 10*: putative hybrid seedlot 3964; *lane 11*: Sitka spruce 4115; *lane 12*: putative hybrid seedlot 4189; *lane 13*: 1 kb ladder (BRL)

of one species to a stand of the other. The conservative nature of the cpDNA and the unique mode of inheritance (i.e., paternal) preclude any ambiquities in the utility of restriction analysis in this case. In fact, due to this conservative nature of cpDNA in most plants and the observed differences among species, restriction analysis has been proven to be a useful phylogenetic tool (e.g., Kaneko et al. 1986).

Although the cpDNA is often regarded as highly conservative, intraspecific variation has been reported in pines (Wagner et al. 1987). In our study it was demonstrated that Sitka and white spruce differ with regard to eight unique fragments (Figs. 1 and 2). The chance of observing all eight fragments collectively in the hybrid seedlot as intra-populational variation seems thus very remote.

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