Theor Appl Genet (1989) 78:768–774

Inheritance of plastids in interspecific hybrids of blue spruce and white spruce

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Received June 6, 1989; Accepted July 31, 1989
Communicated by P. M. A. Tigerstedt

Summary. Chloroplast DNA (cpDNA) was purified from blue spruce (*Picea pungens* Engelm.) and white spruce [*P. glauca* (Moench) Voss], and was digested with several different restriction endonucleases. Restriction fragment length polymorphisms (RFLPs) were identified that differentiated the cpDNA of both species. Intraspecific conservation of the RFLPs that differentiated each species was confirmed by examining trees from across the natural range of each species. Ten F_1 hybrids were examined, and the cpDNA from each showed the banding pattern of the paternal species. Cloned *Petunia* cpDNA containing part of the *rbcL* gene hybridized to polymorphic bands, while a cloned maize mtDNA probe of the *coxII* gene failed to hybridize to any band.

Key words: Paternal Inheritance – RFLPs – Chloroplast DNA – *Picea* – Hybrid identification

Introduction

Most angiosperms exhibit maternal inheritance of the plastids, with approximately one-third having some degree of biparental inheritance (Sears 1980; Whatley 1982). The classic method of studying this characteristic is to follow the inheritance of a plastid mutant (either natural or induced) in reciprocal crosses. There is evidence that among gymnosperms the inheritance of the cytoplasmic organelles may be either strictly or largely paternal. Ohba et al. (1971) followed the inheritance of induced chloroplast mutants in sugi (*Cryptomeria japonica* D. Don) and determined that the plastids were inherited paternally approximately 90%–99% of the time, providing the first genetic evidence for predominantly paternal transmission of plastids. Other evidence for paternal inheritance of cytoplasmic organelles in the Coniferales comes from microscopy studies of fertilization. Cytoplasmic organelles were seen moving through the pollen tube (Maheshwari and Konar 1971), and the neocytoplasm, which formed following fertilization, appeared to exclude the maternal organelles (Willemse 1974).

Restriction analysis of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) is a fairly recent tool for the study of organelle molecular biology. By comparing restriction fragment length polymorphisms (RFLPs) of either cpDNA or mtDNA, it has been possible to ascertain the inheritance of organelles with a high degree of certainty in *Pelargonium* (Metzlaff et al. 1981) and in *Triticum* and *Aegilops* (Bowman et al. 1983).

Recently, there have been several reports that used RFLP analysis of cpDNA to follow the inheritance of chloroplasts in members of the Coniferales. Neale et al. (1986) reported paternal transmission of plastids in 33 of 36 progeny from intraspecific crosses of Douglas fir (*Pseudotsuga menziesii* (Mirb) Franco); the other 3 showed non-parental RFLP types. Szmidt et al. (1987) have reported finding the paternal pattern of cpDNA in three out of six interspecific F_1 hybrid progeny of *Larix*, with one showing the maternal pattern and two showing nonparental patterns. Paternal inheritance of cpDNA has also been demonstrated in interspecific F_1 hybrids of lodgepole pine (*Pinus contorta* Dougl. ex. Loud.) × jack pine (*P. banksiana* Lamb.) (Wagner et al. 1987), and of *Picea sitchensis* (Bong.) Carr. and *P. glauca* (Szmidt et al. 1988). Neale and Sederoff (1988) also reported paternal cpDNA transmission in redwood (*Sequoia sempervirens* D. Don Endl.).

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Table 1. Parentage of blue and white spruce hybrids

Hybrid accession no.[a]	Female parent		Pollen parent	
	Species	Accession no.[a]	Species	Accession no.[a]
720007	*P. glauca*	60.25-3-9	*P. pungens*	310676
720008	*P. glauca*	60.01-NN-10	*P. pungens*	310677
720009	*P. glauca*	60.25-3-5	*P. pungens*	310678
720010	*P. glauca*	60.25-3-7	*P. pungens*	310679
720011	*P. glauca*	60.06-41	*P. pungens*	310678
720017	*P. glauca*	60.06-40A	*P. pungens*	310679
720058	*P. pungens*	Pp-5	*P. glauca*	190061
720059	*P. pungens*	Pp-1	*P. glauca*	190561
720060	*P. pungens*	Pp-1	*P. glauca*	190562
720061	*P. pungens*	Pp-1	*P. glauca*	190560

[a] Add 67,000,000 to obtain the complete MICHCOTIP accession numbers

We report here methods of cpDNA purification from two species of spruce (*Picea*), identification of RFLPs that differentiate both species, and the paternal inheritance of cpDNA in interspecific hybrids.

Materials and methods

Plant material

All trees used in this study are located in the Kellogg Experimental Forest, Augusta/MI, USA. The parentage of the hybrids is listed in Table 1. The blue spruce are located in plantation 70.22, which is a rangewide provenance test established in 1970. The white spruce used are from a rangewide provenance test (plantation 63.05) that was established in 1963. The hybrids of blue and white spruce are located in plantation 70.21, which was established over the 4-year period 1970–1973. Detailed records for each tree are available from the Michigan Cooperative Tree Improvement Program (MICHCOTIP) at Michigan State University.

Trees were sampled by cutting off branch tips containing the current and previous season's growth, with the cut ends placed into distilled water. Each tree was sampled by removing branches from the entire perimeter of the tree, from 1.0 to 1.5 m above the ground. This was done to maximize the likelihood of identifying chimeric individuals. The cut branches were stored in the dark, at 4 °C, for up to 2 weeks.

Chloroplast DNA isolation

The chloroplast DNA (cpDNA) isolation procedures were modified procedures of Palmer (1985). Needles from individual trees were cut from the branches and washed thoroughly with distilled water. Diseased or damaged needles were discarded. Both the current and previous season's needles were used. All of the subsequent steps were carried out either on ice or at 4 °C. All centrifugation runs were done at 4 °C unless otherwise stated. All glassware and pipettes were silanized, prior to use, as outlined in Maniatis et al. (1982).

Between 75 and 100 g of needles were placed in a Waring blender with 10–20 vol (w/v) of semi-frozen homogenization buffer [8% w/v sorbitol, 0.15% w/v polyvinylpyrrolidone MW = 40,000 (PVP), 0.1% w/v bovine serum albumin fraction V (BSA), 10% w/v polyethylene glycol (PEG 6000), 8 m*M* EDTA, 1 m*M* ascorbic acid, 3 m*M* cysteine, 50 m*M* TRIS (pH 7.5), 5 m*M* mercaptoethanol] and were homogenized for 45 s. The homogenate was filtered through a 100-micron mesh nylon screen and then through two layers of Miracloth (Calbiochem). The filtrate was centrifuged for 15 min at 2,000 × g (Sorvall GS-3 rotor). The pellet was then suspended in approximately 20 ml of wash buffer (homogenization buffer minus the PVP) and then layered onto four sucrose step gradients.

The sucrose step gradients were prepared by the procedures of Stine and Keathley (1987) and were composed of five layers containing 80%, 62.5%, 45.0%, 27.5%, and 10.0% w/v sucrose in 50 m*M* TRIS, pH 7.5, 25 m*M* EDTA and 6.0% w/v sorbitol. The samples were centrifuged for 10 min at 18,000 × g in a vertical rotor (Sorvall SV-288) with slow acceleration and deceleration. The chloroplasts were removed from the 27.5%–45.0% sucrose interface with a Pasteur pipette.

The chloroplasts were then diluted with 2–3 vol of 50 m*M* TRIS (pH 8.0)/20 m*M* EDTA, and centrifuged at 18,800 × g for 10 min in a fixed angle rotor (Sorvall SS-34). The pellet was suspended in 2.0 ml of NET buffer (15 m*M* NaCl, 100 m*M* EDTA, 50 m*M* TRIS, pH 9.0), and predigested pronase was added to a final concentration of 100 μg/ml. The mixture was left on ice for 15 min, after which sarkosyl was added, to a final concentration of 1.0% w/v. The lysis mixture was gently shaken for 2–3 h at 4 °C.

Two volumes of a solution of 40 m*M* TRIS, pH 8.0, saturated with cesium chloride (CsCl) was then added to the chloroplast lysate, and the solution was centrifuged at 85,000 × g for 1.5 h at 19 °C in a swing-out rotor (Sorvall AH-650) in an ultracentrifuge. The proteins and other debris on the surface of the lysate were removed. The cleared lysate was then transferred to clean centrifuge tubes, bisbenzimide (Hoechst dye 33258) was added to a final concentration of 20 μg/ml, and the concentration of CsCl was adjusted to a refractive index of 1.3965 ± 0.0005. This mixture was then centrifuged for 14 h in a vertical rotor (Sorvall TV-865) at 155,000 × g and 19 °C.

Following centrifugation, the DNA band was visualized with UV light (366 nm), and was removed with a pipette. The bisbenzimide was extracted from the DNA solution with isopropanol saturated with NaCl. The DNA was precipitated with isopropanol according to Maniatis et al. (1982), and dissolved in sterile TE buffer (10 m*M* TRIS, pH 8.0, 1.0 m*M* EDTA). The hydrated DNA samples were then stored at 4 °C for subsequent digestion with restriction enzymes.

Digestion of DNA samples with restriction endonucleases was carried out according to the directions supplied by the manufacturer of each enzyme. The DNA fragments were separated by standard agarose gel electrophoresis techniques as outlined in Maniatis et al. (1982). The samples were loaded so as to give approximately equal intensity cpDNA bands, with the total amount of DNA per lane varying. Agarose gels (0.8% w/v) were used with TBE buffer (0.089 *M* TRIS, 0.089 boric acid, 2.0 m*M* EDTA, pH 8.0). Ethidium bromide at 0.5 μg/ml was incorporated into both the gel and the TBE buffer. Lambda DNA cut with HindIII in combination with EcoRI provided molecular markers in each gel. Following electrophoresis, the DNA bands were visualized on a 302-nm UV light transilluminator and photographed using Polaroid type 55 film. The DNA in the agarose gels was then transferred to nitrocellulose filters (MSI brand, 0.45 μm pore size) using the procedures of Southern (1975) as described in Maniatis et al. (1982).

A pBR322 clone bank of *Petunia* cpDNA, described in Sytsma and Gottlieb (1986), and a pBR322 clone (pZmE1) of the cytochrome oxidase subunit II (*coxII*) gene from maize mitochondria, described by Fox and Leaver (1981), were purified using the Triton X-100 detergent lysis procedure of Ausubel

et al. (1987). The nomenclature of the cloned cpDNA fragments used here follows that of Sytsma and Gottlieb (1986).

The procedures for random primer labeling of DNA are those as described by the manufacturer of the kit (Boehringer Mannheim Biochemicals). The Southern filters were hybridized according to the procedures of Maniatis et al. (1982), and were incubated overnight at 65 °C with gentle shaking. The hybridized filters were washed according to procedures of Thomashow et al. (1980). The hybridization fluid was removed and the filters were rinsed twice at 23 °C with 2 × SSC. This was followed by four washes with 3 × SSC, 0.2% SDS, and 5.0 m*M* EDTA at 65 °C for 30 min each, when probing with the mtDNA probe. For stringent washing (for the *Petunia* cpDNA probes), this was followed by one 30-min wash at 65 °C with 0.3 × SSC, 0.2% SDS, and 5.0 m*M* EDTA. Two brief 23 °C rinses with 2 × SSC were used to remove the SDS. Autoradiography was carried out at either 23 °C or at −70 °C with DuPont Cronex Lightning Plus intensifying screens. Kodak X-Omat AR X-ray film was exposed for between 1 and 36 h. For subsequent hybridization with different probes, the initial probe was washed off by the procedures of Gatti et al. (1984), followed by those of Thomashow et al. (1980).

Results and discussion

The research described here includes the development of methods to purify spruce cpDNA, the identification of cpDNA RFLPs, and the demonstration of the pattern of inheritance of cpDNA in the spruce hybrids. The use of cpDNA RFLPs allowed chloroplast inheritance patterns to be deduced, and avoided many of the problems of trying to use microscopy to follow the fate of chloroplasts during fertilization. In addition, if biparental transmission of the plastids is followed by differential sorting of the plastids in cell divisions subsequent to fertilization, this approach will be able to identify the final plastome type in the mature plant.

The first step was to obtain reasonably pure samples of cpDNA from the trees to be studied, and to digest them with various restriction endonucleases. Figure 1 shows the pairwise comparison of blue spruce and white spruce cpDNA cut with the enzymes ClaI and AvaI. These two enzymes were chosen because they are methylation-sensitive and infrequently cut nuclear DNA, but freely cut the non-methylated cpDNA. The use of methylation-sensitive enzymes to selectively cut cpDNA in samples containing nucDNA has been reviewed by Palmer (1985). By using methylation-sensitive enzymes, it is easy to visualize the cpDNA bands, even with varying levels of nucDNA contamination. The enzymes EcoRI, BamHI, and HindIII also cut the DNA samples in this study, but the varying levels of background fluorescence from the nucDNA make it difficult to consistently visualize all of the cpDNA bands.

Interspecific RFLPs that differentiate blue spruce cpDNA and white spruce cpDNA are shown in Fig. 1 A. Digestion with ClaI and AvaI results in several polymorphic bands (labeled with arrowheads), which are listed in Table 2. The broad band of fluorescence near the top of each lane is most likely nuclear DNA (nucDNA) that was co-purified with the cpDNA, but was not digested by these methylation-sensitive enzymes. Those bands labeled with a "*" represent ClaI fragments of 4.45 or 2.9 kb in size (white spruce and blue spruce, respectively), which are used in the following figures to demonstrate paternal inheritance of cpDNA.

It was necessary to demonstrate that the RFLPs present in Fig. 1 were cpDNA and not nucDNA or mtDNA. Several approaches to this problem were taken. The first was described earlier, the use of methylation-sensitive enzymes that infrequently cut nucDNA. The second approach was to probe the putative cpDNA samples with known cloned cpDNA from another species. Figure 1 B shows an autoradiograph produced by probing a South-

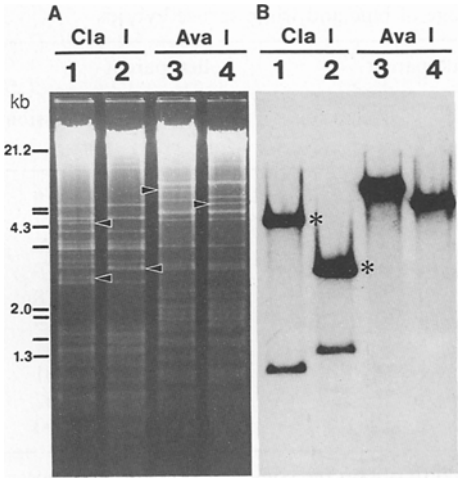

Fig. 1 A and B. Identification of RFLPs that differentiate white spruce and blue spruce. **A** Restriction patterns of cpDNA from white spruce (*lanes 1 and 3*) and blue spruce (*lanes 2 and 4*). The positions of RFLPs that differentiate blue spruce and white spruce are indicated by *arrowheads*. For RFLPs labeled with "*" see text. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.5 V/cm,8 h. **B** Hybridization of ^{32}-P labeled probe P16 to some of the RFLPs identified in Fig. 1A

Table 2. CpDNA RFLPs that differentiate white spruce from blue spruce

Fragment (kb)	White spruce	Blue spruce
ClaI 4.45	+	−
ClaI 2.9	+	++
ClaI 2.8	+	−
AvaI 6.0	+	−
AvaI 5.2	−	+

+ = present; − = absent; ++ = stoichiometrically double in intensity

ern filter, from the gel shown in Fig. 1 A, with a *Petunia* cpDNA fragment (P16) 4.1 kb in size. The probe is from the large single-copy region and contains part of the gene for the large subunit of ribulose bisphosphate carboxylase-oxygenase (*rbcL*). P16 strongly hybridized to the fragments marked with a "*" in Fig. 1 A, and weakly hybridized to a 1.21-kb ClaI fragment in white spruce and to a 1.33-kb ClaI fragment in blue spruce. This probe also hybridized to AvaI fragments of 6.0 or 5.2 kb (white spruce or blue spruce, respectively). Another probe (P3) hybridized to the same bands as P16 in addition to several other fragments (data not shown). P3 is 21.0 kb in size, borders P16 on the cpDNA molecule, and contains the remainder of the *rbcL* gene.

The clone bank used represents approximately 92% of the petunia chloroplast genome, and all 13 cloned fragments hybridized to visible fragments on the filters under high stringency washing conditions. In no instance did the probes hybridize to regions on the filter that did not correspond to visible bands on the gels, demonstrating that the ethidium bromide-stained bands represent cpDNA.

Due to the presence of cpDNA sequences in mitochondria (Sederoff 1987), it is possible that these RFLPs represent mtDNA. This was addressed by using a highly conserved mtDNA probe (pZmE1) which contains the *coxII* gene. The Southern filter from the gel shown in Fig. 1 A was also probed with pZmE1, and it failed to hybridize under low stringency washing conditions (data not shown). Using identical hybridization techniques, pZmE1 will hybridize to Southern filters containing positive control lanes (cloned pZmE1 DNA), and to total DNA preparations from these spruce species (data not shown), indicating that our cpDNA purification techniques do not contain detectable levels of mtDNA.

To establish whether the ClaI RFLPs shown in Fig. 1 are conserved within a species, eight trees from across the natural range of each species were examined. For white spruce, cpDNA was isolated from trees from British Columbia, Saskatchewan, Manitoba, South Dakota, Ontario, New York, New Hampshire and Labrador, and was digested with ClaI. No intraspecific variation in the RFLPs listed in Table 2 was observed (Fig. 2 A). The gel from Fig. 2 A was probed with P16 and is shown in Fig. 2 B. No variation in the 4.45-kb fragment ("*") is apparent. The weakly hybridizing band is variable in size, existing as a 1.21-kb band in trees from Saskatchewan, Manitoba, and South Dakota, and as a 1.33-kb band in the remaining trees.

For blue spruce, two trees from different counties in Arizona, Colorado, and Utah, and one tree each from New Mexico and Wyoming were examined as described for white spruce. Again, as Fig. 3 A demonstrates, no variation was observed in the RFLPs listed in Table 2. When the Southern filter from this gel was probed with

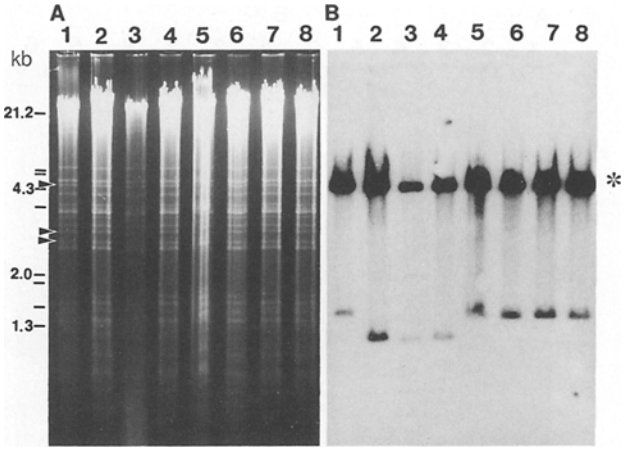

Fig. 2 A and B. White spruce provenance test. **A** ClaI restriction patterns for single trees from British Columbia, Saskatchewan, Manitoba, South Dakota, Ontario, New York, New Hampshire, Labrador (*lanes 1–8*, respectively). *Arrowheads* and "*" indicate bands identified in Fig. 1. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.0 V/cm, 12 h. **B** Hybridization of P16 to a Southern filter from the gel shown in Fig.2A

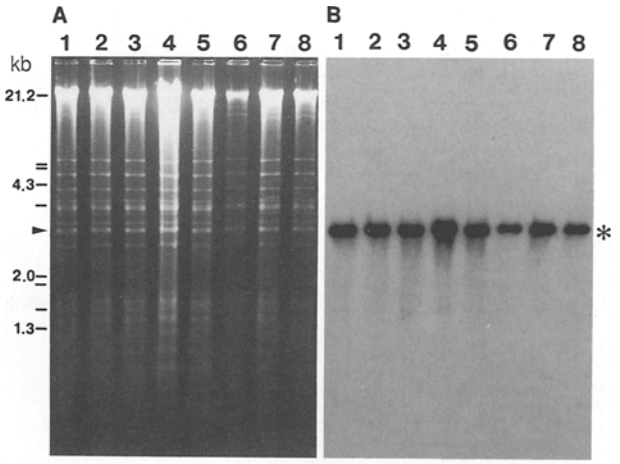

Fig. 3 A and B. Blue spruce provenance test. **A** ClaI restriction patterns of cpDNA from single trees from Arizona (*lanes 1–2*) New Mexico (*lane 3*), Colorado (*lanes 4–5*), Wyoming (*lane 6*), and Utah (*lanes 7–8*). *Arrow* and "*" indicate bands identified in Fig. 1. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.0 V/cm, 12 h. **B** Hybridization of P16 to a Southern filter from Fig. 3A

P16, no variation was observed in either the 2.9-kb band ("*") or in the weakly hybridizing 1.33-kb band.

Based on the results of the provenance tests (Figs. 2 and 3), the RFLPs listed in Table 2 that differentiate blue spruce from white spruce were considered to be invariant within a species. Thus, we felt confident in assuming that these species-specific cpDNA restriction patterns were

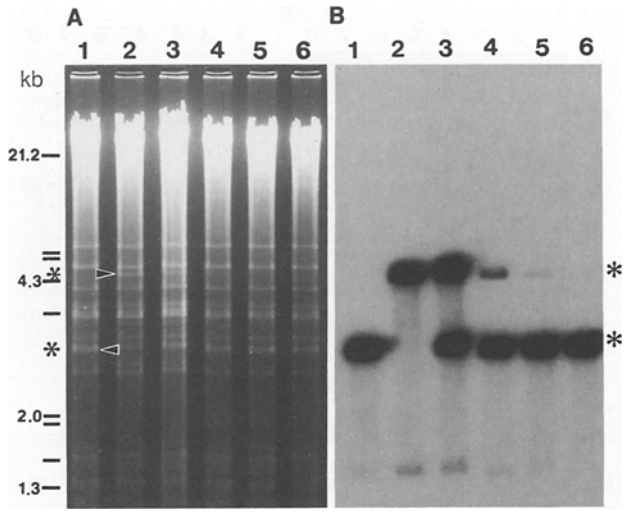

Fig. 4 A and B. Demonstration of autoradiographic techniques to detect under-represented DNA. **A** ClaI restriction fragments of cpDNA from blue spruce (*lane 1*), white spruce (*lane 2*), equal amounts of each (*lane 3*), blue spruce plus 10-fold, 100-fold, and 1,000-fold dilutions of white spruce (*lanes 4–6*, respectively). Fragments were electrophoretically seperated in 0,8% agarose, TBE buffer, 1.5 V/cm, 8 h. **B** Hybridization of P16 to a Southern filter from the gel shown in Fig. 4A

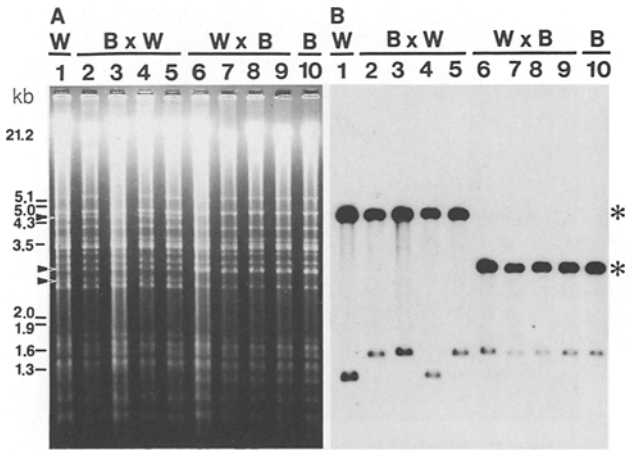

Fig. 5 A and B. Comparison of cpDNA from F_1-hybrids to parental species. **A** ClaI restriction patterns of parental species and hybrids. *Lane 1*, white spruce; *lanes 2–5*, blue spruce × white spruce Hybrids (720058, 720059, 720060, and 720061, respectively); *lanes 6–9* are white spruce × blue spruce hybrids (720007, 720008, 720010, and 720011, respectively); *lane 10*, blue spruce. The restriction fragments were separated in 0.8% agarose, TBE buffer, 0.8 V/cm, 18 h. **B** Hybridization of probe P16 to Southern filter of gel shown in Fig. 5A

also valid for the parents of the hybrids, which are no longer available to assay.

To determine the level of biparental inheritance of cpDNA which was detectable by autoradiography, samples of blue spruce and white spruce cpDNA cut with ClaI were mixed, separated in a gel (Fig. 4A), and probed with P16 (Fig. 4B). The amount of blue spruce cpDNA was kept constant, while the white spruce cpDNA was diluted. In Fig. 4B, the 4.45-kb band can still be seen when diluted 100-fold (lane 5). In overexposed autoradiographs, it was still visible when diluted 1,000-fold. Thus, we should be able to recognize the transmission of cpDNAs from the maternal parent down to approximately one part per thousand.

Figure 5A shows the ClaI restriction patterns of eight F_1 hybrids compared to the parental species. All eight hybrids are from different controlled crosses. In the eight hybrids shown, and two additional (not shown), the restriction pattern was that of the paternal species. A Southern filter from this gel was probed with P16, and is shown in Fig. 5B. Again, the pattern shown is that of the paternal species.

One of the concerns of this study was the possibility of biparental inheritance of the plastids. If this occurred, the different plastid types might sort out into different sectors within one tree, as has been demonstrated in *Pinus* (Govindaraju et al. 1988), or the whole tree might remain heteroplasmic. By sampling the entire crown of each tree at one height (not just one branch), and by using autoradiographic methods, it should be possible to identify chimeric or heteroplasmic individuals among the hybrids, as long as the least abundant DNA accounts for at least 0.1% of the DNA in the sample. In none of the ten hybrids examined did there appear to be any indication of heteroplasmy or chimeric individuals; only the pollen parent cpDNA type was found.

This study differs from Neale et al. (1986) and that of Szmidt et al. (1987), because only the paternal cpDNA restriction patterns were observed in the hybrids. Neale et al. (1986) used cpDNA RFLPs to demonstrate the paternal transmission of plastids in intraspecific crosses of Douglas fir. However, 3 of 36 progeny examined showed nonparental patterns in their cpDNA restriction digests. Szmidt et al. (1987) also reported finding two nonparental cpDNA restriction patterns and one maternal pattern, out of a total of six interspecific hybrids of *Larix*. When examining the cpDNA of eight F_1 hybrids of jack pine × lodgepole pine, Wagner et al. (1987) found only the paternal cpDNA type, a result similar to ours.

When blue spruce cpDNA is digested incompletely, either by diluting the amount of enzyme used or by short digestion periods, a 4.45-kb band that P16 hybridizes to is generated (data not shown), thus indicating that the difference between the blue spruce 2.9-kb band and the white spruce 4.45-kb band may be a ClaI restriction site mutation. We cannot confirm this, however, as we did not prepare restriction site maps for these species. Additionally, when using the *Petunia* probes for heterologous probing, hybridization of one probe to two or more bands in our gels does not necessarily imply that the

fragments are adjacent to each other in spruce cpDNA molecules, as conifer chloroplast genomes have been shown to be extensively rearranged compared to angiosperms (Strauss et al. 1988).

These data provide another piece of evidence which indicates the RFLPs used in this study are not nuclear in origin, but represent organelle DNA. If the RFLPs were nuclear in origin, F_1 progeny of reciprocal crosses should show identical patterns. Since the inheritance of these RFLPs appears to be strictly uniparental, in this case paternal, the RFLPs must represent cytoplasmic DNA.

The results of this study clearly show that the cpDNA in the hybrids of blue spruce and white spruce is inherited from the paternal parent. The mechanism for paternal inheritance of cpDNA in *Picea* is still unclear, though studies using microscopy indicate that during fertilization in the Pinaceae, the plastids from the pollen tube enter the egg cell, and the female plastids are excluded. Studies by Maheshwari and Konar (1971) and Willemse (1974) on fertilization in *Pinus* have shown that the pollen parent plastids enter the cytoplasm of the egg during fertilization, and that the maternal parent chloroplasts are excluded from the neocytoplasm (Willemse 1974). Chesnoy and Thomas (1971), however, could not determine the parentage of plastids in *Pinus nigra* Arnold. Owens and Simpson (1988) have shown that during fertilization of Douglas fir (*Pseudotsuga menziesii*), pollen tube organelles enter the egg cell and a small portion of the pollen cytoplasm migrates along with the male gamete to the female nucleus. For *Biota*, a member of the Cupressaceae, Chesnoy (1969) demonstrated by light microscopy that female plastids are excluded from the neocytoplasm following fertilization.

The ability to hybridize blue spruce and white spruce is well established (Hanover and Wilkinson 1969; Bongarten and Hanover 1982). The trees used in this study have previously been shown to be hybrids by morphological characteristics and monoterpene composition (Hanover and Wilkinson 1969; Bongarten and Hanover 1982). In this study, the cpDNA in the hybrids was always that of the paternal species. This indicates that analysis of cpDNA could aid in the verification of hybrids resulting from controlled pollinations. This would be especially useful if the ranges of morphological characteristics of the parental species overlap.

Acknowledgements. This research was supported by grants from the USDA Forest and Rangeland Renewable Resources, Grant 89-34-158-4231, and from the Michigan Research Excellence Fund. The authors also greatfully acknowledge use of a pBR322 clone bank of *Petunia* cpDNA provided by J. Palmer, c/o D. Neale, Pacific Southwest Forest Range and Experiment Station, Berkeley/CA and the cloned probe of *coxII* (pZmE1) which was provided by T. Fox. Michigan Agricultural Experiment Station Article Number 13150.

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