DEMONSTRATION OF PATERNAL INHERITANCE OF PLASTIDS IN *PICEA* (PINACEAE)

By

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ABSTRACT

DEMONSTRATION OF PATERNAL INHERITANCE
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Chloroplast DNA (cpDNA) was purified from *Picea glauca*, *P. pungens*, *P. engelmannii*, and *P. omorika*, and was digested with several restriction endonucleases. Interspecific restriction fragment length polymorphisms (RFLPs) of cpDNA were identified. The RFLPs were identified as cpDNA by the hybridization of cloned, ^{32}-P labeled, petunia cpDNA to the polymorphic bands, and by the lack of hybridization of a cloned and labeled mtDNA probe from maize. Chloroplast DNA RFLPs that showed no intraspecific variation when examined across the natural range for each species, were used as markers to follow the inheritance of plastids in interspecific hybrids. The inheritance of plastids was determined for F_1-hybrids from reciprocal crosses of *P. glauca* and *P. pungens*, *P. glauca* and *P. omorika*, and F_1-hybrids of *P. engelmannii* x *pungens*. All 31 F_1-hybrids examined showed the cpDNA genotypes of the pollen parent, or the paternal species.

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INTRODUCTION

Most angiosperms show maternal inheritance of plastids, with about one third showing some degree of biparental inheritance (Sears 1980, Whatley 1982). In contrast, for most gymnosperms, there has not been direct genetic evidence which would establish how plastids are inherited. This is primarily due to their long generation time and the lack of available plastid mutants. Most studies have therefore relied on either light, or electron microscopy to examine the fate of plastids following fertilization (Maheswari and Konar 1971; Willemse 1974). Delayed fertilization, following pollination, is a common characteristic of many gymnosperms (Wilson and Burley 1983), which makes it difficult to follow the fertilization process. Furthermore, microscopy destructively samples the fertilized archegonium cell making it impossible to follow the fate of plastids at later points in time. So, even if plastids from the pollen tube are shown to enter the cell, it is not possible to determine whether the cpDNA remains viable, whether the plastids are capable of division, or if differential sorting out of plastids from the two cytoplasms occurs.

The report by Ohba et al. (1971) was the first report in which the inheritance of a plastid mutant was followed in sexual crosses to establish the inheritance of plastids in a gymnosperm. In this report, between 90 and 99% of the progeny showed the plastid phenotype of the pollen parent, with up to 5% chimeric progeny; thus, indicating probable

biparental inheritance. It should be noted that in reciprocal crosses, the rates of paternal transmission varied, with the mutant form showing 90% paternal transmission, and the wild-type showing a 99% paternal transmission rate, respectively.

The use of restriction analysis of chloroplast DNA (cpDNA) to follow the inheritance of plastids has become increasingly popular (Metzlaff et al. 1981; Bowman et al. 1983). This technique has an advantage over the more traditional methods of following either natural or induced chloroplast mutants, in that most cpDNA RFLPs should represent neutral mutations (Beckmann and Soller 1986).

In addition to providing information on the transmission pattern of plastid DNA, RFLP analysis of the cpDNA will provide groundwork for research in a number of areas. These techniques are readily amenable for the use in phylogenetic analysis, either by mapping structural changes in the molecule (Palmer 1985), or by the sequencing of specific genes located on individual fragemnts (Zurawski and Clegg 1987). Many basic questions in gymnosperm taxonomy still exist as illustrated by Florin (1963) describing seven families and 52 genera of conifers, while Silba (1984) and Hart et al. (1987) maintain Florin's seven families, but Silba (1984) accepts 60 genera, and Hart et al. (1987) 63 genera. Meyen (1984) differs even more from Florin (1963) by placing Ginkoales into a different class from Conifierales, and reduces Taxales to family rank.

One of the major problems in the study of evolution and taxonomy of plants is choosing characteristics that show the proper level of variation to resolve groups at the desired level. Mapping of RFLPs of cpDNA has most commonly been used to resolve species and genera (Palmer 1986), but based on recent results these techniques may provide useful and

needed information on gymnosperm taxonmy at the level of family or higher. Until recently, *Ginkgo biloba* was the only gymnosperm for which a map of the cpDNA existed (Palmer 1985). The map of the *Ginkgo* cpDNA molecule reveled a cpDNA structure which included a large inverted repeat region, which has been found in all angiosperms (except the Fabaceae subfamily Papilonoideae), three ferns, and the two bryophytes so far studied (Palmer 1985). Strauss et al. (1988) have demonstrated that Douglas-fir (*Pseudotsuga menziesii*) and Monterey pine (*Pinus radiata*) both lack the inverted repeat structure, which would represent a major difference from *Ginkgo*. Thus, the mapping of cpDNA RFLPs will very likely provide much needed information on the relationships of the gymnosperms, and augment the existing morphological data.

The study of population structure of tree species will also benefit by the determination of plastid and mitochondrial inheritance. When the phenomenon of paternal inheritance of cpDNA, and the probable maternal inheritance of mitochondrial DNA (Neale and Sederoff 1987) is better understood, it may be possible to follow two relatively simple genetic systems in a single species, and to determine how the dispersal mechanism (seed versus pollen) affects gene flow.

Traditional tree breeding will benefit if enough variation in the cpDNA of individual species exists to allow for the identification of races or sub-species. In addition to understanding natural populations, this would help to identify seed sources of planted or exotic species for which records of their origin do not exist.

Advanced generation breeding will be aided by the understanding of cpDNA inheritance in the identification of hybrid progeny, either from

controlled crosses or naturally ocurring putative hybrids. With the paternal transmission of plastids, coupled to the relatively easy task of identification of the species of the tree bearing the ovulate stroboli, all hybrid progeny should show the cpDNA type of the putative pollen donor. The utility of this technique will be limited until it is determined whether cpDNA is strictly paternally inherited, or if there is some level of maternal or biparental inheritance. The usefulness of this technique for identifying hybrids will be inversely proportional to the level of biparental or maternal inheritance of cpDNA.

For the study reported here, it was originally planned that RFLPs would be identified that differentiated each species to be used, and then individual trees would be crossed. The cpDNA restriction patterns of the progeny were then to be compared directly to those of their parents. During the course of this study, very few female stroboli were produced by the species of interest, and it was not possible to complete the reciprocal crosses needed to produce interspecific hybrids. It was then decided to utilize existing *Picea* hybrids at Michigan State University (MSU).

The Department of Forestry at MSU has had a long-standing interest in spruce hybrids (Wright 1955, Hanover and Wilkinson 1969, Bongarten and Hanover 1982, Schaefer and Hanover 1985, Ernst et al. 1988) and as such, a substantial number were readily available for study. The trees used in this study are listed in Tables A1 and A2.

Due to unavoidable mortality, many of the parents of individual hybrids were not available, and thus the direct comparison of the hybrids to their parents was impossible. This necessitated finding RFLPs that differentiated individual species, but were conserved within a single

species. This was accomplished by sampling trees from throughout the natural range of each species, and then checking for conservation of the interspecific RFLPs.

Once interspecific RFLPs that are conserved within a species were identified, representatives of each parental species were compared to the interspecific hybrids. In all 31 F_1-hybrids examined, the cpDNA restriction pattern of the paternal species was observed.

The bands produced by the endorestriction digests of the DNA samples were demonstrated to be cpDNA by: 1) using methylation sensitive restriction endonucleases, which freely cut cpDNA, but only rarely cut nuclear DNA; 2) hybridization of cloned cpDNA to the polymorphic bands; 3) failure of a cloned mtDNA probe to hybridize to restriction fragment bands visible in the gels.

The remainder of this dissertation will present the results of these studies in separate chapters for each hybrid system. Chapter I presents the analysis of blue spruce and white spruce hybrids, and the provenance tests of each species. Chapter II shows the results of Serbian spruce and white spruce hybrids, for which the Serbian spruce parents of all the hybrids examined were available for study. Chapter III presents a more limited study of four Engelmann spruce x blue spruce hybrids, and three putative, naturally occurring interspecific hybrids. A summary and conclusion is presented at the end of the dissertation.

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CHAPTER I

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

ABSTRACT

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.

INTRODUCTION

Most angiosperms exhibit maternal inheritance of the plastids, with approximately one third having some degree of biparental inheritance of plastids (Sears 1980). 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 to 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), or in which 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). The main advantages of this technique are that there is no need to either find or induce chloroplast or mitochondrial mutants, there is no question whether or not the mutation affects the inheritance of the organelles,

and there is no ambiguity as to whether there is differential sorting out of the organelles following fertilization.

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 three showed non-parental RFLP types. Szmidt et al. (1987) have reported finding the paternal pattern of cpDNA in 3 out of 6 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.) x jack pine (*P. banksiana* Lamb.) (Wagner et al. 1987). Neale and Sederoff (1988) also reported paternal cpDNA transmission in redwood (*Sequoia sempervirens* D. Don Endl.).

We report here methods of cpDNA purification from *Picea*, the analysis of the DNA samples, demonstrating that it is cpDNA and not nuclear DNA (nucDNA) or mtDNA, 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, 7060 N. 42nd St., Augusta, MI 49012. The accession number, plantation number, and the row and column numbers for each tree are

listed in Table A1, and 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 four year period, 1970-1973. Detailed records for each tree are available from the Michigan Cooperative Tree Improvement Program (MICHCOTIP) at Michigan State University.

Table 1. Parentage of blue and white spruce hybrids.

Hybrid Accession Number*	Female Parent Species	Female Parent Accession Number	Pollen Parent Species	Pollen Parent Accession Number*
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

* Add 67,000,000 to obtain the complete MICHCOTIP accession numbers.

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 1.0-1.5 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^{o} C, for up to two weeks.

Chloroplast DNA Isolation

The chloroplast DNA (cpDNA) isolation procedures were modified procedures of Palmer (1985) and Stine et al. (1988). Needles from individual trees were cut from the branches and washed thoroughly with distilled water. Any 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^{o} C. All centrifugation runs were done at 4^{o} C unless stated otherwise. 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 to 20 volumes (w/v) of semi-frozen homogenation 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 mM EDTA, 1 mM ascorbic acid, 3 mM cysteine, 50 mM Tris (pH 7.5), 5 mM mercaptoethanol) and were homogenized for 45 seconds. Throughout the cpDNA preparation, each step was monitored microscopically to ascertain the chloroplast's purity.

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 minutes at 2000 x g (Sorvall GS-3 rotor). The pellet was then suspended in approximately 20 ml of wash buffer (homogenation 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 mM Tris, pH 7.5, 25 mM EDTA and 6.0% w./v. sorbitol. The samples were centrifuged for 10

minutes at 18,000 x 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 Pastuer pipette.

The chloroplasts were then diluted slowly with 2 to 3 volumes of 50 mM Tris (pH 8.0)/20 mM EDTA, and centrifuged at 18,800 x g for 10 minutes in a fixed angle rotor (Sorvall SS-34). The pellet was suspended in 2.0 ml of NET buffer (15 mM NaCl, 100 mM EDTA, 50 mM Tris, pH 9.0) and 200 ul of predigested pronase (1.0 mg/ml) was added. The mixture was left on ice for fifteen minutes, after which sarkosyl was added, to a final concentration of 1.0% w/v. The lysis mixture was gently shaken for 2 to 3 hours at 4^{o} C.

Two volumes of a solution of 40 mM Tris, pH 8.0, saturated with cesium chloride (CsCl) were then added to the chloroplast lysate, and the solution was centrifuged at 85,000 x g for 1.5 hours at 19^{o} 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 0.1 mg/ml and the concentration of CsCl was adjusted to a refractive index of 1.3965 $\pm$ 0.0005. This mixture was then centrifuged for 14 hours in a vertical rotor (Sorvall TV-865) at 155,000 x g and 19^{o} 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 and water. This step was repeated until no fluorescence was detected using UV light. The DNA solution was then extracted two additional times. Two volumes of sterile double distilled water and three volumes of isopropanol were

added, and the DNA was precipitated at -20° C over night. The DNA samples were then centrifuged in a microcentrifuge at 13,000 x g at 25° for 10 minutes. The DNA pellet was washed 3X with 70% (v/v) ethanol, centrifuging for 5 minutes at 13,000 g after each washing. The DNA pellet was dried under a stream of filtered air and then dissolved in sterile TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA). The hydrated DNA samples were then stored at 4° C for subsequent digestion with restriction enzymes.

Restriction Analysis and Agarose Electrophoresis

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% or 1.0% w/v) were used with TBE buffer (0.089 M Tris, 0.089 boric acid, 2.0 mM EDTA, pH 8.0). Ethidium bromide at 0.5 ug/ml was incorporated into both the gel and the TBE buffer. Following electrophoresis, the DNA bands were visualized on a 302 nm UV light transilluminator, and photographed using Polaroid type 55 film.

To estimate the size of the individual restriction fragments, Lambda DNA cut with Hind III in combination with Eco RI was used as molecular markers in each gel. The distance of migration of each lambda DNA fragment was plotted in semi-log fashion against the known size of the

fragment, and the size of the cpDNA fragments were then estimated based on their migration.

Southern Transfer

The DNA in the agarose gels was then transferred to nitrocellulose filters (MSI brand, 0.45 um pore size) using the procedures of Southern (1975) and described in Maniatis et al. (1982).

Probe Preparation

A pBR322 clone bank of *Petunia* cpDNA, described in Sytsma and Gottlieb (1986) was provided by J. Palmer c/o D. Neale, Pacific Southwest Forest and Range Experiment Station, 1960 Addison Street, Berkeley, CA 94701, and a pBR322 clone (pZmE1) of the cytochrome oxidase subunit II (coxII) gene from maize mitochondria, described by Fox and Leaver (1981) was supplied by T. Fox. The cloned cpDNA fragments were supplied as stab cultures in Luria-Bertani media with antibiotics and agar. The cloned DNA was purified using 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).

Random Primed Labeling

The procedures for random primed labeling of DNA are those as described by the manufacturer (Boehringer Mannheim Biochemicals) and supplied with the random primed labeling kit. Approximately 2.0 ug of probe DNA was labeled in each reaction.

Hybridization and Autoradiography

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 rinsed twice at 23° C with 2X SSC. This was followed by four washes with 3X SSC, 0.2% SDS and 5.0 mM EDTA at 65° C for 30 minutes each. For stringent washing (for the *Petunia* cpDNA probes), this was followed by one 30 minute wash at 65° C with 0.3X SSC, 0.2% SDS and 5.0 mM EDTA. Two brief 23° C rinses with 2X SSC were used to remove the SDS. The filters were allowed to air dry on Whatman 3M paper. When dry, they were wrapped with cellophane. 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 hours. 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 the pattern of chloroplast inheritance patterns to be deduced, and avoided many of the problems of trying to use microscopy to follow the fate of chloroplasts following fertilization. In addition, if biparental transmission of the plastids is followed by

differential sorting sorting og 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 Cla I and Ava I. 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 methylation insensitive enzymes Eco RI, Bam HI and Hind III will cut the DNA samples in this study, but the varying levels of background fluorescence from the nucDNA makes it difficult to consistently visualize the cpDNA bands.

Interspecific RFLPs that differentiate blue spruce cpDNA and white spruce cpDNA are shown in Fig. 1A. Digestion with Cla I and Ava I results in several polymorphic bands (labeled with arrowheads), and are listed in Table 2. The broad band of fluorescence near the top of each lane is most likely nuclear DNA (nucDNA) that was copurified with the cpDNA. Those bands labeled with a "*" represent Cla I fragments of 4.45 or 2.9-kb in size (white spruce and blue spruce respectively) that are used in the following figures to demonstrate paternal inheritance of cpDNA.

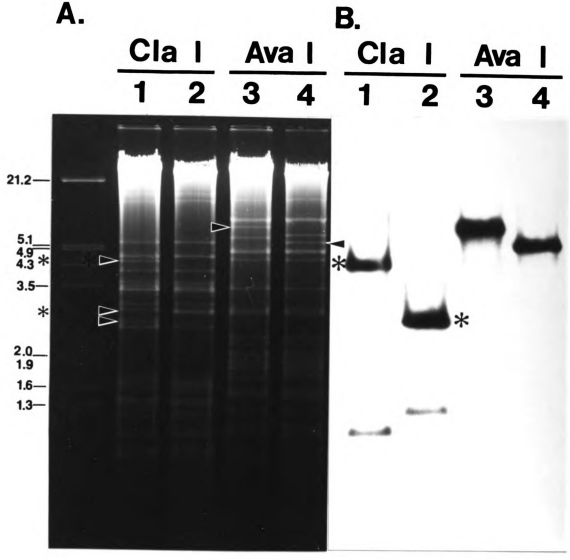

Fig. 1. Identification of RFLPs that differentiate white spruce and blue spruce. A. Restriction patterns of cpDNA from white spruce (lanes 1 and 3) and blue spruce (lanes 2 and 4). RFLPs that differentiate blue spruce and white spruce, are idicated by arrowheads. For RFLPs labeled with "*" see text. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.5 V/cm, 8 hours. 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
Cla I 4.45	+	-
Cla I 2.9	+	++
Cla I 2.8	+	-
Ava I 6.0	+	-
Ava I 5.2	-	+

+ = present - = absent
++ = stoichiometrically double intensity

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 1B shows an autoradiograph produced by probing a Southern filter, from the gel shown in Fig. 1A, with a *Petunia* cpDNA fragment (P16) of 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 in Fig. 1A, and weakly hybridized to a 1.21-kb white spruce Cla I fragment and to a 1.33-kb blue spruce Cla I fragment. This probe also hybridized to Ava I fragments of 6.0 or 5.2-kb (white spruce or blue spruce respectively). Another probe (P3) also hybridized to the same bands as P16 in addition to several other fragments (data not shown). P3 is 21.0-kb in size and borders P16 on the cpDNA molecule and contains the remainder of 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 the bands represent cpDNA.

Due to the presence of cpDNA sequences in mitochondria (Sederoff 1987), it is possibile 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. 1A was also probed with pZmE1, and it failed to hybridize to any band visible in Fig. 1A 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) (data not shown), and to total DNA preparations from these spruce species (David, personal communication).

To establish whether the Cla I 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, Saskatchawan, Manitoba, North Dakota, Ontario, New York, New Hampshire and Labrador, and was digested with Cla I. No intraspecific variation in the RFLPs listed in Table 2 was observed (Fig. 2A). The gel from Fig. 2A was probed with P16 and is shown in Fig. 2B. No variation in the 4.45-kb fragment ("*") is apparent. The weakly hybridizing band is variable, and existing as a 1.21-kb band in trees from Saskatchawan, Manitoba and South Dakota, and as a 1.33-kb band in the remaining trees.

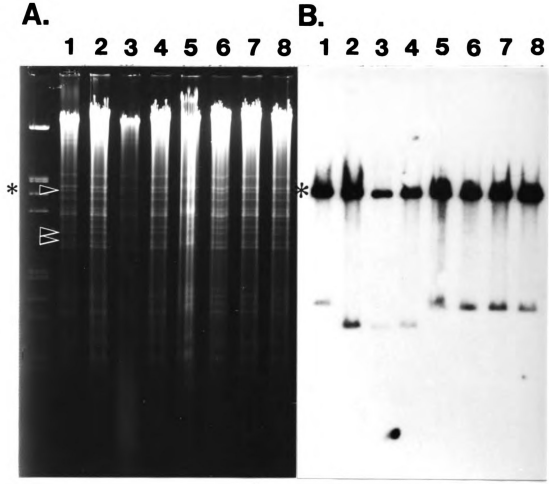

Fig. 2. White spruce provenance test. A. Cla I restriction patterns for single trees from British Columbia, Saskatchawan, Manitoba, S. Dakota, Ontario, New York, New Hampshire, Labrador (lanes 1-8 respectively). Arrowheads and "*" indicate bands identified in Fig. 1. Fragments were electrophoretically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

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. 3A demonstrates, no variation was observed in the RFLPs listed in Table 2. When the Southern filter from this gel was probed with P16, no variation was observed in either the 2.9-kb band ("*"), nor 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 that these species specific cpDNA restriction patterns were also valid for the parents of the hybrids.

To determine the level of biparental inheritance of cpDNA which was detectable by autoradiography, samples of blue spruce and white spruce cpDNA cut with Cla I were mixed and separated in a gel (Fig. 4A) and probed with P16 (Fig. 4B). The amount of blue spruce cpDNA was kept constant, and 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 1000-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 Cla I restriction patterns of six F_1-hybrids compared to the parental species. All six hybrids are from different controlled crosses, and there are no parents in common between them. In the six hybrids shown, and four 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. The blue spruce x

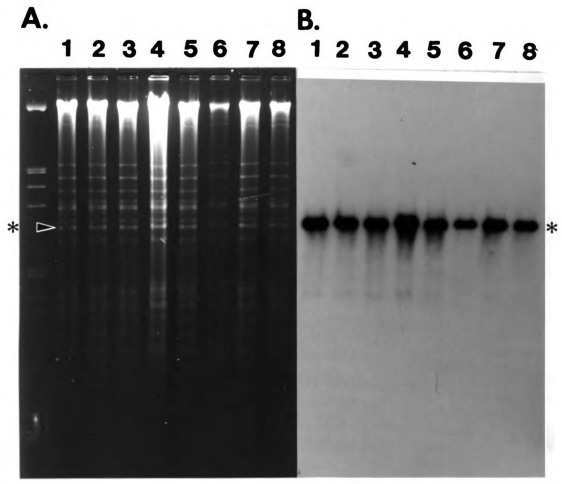

Fig. 3. Blue spruce provenance test. A. Cla I restriction patterns of cpDNA from single trees from Arizona (lanes 1-2), New Mexico (lane 3), Colorado (lanes 4-5), Wyoming (lane 6), and Utah (lanes 7-8). Arrows and "*" indicate bands identified in Fig. 1. Fragments were electrophoretically separated in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

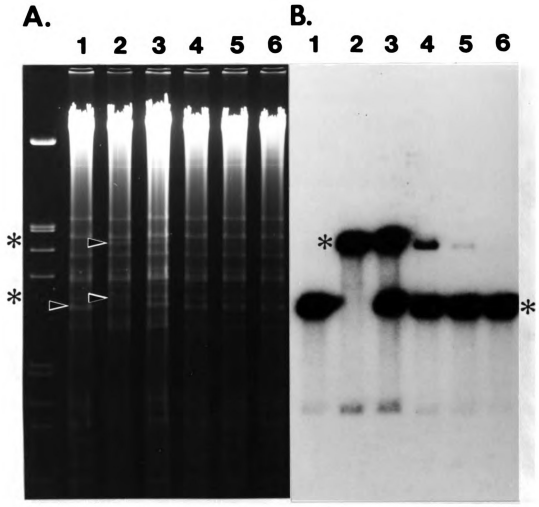

Fig. 4. Demonstration of autoradiographic techniques to detect under-represented DNA. Cla I restiction fragments of cpDNA from blue spruce (lane 1), white spruce (lane 2), equal amounts of each (lane 3), blue spruce plus 10-fold, 100-fold and 1000-fold dilutions of white spuce (lanes 4-6 respectively). Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.5 V/cm, 8 hours. B. Hybridization of P16 to a Southern filter from the gel shown in Fig. 4A.

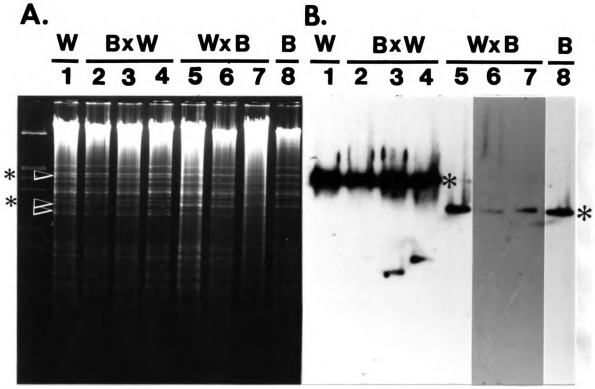

Fig. 5. Comparison of cpDNA from F_1-hybrids to parental species. A. Cla I restriction patterns of parental species and hybrids. Lane 1, white spruce; lanes 2-4 blue spruce x white spruce hybrids (720058, 720060, and 720061 respectively); lanes 5-7 are white spruce x blue spruce hybrids (720007, 720008, and 720011 respectively); lane 8, blue spruce. The restriction fragments were separated in 1.0% agarose, TBE buffer, 1.25 V/cm, 11 hours. B. Hybridization of probe P16 to Southern filter of gel shown in Fig. 5A.

white spruce hybrids (lanes 2-4) showed no variation in the 4.45-kb band ("*") and no evidence of the 2.9-kb band ("*") that would indicate some level of maternal transmission of cpDNA. The filter was then over-exposed, and no evidence of the 2.9-kb band was seen. In these hybrids, the weakly hybrizing band is either 1.21 or 1.33-kb in size, representing the variation demonstrated in Fig. 2.

The white spruce x blue spruce hybrids (lanes 5-7) show the paternal cpDNA type (2.9-kb band). Lane 5 (720007) shows what is apparently a low level of maternal transmission of the 4.45-kb band. The only hybrids that showed any level of putative maternal bands are white spruce by blue spruce. Several "pure" blue spruce also showed low levels of the 4.45-kb band, indicating possibly heteroplasmic or chimeric individuals. Since only the 4.45-kb band was present in low levels, and never the 2.9-kb band in "pure" white spruce or blue spruce x white spruce hybrids, the possiblity of low levels of the 4.45-kb band being due to partial digestion of the DNA samples was investigated.

Blue spruce cpDNA samples were digested with Cla I according to the manufaturers instructions, except the length of digestion was varied. Samples were digested for 1 minute, 10 minutes, 1, 2 or 8 hours, and then electrophoretically separated and transferred to nitrocellulose filters. Probing with P16 yeilded strong signals corresponding to both the 2.9 and 4.45-kb bands at 1 minute. The 4.45-kb band rapidly decreased in intensity with increasing time, and was barely visible in an overexposed autoradiograph in the lane with the sampled digested for 8 hours (data not shown). Since the cpDNA samples were routinely digested for two to four hours, the apparent low level of the 4.45-kb band most likely represents a partial digestion product, rather than a low level of maternal

transmission of cpDNA. To confirm this hypothosis the cpDNA sample 720007 was redigested for eight hours, and the 4.45-kb band showed reduced intensity compared to the stored cut sample used in the gel in Fig. 5 (data not shown). Thus, all ten hybrids examined showed only the paternal cpDNA restriction patterns.

The difference between the blue spruce 2.9-kb band and the white spruce 4.45-kb band appears to be a Cla I restriction site mutation. We cannot confirm this however, as we did not prepare restriction site maps for these species. This was due to the high level of nucDNA in the samples which would have made cloning fragments difficult. 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)

Another piece of evidence which indicates the RFLPs used in this study are not nuclear in origin, but represent organelle DNA, is the non-Mendelian pattern of the inheritance of the RFLPs. If the RFLPs are nuclear in origin F_1-progeny of reciprocal crosses should show identical patterns. Since the inheritance of the RFLPs appears to be strictly uniparental, in this case paternal, the RFLPs must represent cytoplasmic DNA.

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, 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 10 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, three 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 1 maternal pattern, out of a total of six interspecific hybrids of *Larix*. When examining the cpDNA of eight F_1-hybrids of Jack pine x lodgepole pine, Wagner et al. (1987), like this study, found only the paternal cpDNA type.

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 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 monoterpenes 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 range of morphological characteristics of the parental species overlap.

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CHAPTER II

Inheritance of plastids in reciprocal crosses of white spruce and Serbian spruce.

ABSTRACT

Chloroplast DNA (cpDNA) was purified from Serbian spruce (*Picea omorika* (Panic) Purkyne) 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 two species. Intraspecific conservation of the RFLPs that distinguish the two spruce species was confirmed by examining the two Serbian spruces that served as parents of the hybrids. Because the white spruce parents of the hybrids were not available, eight trees from the natural range of white spruce were examined to determine the typical white spruce cpDNA RFLP pattern. The cpDNA from 17 F_1-hybrid trees had the banding pattern of the paternal species. Cloned *Petunia* cpDNA containing part of the rbcL gene hybridized to polymorphic bands; in contrast a cloned maize mtDNA probe of the coxII gene did not hybridize to any band.

INTRODUCTION

Recently we demonstrated paternal inheritance of plastids in interspecific hybrids of blue spruce (*Picea pungens* Engelm.) and white spruce (Stine et al. 1988). In this report, we demonstrate paternal inheritance of cpDNA in hybrids of white spruce and Serbian spruce. This further illustrates how chloroplast inheritance in members of the Coniferales differs from that of the angiosperms, which show either maternal or biparental, but not predominantly paternal inheritance of plastids (Sears 1980, Whatley 1982).

As in Stine et al. (1988), we used restriction analysis of chloroplast DNA (cpDNA) to study chloroplast inheritance. This technique has been used successfully to determine the inheritance of the organelles with a high degree of certainty in *Pelargonium* (Metzlaff et al. 1981) and in *Triticum* and *Aegilops* (Bowman et al. 1983), and is becoming a common method to study organelle inheritance (Palmer 1985). The main advantages of this technique are that there is no need to either find or induce chloroplast mutants, there is no question whether or not the mutation affects the inheritance of the organelles, and there is no ambiguity as to whether there is differential sorting out of the organelles following fertilization. All of the aforementioned are problematic with the long generation time of spruce, and the varying degrees of delayed fertilization following pollination (Wilson and Burley 1985).

Recently, several other reports have used restriction fragment length polymorphism (RFLP) analysis of cpDNA to follow the inheritance of chloroplasts in 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), with the remaining

three having apparently non-parental restriction patterns. Szmidt et al. (1987) reported finding the paternal cpDNA restriction pattern in three of six interspecific F_1-hybrid progeny of *Larix*, with one showing the maternal pattern, and the remaining two having non-parental restriction patterns. Paternal inheritance of cpDNA has also been demonstrated in F_1-hybrids of lodgepole pine *(Pinus contorta* Dougl. ex. Loud.) x jack pine (*P. banksiana* Lamb.) examined (Wagner et al. 1987), and hybrids of white spruce and blue spruce (Stine et al. 1988). Neale and Sederoff (1988) also reported paternal cpDNA transmission in redwood (*Sequoia sempervirens* D. Don Endl.).

This article follows the same approach reported earlier for analysis of white spruce and blue spruce hybrids (Stine and Keathley 1988), and reports the strictly paternal transmission of cpDNA in F_1-hybrids from reciprocal crosses of Serbian spruce and white spruce.

MATERIALS AND METHODS

Plant Material

All trees used in this study are located at the Kellogg Experimental Forest, 7060 N. 42nd St., Augusta, MI 49012, or on the East Lansing campus of Michigan State University (MSU). The accession number, plantation number, and the row and column numbers for each tree are listed in Table A2, and the parentage of the hybrids in Table 1. The two Serbian spruce trees that were used to produce all of the hybrids are located on the the north side of Service Rd. between Farm Lane and Bogue St. on the MSU campus. The two white spruce trees listed in Table 1. were in plantation 70.21 at Kellogg Forest, but were removed in 1986, when this plantation

Table 1. Parentage of white and Serbian spruce hybrids.

Hybrid	Number Examined	Female Parent Species	Female Parent Accession Number*	Pollen Parent Species	Pollen Parent Accession Number*
710003	4	*P. omorika*	270002	*P. glauca*	190423
710004	4	*P. glauca*	190423	*P. omorika*	270001
710005**	5	*P. glauca*	190424	*P. omorika*	270001
				P. omorika	270002
710006	3	*P. omorika*	270001	*P. glauca*	190423
710007	1	*P. glauca*	190423	*P. omorika*	270002

* Add 67,000,000 to obtain the complete MICHCOTIP accession numbers
** 710005 hybrids resulted from pollination with bulked pollen from 270001 and 270002

was converted to a seed orchard for F_2-hybrid seed production. The hybrids of Serbian and white spruce are located in Michigan Cooperative Tree Improvement Program (MICHCOTIP) plantation 78.01, and were planted in 1980. Complete records on these trees can be obtained from MICHCOTIP, Michigan State University, East Lansing, MI 48823.

The entire perimeter of the crown of each tree was sampled at 1.0-1.5 m above ground, and between 75 and 100g of the current and previous season's needles were sampled. The cpDNA was isolated by differential centrifugation following the procedures for cpDNA preparation and analysis described by Stine et al. (1988). Digestion of the DNA samples with restriction endonucleases followed the instructions of the various manufacturers. Standard procedures for agarose gel electrophoresis, and Southern transfer were used (Manniatis et al. 1982). Lambda DNA cut with Hind III and Eco RI was used to provide molecular size markers. *Petunia* cpDNA clones, described in Systma and Gottlieb (1986), were provided by J. Palmer, c/o D. Neale, Pacific Southwest Forest and Range Experiment Station, 1960 Addison St., Berkeley, CA 94701. The cloned probe pZmE1,

described in Fox and Leaver (1981), which contains the gene for cytochrome oxidase subunit II from maize mitochondria DNA (mtDNA), was provided by T. Fox. The probes were labeled by random primed labeling according to the manufacturer (Boehringer Mannheim Biochemicals), and were hybridized to the filters according to the procedures of Maniatis et al. (1982). The filters were washed as described by Stine et al. (1988).

RESULTS AND DISCUSSION

This study is a further illustration of the phenomenon of paternal transmission of chloroplasts in members of the coniferales. Since genetic evidence of the inheritance pattern of organelles in most species of this family is still lacking, it was necessary to rigorously demonstrate that the RFLPs that showed paternal inheritance, are in fact cpDNA. The RFLPs were shown to be cpDNA by several methods.

The RFLPs were generated using restriction enzymes that are methylation sensitive. These enzymes cut cpDNA which is not methylated, but only rarely cut methylated nuclear DNA (Palmer 1985). The enzymes Cla I and Ava I were chosen because they were useful in differentiating cpDNA from white spruce and blue spruce (Stine et al. 1988). Since blue spruce is more closely related to white spruce, than is Serbian spruce (Wright 1955, Liu 1982), we predicted that these enzymes would successfully differentiate white spruce cpDNA from Serbian spruce cpDNA.

Several interspecific cpDNA RFLPs produced by digesting cpDNA with Cla I and Ava I that differentiate Serbian spruce cpDNA and white spruce cpDNA are shown in Fig. 1A. Diagnostic bands are labeled with arrowheads and are listed in Table 2. The broad band of fluorescence near the

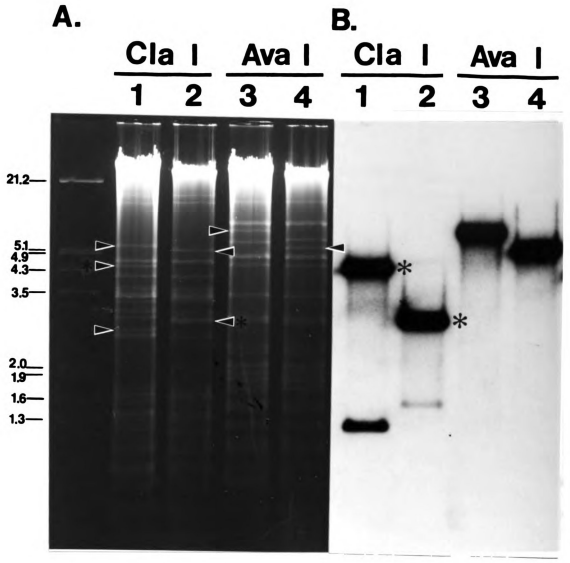

Fig. 1. Identification of RFLPs that differentiate white spruce and Serbian spruce. A. Restriction patterns of cpDNA from white spruce (lanes 1 and 3) and Serbian spruce (lanes 2 and 4). RFLPs that differentiate Serbian spruce and white spruce, are identified by arrows; "*" fragments, see text. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.0 V/cm, 8 hours. B. Hybridization of cloned, ^{32}-P labeled, *Petunia* cpDNA (probe P16) to RFLPs indicated in Fig. 1A.

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

Fragment (kb)	White spruce	Serbian spruce
Cla I 5.2	+	-
Cla I 5.15	-	+
Cla I 4.45	+	-
Cla I 2.9	+	++
Cla I 2.8	+	-
Ava I 6.1	+	-
Ava I 5.2	-	+

+ = present - = absent
++ = a stoichiometrically double intensity band

top of each lane is probably nuclear DNA. Those bands marked with a "*" represent Cla I fragments of 4.45 and 2.9-kb (white spruce and Serbian spruce respectively) which were used to demonstrate the inheritance of cpDNA by autoradiographic methods.

The RFLPs were identified as cpDNA by probing with cloned *Petunia* cpDNA. The clone bank used represents 92% of the petunia chloroplast genome, and all 13 cloned fragments used, hybridized to visible fragments on the filters. In no case did the probes hybridize to regions on the filter that did not correspond to visible bands on the gels. Figure 1B shows an autoradiograph produced by probing a Southern filter from the gel shown in Fig. 1 with a 4.1 kb *Petunia* cpDNA fragment. This cloned fragment (P16) is from the large single copy region of the *Petunia* cp genome, and contains part of the large subunit of ribulose bisphosphate carboxylase-oxygenase (rbcL) gene. Another probe (P3), which contains the rest of the rbcL gene on a fragment of 21.0 kb in size, also hybridizes to these RFLPs, in addition to several other non-polymorphic bands

(data not shown). These results help to demonstrate that these RFLPs represent cpDNA.

The third approach to identify these RFLPs, was to probe using a highly conserved mtDNA probe, in order to demonstrate the presence or absence of mtDNA in our cpDNA preparations. This was necessary because DNA sequences which are putatively of chloroplast origin are commonly found in the mitochondrial genome (Sederoff 1987). The coxII gene from maize mitochondria (probe pZmE1) failed to hybridize to the lanes containing cpDNA digests (data not shown). The probe does hybridize to filters with a positive control lane (pZmE1), and to filters with total DNA preparations of white spruce (David, personal communication). Thus indicating that the cpDNA preparations do not contain mtDNA.

The two Serbian spruce that were used as either the female or pollen parent in all of the hybrids were analyzed for their Cla I cpDNA restriction patterns. The white spruce parents were not available for analysis in this study. The Cla I cpDNA RFLPs in white spruce were previously checked by the analysis of the cpDNA from eight trees from throughout the natural range of white spruce, and were shown to be conserved (Stine et al. 1988).

Figure 2A shows the Cla I restriction patterns of five F_1-hybrids compared to their Serbian parents and an unrelated white spruce. All five hybrids shown are from different controlled crosses. A total of 17 hybrids were examined. In the ten white spruce x Serbian spruce hybrids, only the Serbian parent restriction pattern was found. The seven Serbian spruce x white spruce hybrids showed a typical white spruce cpDNA restriction pattern. A southern filter from this gel was probed with P16 (same as used in Fig. 1B), and the autoradiograph is shown in Fig. 2B.

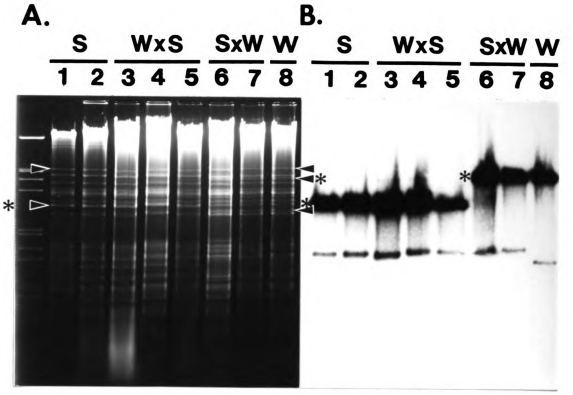

Fig. 2. Comparison of Cla I digests of cpDNA from F_1-hybrids to their parental species. Restriction patterns of cpDNA digested with Cla I. Lanes 1-2, Serbian spruces 270001 and 270002 respectively; lanes 3-5, white spruce x Serbian spruce hybrids 710004, 710005 and 710007 respectively; lanes 6-7, Serbian spruce x white spruce hybrids 710003 and 710006 respectively; lane 8, white spruce. Arrows indicate polymorphisms. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.0 V/cm, 14 hours. B. Hybridization of P16 to some of the RFLPs identified in Fig. 2A.

The bands to which P16 hybridized represent the paternal cpDNA type. The filter was then overexposed to check for possible heteroplasmy or chimeric F_1-hybrids. No evidence of maternal patterns were found (data not shown). All 17 hybrids were examined in the same manner and no evidence for heteroplasmic or chimeric individuals was observed. The probe P16 hybridized weakly to a low molecular weight band in addition to the "*" fragments. In Fig. 2A lanes 1-7 show hybridization to a 1.33-kb band, while lane 8 has a 1.21-kb band. We demonstrated that this fragment shows intraspecific variation previously (Stine et al. 1988). Furthermore, the Serbian spruce and white spruce hybrids (lanes 1-7) are not related to the particular white spruce shown in lane 8, and thus the variation is certainly an RFLP which is individual-specific, rather than species specific.

Another piece of evidence which indicates RFLPs identified in this study are not nuclear in origin, but represent organelle DNA (cpDNA or mtDNA) is that they are uniparentally inherited. If the RFLPs were nuclear DNA, the RFLP patterns would be expected to be identical in F_1-hybrid progeny from reciprocal crosses. Since the inheritance appears to be strictly uniparental, in this case paternal, cytoplasmic DNA is indicated.

The hybrids used in this study are from a continuing spruce breeding program, and while they show intermediate foliage characteristics, the hybrid nature of these trees has not been critically determined. In this study, the cpDNA found in the hybrids was always that of the paternal species. Assuming correct species identification of the female and pollen parents, and the demonstration of conservation of cpDNA restriction

patterns within white spruce, analysis of cpDNA should prove valuable for verification of hybrids in continued breeding of spruces.

In this study, as in our previous study with hybrids of white spruce and blue spruce (Stine et al. 1988), we only found the paternal cpDNA in the progeny. Wagner et al. (1987) also reported finding only the paternal cpDNA type in F_1-hybrids of Jack pine (*Pinus banksiana* Lamb.) and lodgepole pine (*P. contorta* Dougl.). Neale et al. (1986) and Szmidt et al. (1987), however, reported finding nonparental cpDNAs in some progeny. Neale et al. (1986), used cpDNA RFLPs to demonstrate predominantly paternal transmission of plastids in intraspecific crosses of Douglas-fir, although 3 of 36 progeny showed nonparental patterns in their cpDNA restriction digests. Szmidt et al. (1987) also report finding nonparental cpDNA restriction patterns in 2 out a total of 6 interspecific hybrids of *Larix,* in addition to one maternal restriction pattern. Since recombination of cpDNA has not been demonstrated in sexual crosses of higher plants (e.g. Chiu and Sears 1985), there is no reason to expect to find other than parental cpDNA types in the progeny, and the aberrant cpDNA patterns reported by Neale et al. (1986) and Szmidt et al. (1987) likely represent pollen contamination or mutations. However, the occurrence of cpDNA mutations in 8.3% of the progeny of intraspecific hybrids of Douglas-fir, or in 33.3% of interspecific larch hybrids as reported by Neale et al. (1988) and Szmidt et al. (1988) respectively, would not fit the current model of the cpDNA genome as slowly evolving (Zurawski and Clegg 1987).

The mechanism for paternal inheritance in *Picea* is still unclear, though microscopy studies of fertilization in the Pinaceae indicate the pollen tube plastids 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 plastids from the pollen tube enter the egg cytoplasm during fertilization, and Willemse (1974) also demonstrated that probably all plastids from the female parent are excluded from the neocytoplasm. Owens and Simpson (1988) have shown that during fertilization of Douglas-fir (*Pseudotsuga menziesii*), male organelles enter the egg cell, and a small portion of the pollen tube cytoplasm migrates along with the male gamete to the female nucleus. In *Biota*, a member of the Cupressaceae, Chesnoy (1967) demonstrated by light microscopy, the exclusion of female plastids from the neocytoplasm. Thus, in the Coniferales, the mechanism of paternal inheritance of plastids appears to involve plastids of paternal origin entering the archaegonium during fertilization, coupled with the exclusion of maternal plastids from the neocytoplasm.

The evidence for paternal inheritance of plastids in gymnosperms is increasing, with reports showing genetic evidence of paternal inheritance for two genera of the Taxoideacae, *Crytomeria* (Ohba et al 1971), *Sequoia* (Neale and Sederoff 1988); and four genera of the Pinaceae, *Pinus* (Neale et al. 1988, Wagner et al. 1987), *Picea* (Stine and Keathley 1988, and this report), *Pseudotsuga* (Neale and Sederoff 1988) and *Larix* (Szmidt et al. 1987). There are still 10 other families of gymnosperms for which there have been no reports. The further study of plastid inheritance in gymnosperms may elucidate whether paternal inheritance is a derived character or represents the primitive state. It may also lead to a further understanding of why uniparental inheritance of plastids (either maternal or paternal) is much more common than biparental inheritance.

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CHAPTER III

Paternal inheritance of plastids in Engelmann spruce X blue spruce hybrids.

ABSTRACT

Chloroplast DNA (cpDNA) was purified from blue spruce (*Picea pungens* Engelm.) and Engelmann spruce (*P. engelmannii* Parry ex Engelm.), and was digested with several restriction endonucleases. Restriction fragment length polymorphisms (RFLPs) were identified that differentiated both species. Intraspecific conservation of the RFLPs that distinguished each species was confirmed by examining eight trees from the natural range of blue spruce and five of Engelmann spruce. Four Engelmann spruce x blue spruce F_1-hybrids were examined, and the cpDNA from each showed the banding pattern of blue spruce. Three naturally occurring, putative hybrids showed typical Engelmann spruce banding patterns, which is inconsistent with the reported unilateral crossing incompatibility (crosses work only with Engelmann spruce as the female parent), and the apparent paternal inheritance of plastids in *Picea*. These results suggest that these trees are not hybrids. Cloned *Petunia* cpDNA containing part of the rbcL gene hybridized to the polymorphic bands, while a cloned maize mtDNA probe of the coxII gene, failed to hybridize to any bands in the gels.

INTRODUCTION

The classic method for studying the inheritance of plastids is to utilize plastid mutants (either natural or induced) in reciprocal crosses. In such analyses angiosperms exhibit either maternal inheritance or biparental inheritance of the plastids (Sears 1980, Whatley 1982). Among gymnosperms, evidence suggests that the inheritance of the plastids 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 to 99% of the time, providing the first genetic evidence for predominantly paternal transmission of plastids in the gymnospermae.

These genetic data have been supplemented by the application of molecular techniques in the restriction analysis of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA). It has been possible by comparing restriction fragment length polymorphisms (RFLPs) of either cpDNA or mtDNA to ascertain the inheritance of the organelles with a high degree of certainty in *Pelargonium* (Metzlaff et al. 1981) and in *Triticum* and *Aegilops* (Bowman et al. 1983). The advantages of this technique are that there is no need to find or induce chloroplast or mitochondrial mutants, there is no question whether or not the mutation affects the inheritance of the organelles, and there is no ambiguity as to whether differential sorting out of the organelles occurs following fertilization.

Several recent reports have utilized RFLP analysis of cpDNA to determine the inheritance pattern of chloroplasts in members of the Coniferales. Neale et al. (1986) reported predominantly paternal transmission of plastids in intraspecific crosses of Douglas-fir (*Pseudotsuga*

menziesii (Mirb) Franco). Szmidt et al. (1987) also have reported finding the paternal pattern of cpDNA in interspecific F_1-hybrid progeny of *Larix*. Paternal inheritance of cpDNA was demonstrated in F_1-hybrids of lodgepole pine *(Pinus contorta* Dougl. ex. Loud.) x jack pine (*P. banksiana* Lamb.) (Wagner et al. 1987), and in interspecific hybrids of *Picea pungens* (Engelm.) and *P. glauca* (Moench) Voss (Stine et al.) and hybrids of *P. glauca* (Moench) Voss and *P. omorika* (Panic) Purkyne (Stine and Keathley 1988). Neale and Sederoff (1988) have also reported paternal cpDNA transmission in redwood (*Sequoia sempervirens* D. Don Endl.).

We present here an additional demonstration of paternal inheritance of cpDNA in *Picea*. Interspecific F_1-hybrids of *P. engelmannii* x *P. pungens* were examined, as were several putative hybrids from Colorado, in an area sympatric to the two species.

MATERIALS AND METHODS

Plant Material

The blue spruce trees used in this study are located in the Kellogg Experimental Forest, 7060 N. 42nd St., Augusta, MI 49012. Accession number, plantation number, and the row and column numbers for each tree are listed in Table A1, and 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 of blue spruce established in 1970. Detailed records on the trees used in this study can be obtained from the Michigan Cooperative Tree Improvement Program (MICHCOTIP), Michigan State University, East Lansing, MI 48824.

Table 1. Parentage of Engelmann x blue spruce hybrids.

Hybrid Number*	Female Parent Species	Female Parent Accession Number	Pollen Parent Species	Pollen Parent Accession Number
610083	*P. engelmannii*	170509	*P. pungens*	310915
610113	*P. engelmannii*	170517	*P. pungens*	310905
610114	*P. engelmannii*	170517	*P. pungens*	310903
610111	*P. engelmannii*	170516	*P. pungens*	310915

* Add 67,000,000 to obtain the complete MICHCOTIP accession numbers.

The Engelmann spruce, and putative Engelmann and blue spruce hybrids, are located in the Dolores River drainage in southwest Colorado, described by Ernst et al. (1988). The Engelmann spruce x blue spruce hybrids are located at the Tree Research Center, Michigan State University, East Lansing, MI 48824.

The perimeter of the crown of each tree was sampled at least two points, and between 75 and 100g of the current and previous season's needles were sampled. The procedures for chloroplast DNA (cpDNA) preparation and analysis were described previously (Stine et al. 1988). Digestion of the DNA samples with restriction endonucleases followed the instructions of the various manufacturers. Standard procedures for agarose gel electrophoresis, and Southern transfer, as described in Maniatis et al. (1982) were used. *Petunia* cpDNA clones, described by Systma and Gottlieb (1986), were provided by J. Palmer, c/o D. Neale, Pacific Southwest Forest and Range Experiment Station, 1960 Addison St., Berkeley, CA 94701. A maize mtDNA probe (pZmE1), described by Fox and Leaver (1981) and containing the gene for cytochrome oxidase subunit II (coxII), was

provided by T. Fox. The probes were labeled by random primed labeling according to the manufacturer (Boehring Mannheim Biochemicals), and were hybridized to the filters according to the procedures of Maniatis et al. 1982. The filters were washed as described in Stine et al. (1988).

RESULTS AND DISCUSSION

RFLPs that differentiated Engelmann spruce from blue spruce were identified using several enzymes. Figure 1A shows the RFLPs that are produced by the enzymes Cla I and Ava I. Diagnostic bands are labeled with arrows, and are also listed in Table 2. The broad band of fluorescence near the top of each lane is most probably nuclear DNA. Those bands with a "*" represent Cla I fragments of 2.9 and 4.45-kb in size from blue spruce and Engelmann spruce respectively, and are used to demonstrate paternal transmission by autoradiography.

Table 2. Diagnostic cpDNA RFLPs shown in Figure 1.

Fragment (kb)		Blue spruce	Engelmann spruce
Cla I	4.45	-	+
Cla I	2.9	++	+
Ava I	6.1	-	+
Ava I	5.2	+	-

+ = present - = absent
++ = Stoichiometrically double instensity

Figure 1B shows hybridization of a 4.1 kb *Petunia* cpDNA probe (P16) to a Southern filter produced from the gel shown in Fig 1A. P16 contains part of the large subunit of ribulose bisphosphate carboxylase-oxygenase

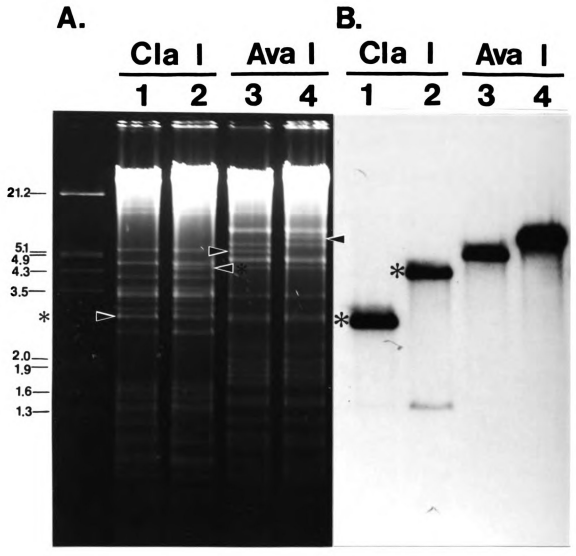

Fig. 1. Identification of RFLPs that differentiate Engelmann spruce and blue spruce. A. Restriction patterns of cpDNA from Engelmann spruce (lanes 1 and 3) and blue spruce (lanes 2 and 4). RFLPs that differentiate blue spruce and white spruce are identified by arrows. For RFLPs labeled "*" see text. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.0 V/cm, for 8 hours. B. Hybridization of P16 to a Southern filter from the gel in A.

(rbcL). Another probe (P3), which contains the rest of the rbcL gene on a fragment of 21.0 kb in size, also hybridizes to these RFLPs, in addition to several other non-polymorphic bands (data not shown).

There are many reports of DNA sequences of putative chloroplast origin being located in the mitochondrial genome (Sederoff 1987). To determine whether our cpDNA preparations contain mtDNA, the southern filter from Fig. 1A was also probed with a 2.1 kb mtDNA probe containing the gene for coxII from maize. The probe failed to hybridize to any band visible in Fig. 1A (not shown), but did hybridize to total DNA preparations from blue spruce (David, personal communication) under identical hybridization and washing conditions.

It has been previously demonstrated that the Cla I cpDNA restriction pattern is conserved in blue spruce throughout its range (Stine et al. 1988). The conservation of the Cla I cpDNA restriction pattern in Engelmann was checked in 5 trees from Colorado, either in or near the Delores river drainage (Fig. 2), and one tree of unknown origin, on the Michigan State University campus (not shown). No variation of the diagnostic Cla I cpDNA RFLPs was observed among these six trees, although the tree shown in lane 1 has a unique band of approximately 2.6-kb in size (lower arrowhead).

Figure 3A shows the Cla I restriction patterns of four F_1-hybrids from controlled crosses, and three putative hybrids, compared to the parental species. All four hybrids are from different controlled crosses. The four F_1-hybrids show the restriction pattern of blue spruce, the paternal species. The putative hybrids show banding patterns characteristic of Engelmann spruce. A Southern filter from this gel was probed with P16 (same probe as used in fig. 1B), and is shown in Fig. 3B.

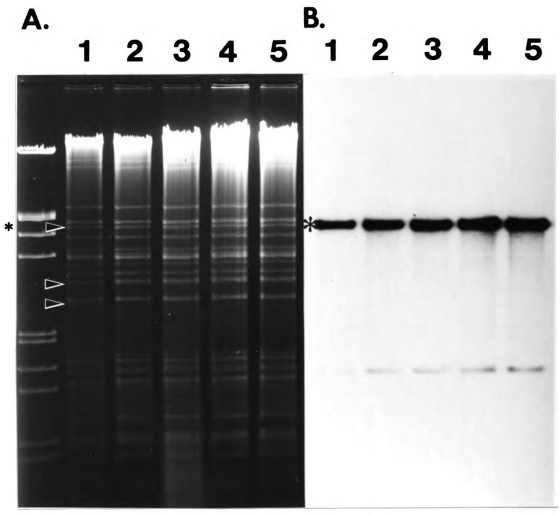

Fig. 2. Conservation of diagnostic cpDNA RFLPs in Engelmann spruce. A. Cla I restriction patterns of Engelmann spruce from within the Delores river drainage (lanes 1, 2 and 4), but outside the sympatric zone. Lanes 3 and 5, Engelmann spruce from outside the Delores river drainage. Fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.5 v/cm, 7 hours. B. Hybridization of P16 to the Southern filter from the gel shown in A.

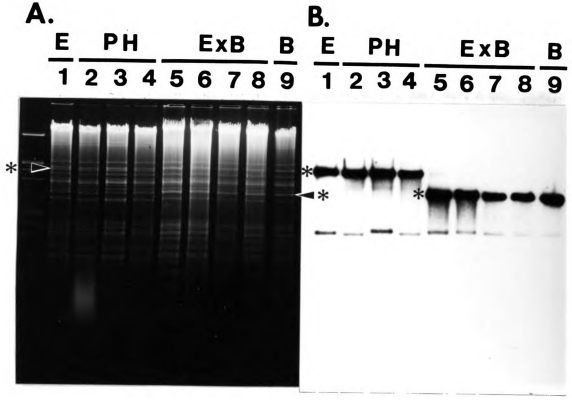

Fig. 3. Comparison of cpDNA from F_1-hybrids and putative hybrids to parental species. A. Cla I restriction patterns of cpDNA from Engelmann spruce (lane 1), putative Engelmann and blue spruce hybrids (lanes 2-4), Engelmann spruce x blue spruce F_1-hybrids 610083, 610113, 610114 and 610111 (lanes 5-8 respectively), blue spruce (lane 9). The restriction fragments were electrophoretically separated in 0.8% agarose, TBE buffer, 1.5 V/cm, 7 hours. B. Hybridization of P16 to a Southern filter of the gel shown in A.

The bands to which P16 hybridized represent the paternal cpDNA type. The filter was then over exposed, to check for possible heteroplasmy or chimeric F_1-hybrids, and no evidence of maternal patterns was observed (data not shown).

Ernst et al. (1988), Fechner and Clark (1969) reported that viable progeny resulted from interspecific hybridization of blue spruce and Engelmann spruce, only when Engelmann spruce was used as the female parent. The three putative hybrids examined were identified as likely hybrids based on discriminant analysis of morphological and monoterpene composition data (Schaefer and Hanover 1985). RFLP analysis of cpDNA from these trees showed typical Engelmann cpDNA patterns. If the unidirectional hybridization pattern found by Ernst et al. (1988) and Fechner and Clark (1988) is correct, and the paternal transmission of cpDNA found in the F_1-hybrids examined also is correct, then we would expect to find blue spruce cpDNA restriction patterns in the putative hybrids. Thus, the putative hybrids are most likely not F_1-hybrids. Possibly these trees represent F_2-hybrids (or later generations) that have backcrossed to Engelmann spruce.

In this study we have confirmed that the bands produced by the digestion of our DNA samples with Cla I or Ava I, are cpDNA by the fact they are produced by methylation sensitive enzymes, cloned cpDNA probes from *Petunia* hybridize to the RFLPs, while a mtDNA cloned probe from maize failed to hybridize to the same Southern filters, and the RFLPs do not assort independently as would be expected for nuclear DNA. As with other interspecific hybrids in spruce, hybrids of white spruce and blue spruce (Stine et al. 1988) and those of Serbian spruce and white spruce (Stine and Keathley 1988), we found the paternal cpDNA restriction

pattern in the F_1-hybrids. Proposed mechanisms for paternal inheritance of plastids have been discussed previously (Stine and Keathley 1988, Stine et al. 1988)

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SUMMARY AND CONCLUSIONS

In these studies, I have been able to identify cpDNA RFLPs that differentiate the four species of spruce under study. By examining the cpDNA from either the actual parents of F_1-hybrids, or from representatives of the natural range of each species, I have been able to identify species-specific plastome types in the F_1-hybrids. In the examination of F_1-hybrids, I have found only the cpDNA restriction pattern of either the actual pollen parent, or the species used as the pollen parent. The results of all three hybrid systems examined are listed in Table 1, and the summary of the pure species examined to assign the species-specific cpDNA restriction pattern are listed in Table 2. The only trees that did not show the expected cpDNA genotypes were three putative hybrids of blue and Engelmann spruce. The hybridization of blue and Engelmann spruces has been successful only when Engelmann spruce has been used as the female parent. The putative hybrids were expected to show a blue spruce cpDNA restriction pattern. Since the opposite result was obtained the hybrid nature of these three trees is left in question, but these findings do not rule out that these trees are F_2-hybrids (or later generations) that resulted from backcrosses with Engelmann spruce as the pollen parent.

The evidence for paternal inheritance of plastids in gymnosperms is increasing, with reports showing paternal inheritance for two genera of the Taxoideacae, *Crytomeria* (Ohba et al 1971), *Sequoia* (Neale and

Table 1. Hybrid spruces examined for plastid inheritance.

Hybrid	Number of Crosses	Total Number of Trees	Plastome Type
White X Serbian	3	10	Serbian
Serbian X White	2	7	White
Blue X White	4	4	White
White X Blue	6	6	Blue
Engelmann X Blue	4	4	Blue
Putative Hybrids		3	Engelmann
Totals	19	34	Paternal

Table 2. Conservation of intraspecific RFLPs in "pure" species.

Species	Number of trees	Variation in RFLPs
White spruce	9	No
Blue spruce	13	No
Serbian spruce	4	No
Engelmann spruce	6	No
Totals	32	No

and Sederoff 1988); and four genera of the Pinaceae, *Pinus* (Neale et al. 1988, Wagner et al. 1987), *Picea* (Stine and Keathley 1988a,b; Stine et al. 1988), *Pseudotsuga* (Neale and Sederoff 1988) and *Larix* (Szmidt et al. 1987). There are still 10 other families of gymnosperms for which there have been no reports. The further study of plastid inheritance in gymnosperms may elucidate whether paternal inheritance is a derived character or represents the primitive state. It may also lead to a further understanding of why uniparental inheritance of plastids (either maternal or paternal) is much more common than biparental inheritance.

Another example of paternal inheritance in the Pinaceae was reported by Allen (1976), who showed that hybrids of either longleaf pine (*Pinus palustris* Mill.) or slash pine (*P. elliottii* Engelm.) with loblolly pine (*P. taeda* L.) or shortleaf pine (*P. echinata* Mill.) showed rates of O_2 evolution in the dark, and stomate closure patterns that were similar to the paternal parent in reciprocal crosses.

While there is still very limited information on the chloroplast genome of gymnosperms, there is even less known about the mitochondrial genome of gymnosperms. Neale and Sederoff (1988) demonstrated the maternal inheritance of mtDNA in loblolly pine (*Pinus taeda*) by the RFLP analysis of mtDNA, which is to the author's knowledge the only report on the analysis of mtDNA in a gymnosperm. If paternal inheritance of plastids, and maternal inheritance of mitochondria, prove the rule, then several very interesting questions can be addressed.

First, it will be possible to do phylogenetic analysis of the gymnospermae through the analysis of two relatively simple genomes, and to simutaneously develop both maternal and paternal lineages. Theoretically, if the molecular clock hypothesis is correct, then both phylogenies should be similar. If rates of molecular evolution are not constant within both genomes, but vary independently, then very different phylogenies may be obtained.

It will also be possible to follow gene flow in populations for both maternally and paternally inherited traits. This will allow differences in dispersal mechanisms to be studied, ie. seed verses pollen.

Analysis of cpDNA could also be of use in confirming the hybrid nature of F_1-hybrids, and for identifying the male parent species of progeny resulting from open pollination of F_1-hybrids.

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APPENDIX

Table A1. Location of blue spruce, white spruce, and hybrid spruce trees used.

Species	Origin	Accession Number*	Plantation Number	Row	Column
P. pungens	Utah	310070	70.22	24	GG
P. pungens	N. Mexico	310045	70.22	24	FF
P. pungens	Utah	310068	70.22	24	EE
P. pungens	Colorado	310106	70.22	24	CC
P. pungens	Wyoming	310128	70.22	24	BB
P. pungens	Arizona	310192	70.22	23	DD
P. pungens	Colorado	310246	70.22	24	DD
P. pungens	Arizona	310193	70.22	23	AA
P. glauca	Ontario	191687	63.05	B	2
P. glauca	New York	191644	63.05	B	4
P. glauca	Manitoba	191631	63.05	B	5
P. glauca	S. Dakota	191628	63.05	B	6
P. glauca	Labrador	191657	63.05	B	7
P. glauca	N. Hampshire	191649	63.05	B	8
P. glauca	B. Columbia	191672	63.05	B	9
P. glauca	Saskacthewan	191665	63.05	B	10
P. glauca x pungens		720007	70.21	8	27
P. glauca x pungens		720008	70.21	7	36
P. glauca x pungens		720009	70.21	8	36
P. glauca x pungens		720010	70.21	8	39
P. glauca x pungens		720011	70.21	8	41
P. glauca x pungens		720017	70.21	7	29
P. pungens x glauca		720058	70.21	21	21
P. pungens x glauca		720059	70.21	25	23
P. pungens x glauca		720060	70.21	23	33
P. pungens x glauca		720061	70.21	25	33

* Add 67,000,000 to obtain the complete MICHCOTIP accession numbers.

Table A2. Location of white spruce, serbian spruce, and hybrid spruce trees used.

Species	Accession Number*	Plantation Number	Row Number	Column Number
Hybrid	710003	78.01	4	22
Hybrid	710003	78.01	25	20
Hybrid	710003	78.01	14	22
Hybrid	710003	78.01	57	22
Hybrid	710004	78.01	11	21
Hybrid	710004	78.01	32	21
Hybrid	710004	78.01	13	21
Hybrid	710004	78.01	12	20
Hybrid	710004	78.01	30	21
Hybrid	710005	78.01	25	20
Hybrid	710005	78.01	8	21
Hybrid	710005	78.01	58	22
Hybrid	710005	78.01	40	22
Hybrid	710006	78.01	7	22
Hybrid	710006	78.01	44	22
Hybrid	710006	78.01	41	22
Hybrid	710006	78.01	39	22
Hybrid	710007	78.01	38	22
P. omorika	270002	**	**	**
P. omorika	270003	**	**	**
P. glauca	190423	--	--	--
P. glauca	190424	--	--	--

* Add 67,000,000 to obtain the complete Michigan cooperative tree improvement program (MICHCOTIP) accession numbers.

** See text for locations.