



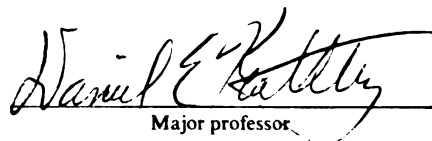
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SOUTHWESTERN COLORADO: GENETIC EVIDENCE FROM
RFLP ANALYSIS OF MITOCHONDRIAL AND CHLOROPLAST DNA

presented by

Andrew Joseph David

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SOUTHWESTERN COLORADO: GENETIC EVIDENCE FROM
RFLP ANALYSIS OF MITOCHONDRIAL AND CHLOROPLAST DNA

By

Andrew Joseph David

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ABSTRACT

NATURAL HYBRIDIZATION BETWEEN ENGELMANN AND BLUE SPRUCE IN SOUTHWESTERN COLORADO: GENETIC EVIDENCE FROM RFLP ANALYSIS OF MITOCHONDRIAL AND CHLOROPLAST DNA

By

Andrew Joseph David

Engelmann spruce (*Picea engelmannii*) and blue spruce (*P. pungens*) are two western spruce species that are morphologically very similar and usually separated by differences in elevation. Occasionally, the two species are found in parapatry where they may hybridize. There were 4 main objectives of this research. 1) Determine the inheritance of mitochondrial DNA (mtDNA) in *Picea*, 2) investigate the stability of the mechanism that controls inheritance of mtDNA in *Picea*, 3) ascertain the suitability of Restriction Fragment Length Polymorphism (RFLP) analysis, using mtDNA and chloroplast DNA (cpDNA) probes, as a technique for identifying natural hybrids, and 4) investigate a putative hybrid zone between Engelmann and blue spruce in the Scotch Creek drainage of southwestern Colorado.

RFLP analysis of total DNA from foliage samples of Serbian, white spruce and fifteen interspecific hybrids representing 5 separate crosses, including reciprocals, was used to demonstrate the maternal inheritance of mtDNA in *Picea*. Maternal inheritance was indicated in all hybrids for nine diagnostic enzyme/probe combinations (AvaI, BamHI,

ClaI, EcoRI, or ScaI/ATPase α and ClaI, EcoRI, ScaI, or PstI/COXII) and no paternal or non-parental bands were detected. Thus, the mechanism that controls the inheritance of mitochondria in *Picea* is functional even in wide interspecific crosses.

The next series of experiments utilized mtDNA and cpDNA probes and RFLP analysis to differentiate 14 of 14 artificial Engelmann X blue spruce F₁ hybrids from their parents. This was possible due to the uniparental inheritance of organelles in *Picea*, which dictates that mitochondria are maternally inherited while chloroplasts are paternally inherited, and the unilateral crossing incompatibility between Engelmann and blue spruce where hybrids are successful only when Engelmann spruce is the female parent. Finally, the same enzyme/probe combinations (BamHI/COXII and ClaI/P16) used above, plus two new combinations (AvaI/ATPase α and SmaI/P3) were used in the investigation of a putative hybrid zone which resulted in the discovery of four naturally occurring interspecific hybrids (one of which was heteroplasmic) and a potential introgressant. A fire disturbance model for the Scotch Creek drainage is presented that allows for the continued integrity of the two species despite high levels of natural hybridization and introgression.

**This dissertation is dedicated to my parents
Margaret and Donald David.**

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I greatly acknowledge T. Fox, C.S. Levings III, and J. Palmer for their generous donation of clones for COXII, ATPase α , and P16 and P3, respectively.

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INTRODUCTION

Historically, hybrid zones have been the subject of intense scrutiny because they represent an opportunity to test hypotheses about species interactions, gene flow, selection, drift, speciation, and reproductive isolation in a natural setting. But calling these areas hybrid zones without further explanation is somewhat misleading. Hybridization has been defined as "... the interbreeding of individuals from two populations or groups of populations, which are distinguishable on the basis of one or more heritable characters" (Harrison 1990). Although this adequately describes a hybrid zone in the strictest sense it does not describe F_2 or BC generations and so ignores the process of introgression. It is introgression, which is the movement of genes between the gene pools of two populations through hybridization and backcrossing, that creates the primary interest among researchers. Therefore, even though they are called hybrid zones, in reality the meaning is usually closer to introgression zones and is inclusive of advanced generation hybrids and backcrosses. Regardless of what they are called the potential importance of these areas has been recognized for some time. Over 45 years ago Anderson (1949) described introgression as "a vehicle for providing an increase in genetic variability in the gene pool of a population" and went on to state how this increase

in genetic variability, together with mutation, was all important in the evolution of the species.

Hybrid Zones

By definition, hybridization requires the two differentiated populations to be both in close physical proximity, either parapatric or sympatric, and genetically interfertile. When a hybrid zone forms between two species there are three possible outcomes 1) hybridization occurs forming a hybrid zone but there is no introgression; 2) asymmetrical or bidirectional introgression occurs; or 3) there is an increase in the reproductive isolation of the two species through reproductive character displacement (Arnold et al 1990a).

Because these three possibilities may seem at odds with each other each one will be considered separately.

Hybridization and the formation of hybrids may occur but because of hybrid inviability the F_1 s are not capable of crossing with themselves or either of the parental types. This creates a hybrid zone, or hybrid swarm, but no introgression. Intense selection against the hybrids may also render them incapable of reproducing if they are eliminated before they reach maturity. In general these possibilities are considered relatively rare and more common is the occurrence of some form of introgressive hybridization. One notable exception is *Quercus x morehus*

Kell. the F_1 hybrid between *Quercus kelloggii* and *Q. wislizenii*. An investigation into the nature of selected hybrid individuals determined that all were representative of an F_1 hybrid class and not advanced generation backcrosses or F_2 generation individuals (Nason et al 1992).

Introgressive hybridization occurs when any of the filial generations hybridize with one or both of the parental species (Anderson and Hubrecht 1938). Asymmetrical or unidirectional introgression occurs when the hybrids backcross to only one of the parents as in the case of *Populus fremontii* and *P. angustifolia* where the hybrid is involved in backcrosses only to *P. angustifolia* (Keim et al 1989). Bidirectional introgression occurs when the hybrids cross to both parental types, although it does not have to cross with an equal frequency to both parents. A good example of this is the introgressive hybridization between *Iris fulva* and *I. hexagona* in Louisiana, where both species possess ribosomal DNA and isozymes indicative of the other (Arnold et al 1990a, 1990b).

Hybridization can also cause an increase or decrease in the reproductive isolation of the two species through a process called reproductive character displacement. In this process certain individuals of each species are capable of creating hybrids while others are not. When these individuals do hybridize there are fewer opportunities for their genes to be passed on within their own taxon. This

can lead to an overall increase in the reproductive isolation of the two species, especially if there is selection pressure against the hybrids or selection pressure in conjunction with partial sterility (Levin 1985).

One factor that is important to the formation of hybrids is the availability of suitable habitat. Often this means habitat that is ecologically intermediate between the habitat requirements of the two parents. Although suitable habitat for hybrids can be formed by natural processes (fire, flood, landslides, etc) quite frequently it is human intervention, especially agriculture and development practices, that "hybridize the habitat" creating suitable combinations of light, moisture and soil conditions (Anderson 1948).

Introgression Zones

Although the formation of a hybrid zone does not necessarily imply that introgression will occur, it is the most common of the three possibilities mentioned above. The possible consequences of introgression are: 1) an increase in genetic diversity; 2) a decrease in reproductive isolation; 3) range expansion of the introgressed species, and 4) speciation (Arnold et al 1991; Reiseberg and Wendel 1993). Evidence for introgression in plants is not uncommon (for reviews see Knobloch 1972; Heiser 1973; Reiseberg and

Wendel 1993) but unambiguous evidence for each of the various consequences of introgression is rare.

Increases in the genetic diversity is one of the principle tenets of introgressive hybridization. Once the genetic bridge between the two species is made, gene flow can occur. In fact Anderson and Hubrecht (1938) defined introgressive hybridization as "the infiltration of germplasm from one species into another through repeated backcrossing of the hybrids to the parental species." It is upon this assumption that the other consequences of introgression are based. The obvious sources of an increase in genetic diversity are the alleles from the introgressing parent. What is overlooked is the potential for new alleles through intergenomic recombination (Golding and Strobeck 1983) and the expression of novel traits in the hybrid class (Cruzan and Arnold 1993, Rieseberg and Wendel 1993). It is this increase in genetic diversity that impacts the increase or decrease in relative fitness of the introgressed taxon.

Introgression can increase reproductive isolation in the same manner that a strict hybrid zone might but it can also decrease reproductive isolation. Some of the best studies that describe a breakdown in barriers to reproduction look at pollen viability in hybridizing populations over an extended period of time. In one case that spanned 30 years, pollen viabilities were measured for known first year hybrids and hybrid populations of *Salvia*

apiana and *S. mellifera*. First generation hybrids had a pollen viability of less than 50% whereas the hybrid populations stained at the same or greater level than the parental populations (Meyn and Emboden 1987). Taken to the extreme, a decrease in reproductive isolation between introgressing species could allow for one species to merge into the other creating a localized extinction.

Range expansion is also considered a possible outcome of introgression. In this case the introgressed species acquires the traits of a locally adapted species and uses these to extend its range. Theoretically this sounds very clean but experimentally it is extremely difficult to prove, the chief consideration being the ability to differentiate between similar responses of related species to the same selection pressure and the acquisition of adaptive traits.

Speciation is another potential consequence of introgression and one for which several examples exist. Grant and Grant (1964) have demonstrated the existence of different levels of allopatric speciation in *Gilia*, and Arnold and Bennett (1993) have shown that *Iris nelsonii* is a stabilized hybrid species of *I. hexagona*, *I. fulva*, and *I. brevicaulis*. Despite the fact that references in the literature to speciation are more numerous than those detailing increases in genetic variability, range expansion, or decreases in reproductive isolation barriers, it is plausible that the other possibilities are occurring as

often as speciation and it is the difficulty in their detection that keeps them from being reported. Equally plausible is the notion that multiple scenarios are being played out at any one time in a newly formed introgression zone. In all likelihood, with the exception of instantaneous speciation, introgression zones are as much a blending of the different possibilities as they are a blending of matings between individuals. It is only with time that one outcome evolves.

The remainder of this dissertation will be concerned with species in *Picea*. The first chapter is an investigation into the nature of mitochondrial inheritance in *Picea* and a determination of the effectiveness of the mechanism that controls mitochondrial inheritance in a wide interspecific cross within the genus. The second chapter is a determination of the feasibility of using organelle molecular markers to identify F_1 hybrids resulting from the controlled crosses of Engelmann and blue spruce parents. The final chapter is an investigation of a putative Engelmann spruce/blue spruce hybrid zone in the Scotch Creek drainage of southwestern Colorado.

LITERATURE CITED

- Anderson, E 1948. Hybridization of the habitat. *Evolution* 2:1-9.
- Anderson, E 1949. Introgressive hybridization. John Wiley and Sons, Inc., New York.
- Anderson, E and Hubrecht, L 1938. Hybridization in *Tradescantia*. III. The evidence for introgressive hybridization. *Amer. J. Bot.* 25:396-402.
- Arnold, ML and Bennett, BD 1993. Natural hybridization in Louisiana irises: genetic variation and ecological determinants. In: Hybrid zones and the evolutionary process. RG Harrison, ed., Oxford University Press.
- Arnold, ML, Burnett, BD, and Zimmer, EA 1990a. Natural hybridization between *Iris fulva* and *Iris hexagona*: pattern of ribosomal DNA variation. *Evolution* 44:1512-1521.
- Arnold, ML, Hamrick, JL, and Bennett, BD 1990b. Allozyme variation in Louisiana irises: a test for introgression and hybrid speciation. *Heredity* 65:297-306.
- Arnold, ML, Buckner, CM and Robinson, JJ 1991. Pollen-mediated introgression and hybrid speciation in Louisiana irises. *Proc. Natl. Acad. Sci. USA* 88:1398-1402.
- Cruzan, MB and Arnold, ML 1993. Ecological and genetic associations in an *Iris* hybrid zone. *Evolution* 47:1432-1445.
- Golding, GB and Strobeck, C 1983. Increased number of alleles found in hybrid populations due to intragenic recombination. *Evolution* 37:17-29.
- Grant, V and Grant, A 1964. Genetic and taxonomic studies in *Gilia*. XI Fertility relationships of the diploid cobwebby *Gilias*. *Evolution* 18:196-212.
- Harrison, R 1990. Hybrid zones: windows on evolutionary process. In: *Oxford Surveys in Evolutionary Biology* 7:69-128.
- Heiser, CB 1973. Introgression re-examined. *Bot. Rev.* 39:347-366.

- Keim, P, Paige, KN, Whitham, TG, and Lark, KG. 1989.
Genetic analysis of an interspecific hybrid swarm of
Populus: occurrence of unidirectional introgression.
Genetics 123:557-565.
- Knobloch, IW 1972. Intergeneric hybridization in flowering
plants. *Taxon* 21:97-103.
- Levin, DA 1985. Reproductive character displacement in
Phlox. *Evolution* 39:1275-1281.
- Meyn, O and Emboden, WA 1987. Parameters and consequences
of introgression in *Salvia apiana* X *S. mellifera*
(Lamiaceae). *Syst. Bot.* 12:390-399.
- Nason, JD, Ellstrand, NC, and Arnold, ML 1992. Patterns of
hybridization and introgression in populations of oaks,
manzanitas, and irises. *Am. J. Bot.* 79:101-111.
- Rieseberg, LH and Wendel, JF 1993. Introgression and its
consequences in plants. In: *Hybrid zones and the
evolutionary process*. RG Harrison, ed., pp70-109.
Oxford University Press, NY.

CHAPTER I

Inheritance of Mitochondrial DNA in Interspecific Crosses of *Picea glauca* and *P. omorika*

ABSTRACT

Fifteen interspecific hybrids of Serbian (*Picea omorika* (Panc) Purkyne) and white spruce (*P. glauca* (Moench) Voss) representing 5 separate crosses, including reciprocals, were used to demonstrate maternal inheritance of mitochondrial DNA. Total DNA was extracted from foliage samples of Serbian spruce (S), white spruce (W), and both S x W and W x S hybrids, digested and probed with one of two maize mitochondrial genes, ATPase α or COXII. ATPase α generated diagnostic Serbian and white spruce genotypes for all five enzymes tested while COXII differentiated between the two species for four of five enzymes. Maternal inheritance was indicated in all hybrids for every diagnostic enzyme/probe combination. No paternal or nonparental bands were detected. A dilution experiment indicated that the Serbian and white spruce mitochondrial DNA RFLPs could be detected in as little as 60 ng and 500 ng of total DNA respectively. It appears that the mechanism that controls the inheritance of mitochondria in *Picea* is still functional in wide interspecific crosses.

INTRODUCTION

Organelle inheritance within the angiosperms is primarily maternal (Sears 1980; Palmer 1987; Sederoff 1987) but among gymnosperms inheritance of organellar DNA varies. In *Picea*, inheritance of chloroplast DNA is paternal (Stine et al. 1989; Stine and Keathley 1990; Sutton et al. 1991) and mitochondrial DNA inheritance is maternal (Sutton et al. 1991; Stine et al. 1991) as it is for *Pinus* (Neale and Sederoff 1989), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Neale et al. 1986; Marshall and Neale 1992) and *Larix* (DeVerno et al 1993). However, in redwood (*Sequoia sempervirens* (D. Don) Endl.) (Neale et al. 1989) and incense cedar (*Calocedrus decurrens* (Torr.) Florin) (Neale et al. 1991) both organelles are inherited paternally.

Recent work suggests that organelles are not always inherited uniparentally in the Pinaceae. Cytological analysis of pre- and postfertilization events in Douglas-fir and western white pine indicated that leakage of paternal mitochondria could occur. In these species, the neocytoplasm, which includes some paternal mitochondria, migrates with the free nuclei towards the chalazal end of the archegonium prior to proembryo formation (Bruns and Owens 1989; Owens and Morris 1991) resulting in a potential for paternal or biparental inheritance of mitochondria.

Recent molecular studies support the occurrence of occasional paternal mitochondrial inheritance.

Approximately 7.1% of the progeny from diagnostic controlled crosses, either between jack pine (*Pinus banksiana* Lamb.) and lodgepole pine (*Pinus contorta* Dougl. ex Loud.) or among lodgepole pine, demonstrated paternal mitochondrial DNA inheritance (Wagner et al. 1991). Furthermore, if only diagnostic interspecific crosses of jack and lodgepole pine are considered, 10.9% of the seedlings indicated paternal or nonparental inheritance of mitochondrial DNA (Wagner et al 1991). Analysis of other interspecific crosses, particularly those that span section boundaries due to their greater potential divergence, may offer insights into the mechanism and efficiency of mitochondrial inheritance in the Pinaceae.

Picea is divided into three subsections, most commonly Eupicea, Casicta and Omorika, with white spruce placed in the subdivision Eupicea, and Serbian spruce in Omorika (Dallimore et al. 1967). Analysis of interspecific hybrids resulting from reciprocal crosses of Serbian and white spruce was undertaken to investigate the efficiency of mitochondrial inheritance in *Picea*.

MATERIALS AND METHODS

Plant Material

Fifteen interspecific hybrids from five different controlled crosses of Serbian and white spruce, along with

the two Serbian parents and eight white spruce, were used in this study. Two representative white spruce were included in hybrid analysis, following determination that white spruce was monotypic for the RFLPs of interest. This was necessary because the original parents were rogued from a progeny trial upon conversion to a seed orchard. The Serbian spruce used for this study were located on the campus of Michigan State University, East Lansing, Michigan. All other trees were located at the W.K. Kellogg Experimental Forest near Augusta, Michigan.

Four samples of current and previous years' foliage from points equidistant around the crown were collected for each tree at an approximate height of 1.3 meters and immediately placed on ice for transport to the laboratory. Foliage samples were stored in water at 4°C for 1-15 days.

Total DNA Extractions and Digestion

Needles were pooled for each tree and total DNA extractions were performed per Neale (personal communication) except the Extraction and Wash Buffers were optimized for spruce with 22 mM EDTA. Ten grams of freshly cut needle tissue were ground using a Tekmar Tissumizer (Tekmar Corporation, Cincinnati, OH 45222) in 100 ml of cold Extraction Buffer (50 mM Tris pH 8.0, 22 mM EDTA, 0.35 M sorbitol, 0.1% BSA, 10% PEG 3350, 0.1% B-mercaptoethanol) and filtered on ice through 2 layers of Miracloth

(Calbiochem, La Jolla, CA 92037) and a 400 μ mesh screen. The filtrate was centrifuged in a GSA rotor at 13,180 x g for 15 minutes at 4°C and the pellet resuspended in 5 ml of Wash Buffer (50 mM Tris pH 8.0, 22 mM EDTA, 0.35 M sorbitol, 0.1% B-mercaptoethanol). One-fifth volume of 5% sarkosyl was added, the solution gently swirled, and allowed to rest for 15 minutes at room temperature. One-seventh volume of 5 M NaCl was added and again swirled gently before 1/10 volume of CTAB solution (8.6% CTAB, 0.7 M NaCl) was added and swirled to completely mix. The entire solution was incubated in a water bath at 60°C for 15 minutes before 10 ml of chloroform:octanol (24:1) was added and an emulsion formed by inverting the test tube approximately 20 times. The sample was then centrifuged in a HB-4 rotor at 5000 x g for 10 minutes at room temperature. The upper aqueous layer was saved, 5 mls isopropanol was added and the solution swirled to precipitate the DNA. The DNA was hooked out using a modified Pasteur pipette and placed in 20 ml of 10 mM NH₄Ac in 76% EtOH, for 20 minutes. Afterwards the DNA was hooked out, transferred to a 1.5 ml microfuge tube containing 1.0 ml of TE, and allowed to redissolve at 4°C overnight, or briefly at 65°C if necessary, before being stored at 4°C.

Total DNA was digested with AvaI, BamHI, ClaI, EcoRI, ScaI or PstI according to manufacturers directions (BMB, Indianapolis, IN 46209) for 1-3 hours and stored at 4°C.

Southern Analysis

A total of 2.0 μ g of digested DNA, as determined by a Perkin Elmer Lambda 4B Spectrophotometer, was run per lane on 0.8% agarose gels at 1.0 v/cm for approximately 15 hours and blotted onto ZetaProbe nylon membranes (BioRad Industries, Hercules, CA 94547) using a 0.4 N NaOH buffer solution.

COXII probe DNA was made using the 2.1 kb maize cytochrome oxidase II mitochondrial gene insert from pZmE1, obtained from T. Fox (Fox and Leaver 1981). The pZmE1 was amplified and harvested according to the alkaline lysis method of Sambrook et al. (1989). The insert was cleaved from the plasmid using EcoR1, following manufacturer's (BMB) directions, concentrated by ethanol precipitation, and run overnight at 4°C on a 1.0% low melting agarose gel at 1.7 v/cm. The 2.1 kb insert fragment was excised from the gel and stored at 4°C with 1.5 volumes of double distilled water.

The ATPase α probe DNA was created using pTA22 containing the F₁-ATPase α subunit gene from cms-T maize mitochondria donated by C.S. Levings III (Braun and Levings 1985). The plasmid was amplified and harvested as above, digested with HindIII, according to manufacturer's (BMB) directions, and run on a 0.8% agarose gel for five hours at 2.1 v/cm. The 4.2 kb insert containing the ATPase α gene was

excised from the gel using a FMC (Rockland, ME 04841) SpinBind DNA recovery kit.

Creation of the radioactive probe was accomplished with the aid of a BMB random primer labeling kit following the directions for probe DNA isolated from low melting agarose. Each reaction used 3000Ci/mmol dCTP (DuPont NEN, Boston, MA 02118) along with approximately 20 ng of probe DNA. Hybridization solution was created with a minimum of 1×10^6 cpm/ml and hybridization proceeded at 65°C for at least 18 hours. Post hybridization washes were according to the ZetaProbe protocol, except that wash times were decreased to 20 minutes and only one wash with 1.0% SDS, 40 mM NaH_2PO_4 , 1 mM EDTA pH 7.2 was performed.

Southern filters were allowed to expose Kodak XAR-5 film at -70°C with DuPont Cronex Lightning Plus intensifying screens for 1-6 days. Filters were stripped according to directions for Zetaprobe nylon membranes (BioRad Industries).

RESULTS

Total DNA preparations from both S x W and W x S hybrids, when digested and probed with the nine diagnostic enzyme/probe combinations, always retained the maternal genotype (Table 1). No paternal or nonparental bands were detected in any hybrid.

A rangewide provenance of white spruce digested and probed with the ten enzyme/probe combinations showed conservation of a white spruce mitochondrial genotype. One white spruce from the provenance (#191687), originating from Kakabeka Falls, Ontario, Canada, possessed an additional RFLP for two enzyme/probe combinations. With *AvaI*/ATPase α #191687 possessed the white spruce RFLP and a second band that comigrated with the Serbian spruce 9.75 kb fragment. In the second case, *ScaI*/ATPase α , the same white spruce had the typical white spruce genotype and an additional 12.5 kb fragment. For the other eight enzyme/probe combinations the banding patterns for #191687 and all the other white spruce in the provenance were identical (Table 1).

Total DNA from Serbian spruce and S x W hybrids digested with *EcoRI* and probed with the maize mitochondrial gene COXII, (Fox and Leaver 1981) retained the diagnostic 7.8 kb Serbian RFLP (Figure 1). Similarly, white spruce and all W x S hybrids possessed a white spruce 4.85 kb RFLP (Figure 1). A 3.2 kb band common to both Serbian parents, all S x W hybrids, and two W x S hybrids was present when probed with COXII (Figure 1). However, stripping and probing the filter used in Figure 1 with pBR322 showed that the 3.2 kb band resulted from hybridization to the vector, not the COXII insert (Appendix A).

A dilution experiment of Serbian and white spruce DNA was performed which represented a range of 0.06 μ g - 2.0 μ g,

or 3% - 100% of the total DNA per lane in Figure 1. Serbian and white spruce bands were detectable to levels of 60 ng and 500 ng, or 3% and 25%, respectively (data not shown).

Table 1. Mitochondrial DNA genotypes for the white spruce provenance (W), Serbian spruce parents (S), and W x S and S x W hybrids.

Mt probe	Enzyme	White spruce provenance ^a	W x S hybrids ^a	S and S x W hybrids ^a
ATPase α	Ava I	3.65 ^b	3.65	9.75
	Bam HI	4.0	4.0	3.0 2.75
	Cla I	7.8	7.8	6.9
	Eco RI	11.5	11.5	1.75
	Sca I	15.0 ^c	15.0	8.8
COXII	Bam HI	2.6	2.6	2.6
	Cla I	8.0	8.0	8.0
		3.0	3.0	5.65
	Eco RI	5.35	5.35	7.8
		4.85	4.85	5.5
	Sca I	6.85	6.85	25.0
				23.1
				6.3
	Pst I	8.75	8.75	15.5
		4.0	4.0	8.75 4.0

^a All values are given in kilobase pairs.

^b White spruce #191687 possessed an additional 9.75 kb RFLP.

^c White spruce #191687 possessed an additional 12.5 kb RFLP.

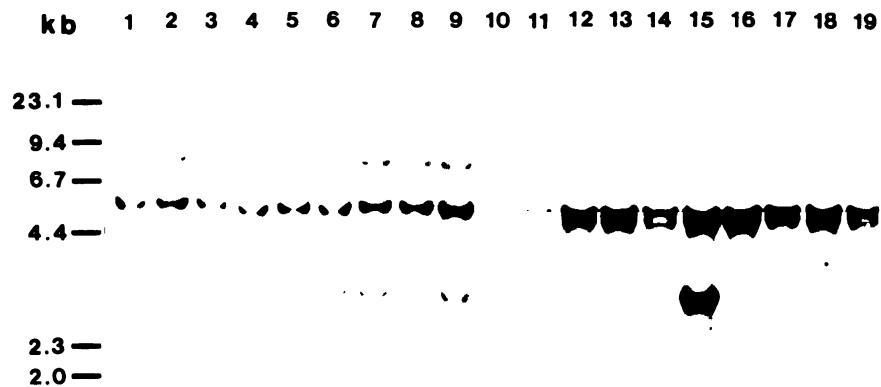


Figure 1. Hybridization of the mitochondrial gene COXII to EcoRI digested total DNA extracted from Serbian, white and interspecific hybrids of Serbian and white spruce. Lanes 1 and 2 are Serbian parents. Lanes 3-9 are Serbian x White hybrids from two separate crosses. Lanes 10 and 11 are representative white spruce. Lanes 12-19 are White x Serbian hybrids from 3 separate crosses. Molecular weight markers are λ HindIII.

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DISCUSSION

Information on the inheritance of mitochondria and the efficiency of the mechanism of mitochondrial inheritance in *Picea* has implications for breeding programs, phylogenetic studies, and research on the genetic structure of *Picea* populations. Differential organelle inheritance could impact the selection of parents in seed orchards or provide a mechanism, in conjunction with Southern analysis, to verify the status of commercially important hybrid seedling varieties. Because of its non-Mendelian inheritance, mitochondrial DNA can be used in phylogenetic studies and may be more accurate than plastid data due to lower nucleotide substitution rates in mitochondrial versus plastid genes (Palmer and Herbon 1988; Palmer 1990). Maternal inheritance of mitochondrial DNA also provides a method for comparing the results of plastid DNA based phylogenetic studies.

This study demonstrates that mitochondria are inherited maternally in *Picea*, even in a wide interspecific cross. Thus it appears that the mechanism that controls the maternal inheritance of mitochondria is still functional. No evidence of paternal inheritance was found in the hybrid progeny as there was in controlled crosses between jack and lodgepole pine (Wagner et al. 1991) although the sample size was low due to the small number of available interspecific *Picea* hybrids. No nonparental bands were detected, which

substantiates recent work on mitochondrial inheritance in pines (Neale and Sederoff 1989) Douglas-fir (Marshall and Neale 1992) and spruce (Sutton et al. 1991) although Wagner et al. (1991) reported a single instance of nonparental bands in an interspecific hybrid of lodgepole and jack pine.

In addition, no heteroplasmic individuals were detected even though a dilution experiment indicated that paternal bands could be detected, if any existed, to a level ranging from 3% to 25% of the total DNA in the lane. This means that if individual cells were heteroplasmic the paternal DNA type would be detectable if it comprised at least 25% of the mitochondrial DNA in the cell. Or, if individual branches on a tree were homoplasmic for maternal or paternal DNA types, then the paternal DNA type would be detectable if one of the four pooled samples contained the paternal DNA type.

The lack of heteroplasmic individuals or individuals showing paternal inheritance was interesting given the paternal inheritance of mitochondrial DNA in both inter- and intraspecific crosses in *Pinus* (Wagner et al. 1991) and cytological studies of archegonial and proembryo development in the Pinaceae suggesting that some paternal inheritance of mitochondria may be possible (Bruns and Owens 1989; Owens and Morris 1990; Owens and Morris 1991). The lack of genetic evidence in Douglas-fir (Marshall and Neale 1992) may have been due to a small sample size, the inability of Southern analysis to detect heteroplasmy at low levels, or

an enlarged perinuclear zone relative to *Pinus* (Owens and Morris 1991). This increased width could carry more maternal mitochondria and exclude paternal mitochondria during proembryo formation (Owens and Morris 1991).

Intraspecific variation at the ATPase α locus has been observed previously in Sitka but not white or Engelmann spruce (Sutton et al 1991). In conjunction with evidence for intraspecific variation in white spruce from this study and a lack of intraspecific variation at the COXII locus in both studies this may indicate that mutation and/or insertion/deletion events in the spruce mitochondria genome are more common near the ATPase α locus than the COXII locus.

The consistency of the results reported here lend confidence to ecological genetic studies which utilize differential organelle inheritance to identify naturally occurring F₁ hybrids and their corresponding introgression zones (Szmidt et al. 1988; Sutton et al. 1991), and provide a technique to answer questions about the genetic structure and evolution of natural and managed *Picea* populations.

LITERATURE CITED

- Braun, C.J., and Levings III, C.S. 1985. Nucleotide sequence of the F₁-ATPase α subunit gene from maize mitochondria. *Plant Physiol.* 79: 571-577.
- Bruns, D., and Owens, J.N. 1989. Mechanisms of cytoplasmic inheritance in western white pine. In *Proceedings of the 47th Annual Meeting of the Electron Microscopy Society of America*. Edited by G.W. Bailey. San Francisco Press, Inc. San Francisco, CA, pp. 766-767.
- Dallimore, W., Jackson, A.B., and Harrison, S.G. 1967. *A Handbook of Coniferae and Ginkgoaceae*. St. Martins Press, New York.
- DeVerno, L.L., Charest, P.J., and Bonen, L. 1993. Inheritance of mitochondrial DNA in the conifer *Larix*. *TAG* 86:383-388.
- Fox, T.D., and Leaver, C.J. 1981. The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. *Cell* 26 :315-323.
- Marshall, K.A., and Neale, D.B. 1992. The inheritance of mitochondrial DNA in Douglas-fir (*Pseudotsuga menziesii*). *Can. J. For. Res.* 22: 73-75.
- Neale, D.B., Wheeler, N.C., and Allard, R.W. 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. *Can. J. For. Res.* 16:1152-1154.
- Neale, D.B., Marshall, K.A., and Sederoff, R.R. 1989. Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. *Proc. Natl. Acad. Sci. USA.* 86: 9347-9349.
- Neale, D.B., and Sederoff, R.R. 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theor. Appl. Genet.* 77: 212-216.
- Neale D.B., Marshall, K.A., and Harry, D.E. 1991. Inheritance of chloroplast and mitochondrial DNA in incense-cedar (*Calocedrus decurrens*). *Can. J. For. Res.* 21: 717-720.
- Owens, J., and Morris, S. 1990. Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*: I. Pollen tube and archegonial development. *Am. J. Bot.* 77: 433-445.

- Owens, J., and Morris, S. 1991. Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*: II. Fertilization and proembryo development. *Am. J. Bot.* 78: 1515-1527.
- Palmer, J.D. 1987. Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *Am. Nat.* 130: S6-S29.
- Palmer, J.D. 1990. Contrasting modes and tempos of genome evolution in land plant organelles. *TIG* 6: (4)115-120.
- Palmer, J.D., and Herbon, L.A. 1988. Plant mitochondrial DNA evolves rapidly in structure, but slowly in sequence. *J. Mol. Evol.* 28: 87-97.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning a Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sears, B.B. 1980. Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. *Plasmid* 4: 233-255.
- Sederoff, R.R. 1987. Molecular mechanisms of mitochondrial genome evolution. *Am. Nat.* 130: S30-S45.
- Stine, M., Sears, B.B., and Keathley, D.E. 1989. Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. *Theor. Appl. Genet.* 78: 768-774.
- Stine, M., and Keathley, D.E. 1990. Paternal inheritance of plastids in Engelmann spruce X blue spruce hybrids. *J. Hered.* 81: 443-446.
- Stine, M., David, A.J., and Keathley, D.E. 1991. Inheritance of plastids and mitochondria in spruce. *Plant Genetics Newsletter* 8: 2-4.
- Sutton, B.C.S., Flanagan, D.J., Gawley, J.R., Newton, C.H., Lester, D.T., and El-Kassaby, Y.A. 1991. Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. *Theor. Appl. Genet.* 82: 242-248.
- Szmidt, A.E., El-Kassaby, Y.A., Sigurgeirsson, A., Alden, T., Lindgren, D., and Hallgren, J-E. 1988. Classifying seedlots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA. *Theor. Appl. Genet.* 76: 841-845.

Wagner, D.B., Dong, J., Carlson, M.R., and Yanchuk, A.D.
1991. Paternal leakage of mitochondrial DNA in *Pinus*.
Theor. Appl. Genet. 82: 510-514.

CHAPTER II

Identification of *Picea engelmannii* Parry X *Picea pungens* Engelm. F₁ Hybrids Using Organelle Molecular Markers

ABSTRACT

Fourteen artificial Engelmann x blue (E x B) interspecific hybrids and their six Engelmann and eight blue spruce parents were used to demonstrate the maternal inheritance of mitochondrial (mt) DNA and paternal inheritance of chloroplast (cp) DNA. Total DNA was extracted from the current years' needles, digested with BamHI and probed with the maize mt gene cytochrome oxidase II, or digested with ClaI and probed with the *Petunia* cp clone P16. Inheritance of organelle DNA from the fourteen hybrids was consistent with the unilateral crossing incompatibility for these two species, controlled crosses are successful only when Engelmann spruce is used as the female parent, and the differential inheritance of organelles in *Picea*. The superiority of using organelle molecular markers over morphological, isozyme or chemical analysis to detect naturally occurring F₁ hybrids, and nth generation hybridization events is discussed.

INTRODUCTION

The question of natural hybridization and introgression between blue and Engelmann spruce has never been fully answered. Based on their similar morphology (Wright 1955) and sympatric ranges, it is widely believed that blue spruce is derived from Engelmann spruce (Daubenmire 1972), thus generating speculation on the possibility of interspecific hybridization. This debate is further fueled by the high degree of overlapping expression of many morphological and biochemical traits in these two species. However, there is cytological and germination evidence to suggest that natural hybrids between the two would be a rare occurrence (Kossuth and Fechner 1973, Ernst et al. 1990). Several efforts have been made to determine if natural hybridization does exist. Early work focused on morphological traits and/or chemical analysis to separate the two species and identify potential hybrids by their intermediate characteristics but the results were inconclusive (Daubenmire 1972, Taylor et al. 1975, Mitton and Andalora 1981, Schaefer and Hanover 1985, 1986, 1990).

More recently isozyme analysis was used in an attempt to demonstrate introgression between Engelmann and blue spruce (Ernst et al. 1990). Although both the Pgi-2 and Got-3 isozyme markers differentiated reference populations of Engelmann and blue spruce, classifying individuals sampled from a suspected introgression zone proved

problematic. Two individuals were detected, one heterozygous at the Pgi-2 locus with Engelmann and blue spruce alleles, the other heterozygous at the Got-3 locus, but they did not exhibit a hybrid isozyme pattern for any other isozyme system examined, therefore they could not accurately assess natural hybridization. However, because the Pgi-2 and Got-3 isozyme markers could separate Engelmann and blue spruce reference populations they were used to identify artificial hybrids.

The use of organelle molecular markers appears appropriate for determining hybridization between Engelmann and blue spruce for two reasons. First, controlled crosses between Engelmann and blue spruce have shown that viable seed is produced only when Engelmann spruce is used as the female parent (Fechner and Clark 1969; Ernst et al. 1990). Secondly, in *Picea* organelles are inherited differentially; mitochondria are maternally inherited (Stine et al. 1991; Sutton et al. 1991) and plastids are paternally inherited (Stine and Keathley 1990). This means that any F₁ hybrid would be the result of an E x B cross and would be expected to have mitochondria derived from the Engelmann parent and plastids from the blue parent.

The goal of this study was to determine, using E x B hybrids from controlled crosses and their respective parents (Ernst et al. 1990), if organelle molecular markers could be

used to detect a hybridization event between Engelmann and blue spruce.

MATERIALS AND METHODS

Foliage Collection

Thirteen of the fourteen artificial Engelmann x blue F_1 hybrids used in this study, and the eight Engelmann spruce that represent the southwest Colorado provenance, are growing in plantations located on the campus of Michigan State University, East Lansing, Michigan. The seven blue spruce that form the rangewide provenance are located at Kellogg Experimental Forest near Augusta, Michigan. The fourteenth Engelmann x blue hybrid is located in an experimental plantation at 3,200 m elevation in the Scotch Creek drainage south of Rico, Colorado. The Engelmann and blue spruce parents are located along the Dolores River near Rico, Colorado. The Michigan Cooperative Tree Improvement Program (MICHCOTIP) at Michigan State University was responsible for initiating the plantations and has detailed records of all trees used in this study.

Four current year foliage samples per tree from points equidistant around the crown were collected at a height of 1.3 meters where possible and immediately placed on ice for transport to the laboratory. Foliage samples were then placed in water and stored at 4°C for 1-15 days.

DNA Extraction, Digestion and Southern Analysis

Procedures for total DNA extraction, digestion and Southern analysis followed those of David and Keathley (in press) with the following exceptions: for cp probe P16, a gift of J. Palmer (Sytsma and Gottlieb 1986), the vector and insert were not separated by low melting agarose, and creation of the P16 probe followed standard directions for the BMB random primer labeling kit.

Southern filters exposed Kodak XAR-5 film at -70°C with DuPont Cronex Lightning Plus intensifying screens for 12 hours to 3 days.

RESULTS

A rangewide provenance of blue spruce and a provenance of southwestern Colorado Engelmann spruce total DNA's were digested with BamHI and probed with the maize mt gene cytochrome oxidase II (Figure 1), and also digested with ClaI and probed with the petunia cp clone P16 (Figure 2). Distinct RFLP patterns were conserved for each species/enzyme/probe combination and are summarized in Table 1.

All fourteen E x B F_1 hybrids, when digested with BamHI and probed with COXII, possessed a strong 2.6 kb RFLP band and maintained the 8.1 kb, 6.75 kb and 1.2 kb RFLP bands of their Engelmann parents, in addition to inheriting

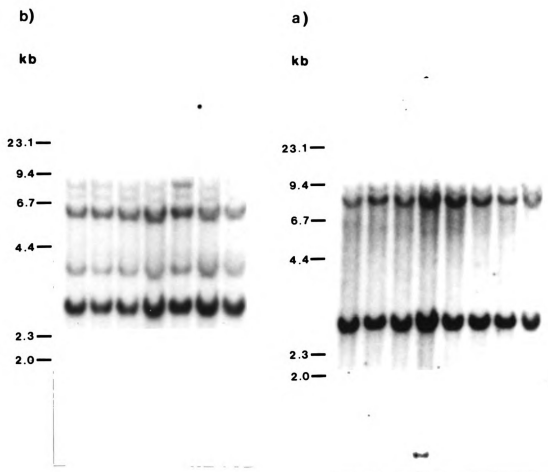
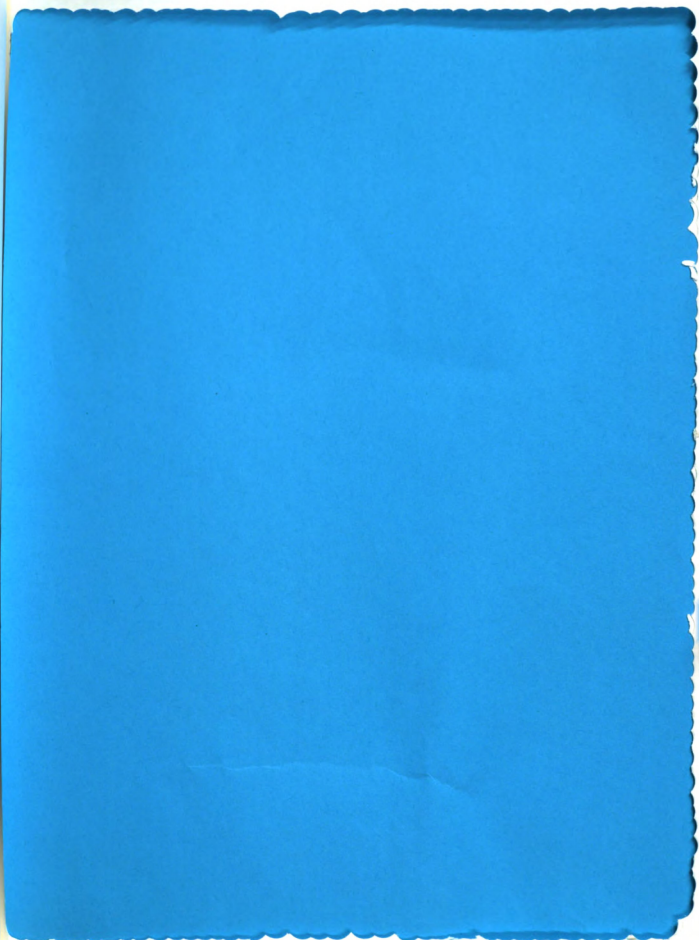


Figure 1. Conservation of RFLPs when digested with BamHI and probed with the mt gene COXII a) Engelmann spruce provenance of southwestern Colorado sources, b) Blue spruce rangewide provenance.





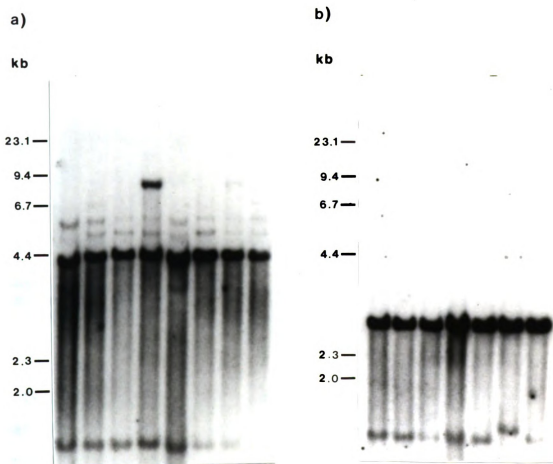


Figure 2. Conservation of RFLPs when digested with *Cla*I and probed with the cp clone P16 a) Engelmann spruce provenance of southwestern Colorado, b) Blue spruce rangewide provenance.

Table 1. Diagnostic RFLPs of blue and Engelmann spruce provenances for the enzyme/probe combinations BamHI/COXII and ClaI/P16. (Summary of Figures 1 and 2)

RFLP size (kb)	BamHI/COXII	
	Engelmann	Blue
8.1	*	
7.5		*
6.75	**	
6.5		*
5.6		**
3.5		*
2.6	***	***
1.25	*	*
	ClaI/P16	
	Engelmann	Blue
6.1	*	
5.2	*	
4.35	***	*
2.73		***
1.5	**	**

* weak hybridizing fragment

** moderate hybridizing fragment

*** strong hybridizing fragment

the faint 3.5 kb blue spruce RFLP (Figure 3). Maternal inheritance of mt DNA was confirmed because the E x B hybrids had the three Engelmann RFLPs and lacked the 7.5 kb, 6.5 kb, 5.6 kb, and 1.25 kb RFLPs common to blue spruce. The stronger hybridizing 5.6 kb blue spruce RFLP is not present in any of the hybrids, indicating that the 3.5 kb blue spruce band the 14 E x B F₁ hybrids did possess is not

the result of a failure of the mechanism that controls mitochondrial inheritance, and cannot be ascribed to the blue spruce mt genome. Subsequent work has shown that the presence or absence of this 3.5 kb fragment is perfectly correlated with the presence or absence of a blue spruce cp background (David Chapter 3). Thus the presence of the 3.5 kb band is not a result of leakage.

When digested with ClaI and probed with the P16 petunia cp probe, all fourteen E x B F₁ hybrids demonstrated paternal inheritance of cp DNA by retaining the strong 2.73 kb and faint 4.35 kb RFLPs of their blue parent (Figure 4). Although the faint hybridizing band comigrates with the strong hybridizing 4.35 kb Engelmann RFLP, because of its hybridization intensity it is considered indicative of blue spruce based on the rangewide provenance test (Figure 2). Moreover, leakage of cp DNA from the Engelmann parent was not indicated because there was no increase in the hybridization intensity of the 4.35 kb RFLP relative to the blue parent and the presence of the 6.1 kb and 5.2 kb Engelmann spruce RFLPs was never demonstrated, despite overexposure of the autoradiograms (data not shown).



Figure 3. Hybridization of COXII to BamHI digested total DNA extractions of E x B F₁ hybrids and their Engelmann and blue spruce parents. Each group of three from left to right is: Engelmann parent, E x B F₁ hybrid, blue parent.



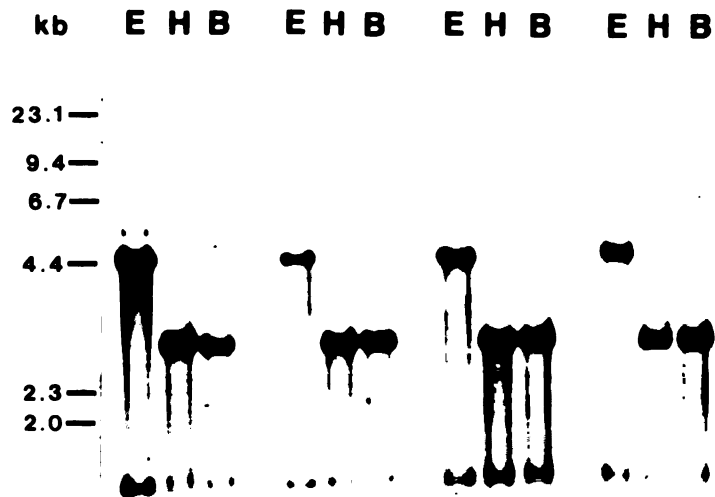


Figure 4. Hybridization of P16 to *Cla*I digested total DNA extractions of $E \times B$ F_1 hybrids and their Engelmann and blue spruce parents. Each group of three from left to right is: Engelmann parent, $E \times B$ F_1 hybrid, blue parent.

DISCUSSION

Organelle molecular markers can be used to accurately detect F_1 hybrids from controlled crosses of Engelmann X blue spruce if the inheritance patterns and intensities of all the RFLPs are considered. The faint 3.5 kb blue spruce RFLP that is generated when blue spruce or an E x B hybrid is digested with BamHI and probed with COXII is not of mt origin because of the lack of inheritance patterns of the other blue spruce RFLPs in the E x B hybrids. Additional work presented elsewhere has shown that the origin of this fragment is in the blue spruce cp genome (David Chapter 3). This indicates that there is homology between the COXII mt probe from maize and the blue spruce cp genome, although the level of hybridization intensity suggests that the sequence similarity is low. Cross homologies between mt and cp genomes in plants have been noted previously (Stern and Palmer 1984, Levings and Brown 1989) but probing the petunia cp clone bank with COXII did not generate a hybridizing fragment (data not shown).

Because organelle molecular markers can be used to detect E x B F_1 hybrids, these markers could also be used to identify naturally occurring F_1 hybrids and locate zones of introgression. Unlike morphological traits, whose expression may be effected by environmental conditions and for which there may be an overlap of traits between species, molecular markers are unaffected by the environment.

Using organelle molecular markers to detect introgression between Engelmann and blue spruce also has advantages over isozyme analysis. Not only can organelle molecular markers be used to detect F_1 hybrids, but they may be capable of detecting past introgression events as well. Because organelle inheritance is differential and non-Mendelian, the organelle background of an F_1 hybrid would be preserved as long as backcrosses were made to a female Engelmann or male blue spruce parent. In this case, organelle molecular markers would be able to detect a n^{th} generation hybridization event. With an increasing number of backcrosses to the same parental type the ability of isozyme analysis to detect a hybridization event decreases as the nuclear genome is recombined every generation.

This may be the explanation for one tree sampled by Ernst et al. (1990). This tree was heterozygous for Got-3, with one typical blue and one typical Engelmann allele, but was morphologically blue, and all other isozyme loci tested resembled blue spruce. This typical Engelmann isozyme band in an otherwise blue spruce population may be the result of nuclear DNA mutation from the blue to Engelmann spruce allele. It is also possible that this typical Engelmann allele is the result of a previous hybridization event that cannot be discerned by isozyme analysis because of successive backcrosses to blue spruce parents. An analysis of this tree using organellar molecular markers may be able

to determine if this individual commemorates a former interspecific hybridization event. Unfortunately, the sole reference to this tree lists its location as "...from the lower elevation zone of the Dolores River..." (Ernst et al 1990), making positive identification impossible.

It should be noted that a single crossing event other than a female Engelmann or male blue spruce would change the organellar background and render detection of the original interspecific hybridization event impossible. Although the fertility or backcrossing ability of $E \times B F_1$ hybrids has not been investigated, quantitative analysis of intraspecific crosses of Engelmann and blue spruce in a parapatric region of southwest Colorado revealed that maternal effects were very important for seed viability in blue spruce but almost nonexistent in Engelmann spruce. In addition, Engelmann spruce had a higher percentage of germinated seed and was more successful in self crosses than blue spruce (Ernst et al. 1988). Collectively this information may help to explain the unilateral crossing incompatibility, and suggests that there may be a lower barrier to interspecific or backcross hybridization when Engelmann spruce is the female parent. However, because the exact nature of the incompatibility is unknown, and the existing F_1 hybrids are not yet mature, it is impossible to make predictions about their fertility or backcrossing ability.

Because of the unilateral crossing incompatibility between Engelmann and blue spruce and the differential inheritance of organelles in *Picea*, organelle molecular markers can be used to identify F₁ hybrids of Engelmann and blue spruce. Because these markers are more reliable than morphological traits and in some circumstances may be more informative than isozyme or chemical analysis, they are well suited for identifying interspecific hybridization events in sympatric regions of blue and Engelmann spruce.

LITERATURE CITED

- Daubenmire, R 1972. On the relation between *Picea pungens* and *Picea engelmannii* in the Rocky Mountains. Can. J. Bot. 50:733-742.
- David, AJ and Keathley, DE 199x. Inheritance of mitochondrial DNA in interspecific crosses of *Picea glauca* and *P. omorika*. Can. J. For. Res. (in press).
- Ernst, SG, Hanover, JW, Keathley, DE and Mao, IL 1988. Genetic variation and control of intraspecific crossability in blue and Engelmann spruce. Sil. Genet. 37(3-4):112-118.
- Ernst, SG, Hanover, JW, and Keathley, DE 1990. Assessment of natural interspecific hybridization of blue and Engelmann spruce in southwestern Colorado. Can. J. Bot. 68:1489-1496.
- Fechner, GH and Clark, RW 1969. Preliminary observations on hybridization of Rocky Mountain spruces. Proc. Comm. For. Tree Breed. Can. 11:237-247.
- Kossuth, SV and Fechner, GH 1973. Incompatibility between *Picea pungens* Engelm. and *Picea engelmannii* Parry. For. Sci. 19:50-60.
- Levings III, CS and Brown, GG 1989. Molecular biology of plant mitochondria. Cell 56:171-179.
- Mitton, JB and Andalora, R 1981. Genetic and morphological relationships between blue spruce, *Picea pungens*, and Engelmann spruce, *Picea engelmannii*, in the Colorado Front Range. Can. J. Bot. 59:2088-2094.
- Schaefer, PR and Hanover, JW 1985. A morphological comparison of blue and Engelmann spruce in the Scotch Creek drainage, Colorado. Sil. Genet. 34(2-3):105-111.
- Schaefer, PR and Hanover, JW 1986. Taxonomic implications of monoterpene compounds of blue and Engelmann spruces. For. Sci. 32:725-734.
- Schaefer, PR and Hanover, JW 1990. An investigation of sympatric populations of blue and Engelmann spruces in the Scotch Creek drainage, Colorado. Sil. Genet. 39(2):72-81.

- Stern, DB and Palmer, JD 1984. Extensive and widespread homologies between mitochondrial DNA and chloroplast DNA in plants. *Proc. Natl. Acad. Sci. USA* 81:1946-1950.
- Stine, M and Keathley, DE 1990. Paternal inheritance of plastids in Engelmann spruce X blue spruce hybrids. *J. Hered.* 81:443-446.
- Stine, M, David, AJ, and Keathley, DE 1991. Inheritance of plastids and mitochondria in spruce. *Plant Genetics Newsletter* 8:2-4.
- Sutton, BCS, Flanagan, DJ, Gawley, JR, Newton, CH, Lester, DT and El-Kassaby, YA 1991. Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. *Theor. Appl. Genet.* 82:242-248.
- Sytsma, KJ and Gottlieb, LD 1986. Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* sect. *Peripetasma* (Onagraceae). *Evol.* 40:1248-1261.
- Taylor, RJ, Williams, S and Daubenmire, R 1975. Interspecific relationships and the question of introgression between *Picea engelmannii* and *Picea pungens*. *Can. J. Bot.* 53:2547-2555.
- Wright, J 1955. Species crossability in spruce in relation to distribution and taxonomy. *For. Sci.* 1:319-349.

CHAPTER III

Evidence of Natural Hybridization Between Engelmann Spruce (*Picea engelmannii*) and Blue Spruce (*Picea pungens*) in Southwestern Colorado.

ABSTRACT

Genetic markers for both mitochondrial (mt) and chloroplast (cp) genomes were used to provide evidence of hybridization between Engelmann and blue spruce in the Scotch Creek drainage of southwestern Colorado. Similarity of RFLP banding patterns between previously studied Engelmann x blue spruce artificial F₁ hybrids and tree 574 and seedlings II5 and III5 for four enzyme/probe combinations indicated that these sampled trees were natural hybrids. A 3.5 kb RFLP generated by BamHI/COXII was shown to be the result of weak homology to a sequence in the blue spruce cp genome. Cones were collected from tree 574, the only mature tree to genetically resemble a hybrid, and measured for cone length and cone scale length and size. The measurement of these traits, considered diagnostic for Engelmann and blue spruce in this drainage, supported the molecular data indicating that this tree is a first generation hybrid. Two seedlings possessed organelle profiles that could not be classified as Engelmann, blue, or Engelmann x blue hybrid. Seedling IV3 generated an

organellar background that suggested it was the result of an advanced generation backcross, and seedling II1 was heteroplasmic by branch sample for Engelmann and blue spruce. A fire disturbance model, which takes into account a high level of hybridization, potential introgression, continued species integrity, and the relatively young age of the forest, is presented to describe the role fire may play in controlling hybridization between Engelmann and blue spruce in this drainage. The availability of organelle specific genes and the differential non-Mendelian inheritance of organelles in the genus *Picea* is a powerful combination for analyzing the genetic structure of natural and managed populations.

INTRODUCTION

Natural hybridization between congeneric forest species is possible in a wide range of angiosperms and gymnosperms, including both wind pollinated and vector pollinated species (*Aesculus*, de Pamphilis and Wyatt 1989; *Alnus*, Bousquet et al 1990; many species in *Quercus*, Palmer 1948, Whittemore and Schaal 1991, and Jensen et al 1993; *Pinus*, Lanner and Phillips 1992, Wheeler and Guries 1987; and *Populus*, Keim et al 1989).

Of the eight native species of *Picea* in North America natural hybridization has been known to occur between white

Engelmann (Daubenmire 1974), red and black (Morgenstern and Farrar 1964, Manley 1972) and a single verified example of black and white (Little and Pauley 1958, Riemenschneider and Mohn 1975). Natural hybridization between Engelmann and blue has been suggested by Habeck and Weaver (1969) who concluded, on the basis of monoterpene analysis, that three trees from Fremont County, Wyoming might be natural hybrids. All other references are based solely on field observations (for a review see Daubenmire 1972).

Because Engelmann and blue spruce are morphologically similar (Jones and Bernard 1977), species identification can be problematic, making hybrid identification in sympatric zones very difficult. Early efforts used multiple morphological traits and hybrid indices to distinguish Engelmann from blue (Daubenmire 1972). Later work utilized morphological traits and chemical markers (Taylor et al 1975) or morphological traits and isozyme analysis (Mitton and Andalora 1981) in an attempt to identify natural hybrids. Schaefer and Hanover (1985) used 43 morphological traits to positively identify Engelmann and blue spruce at opposite zones in the Scotch Creek drainage of Colorado and followed that with monoterpene analysis, resulting in an unambiguous classification of trees in the same drainage (Schaefer and Hanover 1986). A combination of these two analyses gave little evidence to suggest that any

hybridization was occurring in the Scotch Creek drainage (Schaefer and Hanover 1990).

Extensive isozyme analysis of open pollinated and intra and interspecific controlled crosses of Engelmann and blue spruce parents in the Dolores River drainage near Rico, Colorado, plus 20 trees from the region of sympatry in Scotch Creek, indicated that although isozyme identification of the interspecific F_1 s was possible, no F_1 hybrids were detected (Ernst et al 1990).

Previous work has determined species specific mt and cp DNA markers for Engelmann and blue spruce, and shown that F_1 hybrids from controlled crosses can be positively identified based on these markers (David Chapter 2). Unambiguous determination of the F_1 hybrids is possible for three reasons: First, the unilateral crossing incompatibility between Engelmann and blue spruce means that controlled crosses are successful only when Engelmann spruce is the female parent (Fechner and Clark 1969; Ernst et al 1990). Second, the differential inheritance of organelles in *Picea* means that mt are maternally inherited (Sutton et al 1991; Stine et al 1991) and cp are paternally inherited (Stine and Keathley 1990; Sutton et al 1991). Therefore all F_1 hybrids are the result of an Engelmann x blue cross, and possess an Engelmann mt and a blue cp backgrounds. Thirdly, total DNA digested with BamHI and probed with COXII results in an unique banding pattern for F_1 hybrids (David Chapter 2).

This paper demonstrates the use of organelle molecular markers in conjunction with the differential inheritance of organellar DNA in spruce and the unilateral crossing incompatibility to positively identify natural hybridization events between Engelmann and blue spruce.

MATERIALS AND METHODS

Plant Material

The Serbian spruce, thirteen of the fourteen artificial Engelmann x blue F_1 hybrids, and the eight Engelmann spruce that represent the southwest Colorado provenance, are growing in plantations located on the campus of Michigan State University, East Lansing, Michigan. The seven blue spruce that form the rangewide provenance are located at Kellogg Experimental Forest near Augusta, Michigan, as are the white spruce that form the provenance and the 15 white x Serbian and Serbian x white hybrids. The fourteenth artificial Engelmann x blue hybrid is located in an experimental plantation at 3,200 m elevation in the Scotch Creek drainage south of Rico, Colorado. The Engelmann and blue spruce parents are located along the Dolores River near Rico, Colorado. The Michigan Cooperative Tree Improvement Program (MICHCOTIP) at Michigan State University was responsible for initiating the plantations and has detailed records of all trees used in this study.

Experiment Location and Site Description

Scotch Creek empties into the Dolores River approximately 5 kilometers south of Rico, Colorado along state highway 145. Elevation in the drainage runs from greater than 3050 m at the upper end to 2590 m where Scotch Creek empties into the Dolores River. From the lower to the upper end of the drainage there is a continuum of spruce such that blue spruce is the predominant tree at the lower elevations and Engelmann spruce, or Engelmann in association with subalpine fir (*Abies lasiocarpa*), is the predominant tree at the upper elevations. In addition, Scotch Creek flows east to west creating aspects of a roughly north/south nature; blue spruce inhabiting the hotter, drier slope and Engelmann spruce inhabiting the cooler, moister slope. The combination of elevational and site preferences for Engelmann and blue spruce provide an area of sympatric contact between the two species, especially in middle regions (zones 2 and 3) where Engelmann and blue spruce grow side-by-side. For a more detailed description of the Scotch Creek drainage see Schaefer and Hanover (1985; 1990).

Scotch Creek Plant Material

The Scotch Creek collections were made in the summer of 1990. All trees and seedlings were selected randomly from throughout each zone. Based on needle stiffness, needle sharpness, needle angle from twig, twig pubescence, the

presence/absence of epicormic branches and cone length (where applicable) five large blue spruce (dbh $\geq$ 46 cm) and five small blue spruce ($\leq$ 2.54 cm at 15 cm above ground) for a total of 10 per zone were selected in each of zones 1 through 3. In zone 4, five mature trees were located, but only three seedlings could be found that met the requirements for blue spruce, for a total of 8 individuals from this zone.

Five current year foliage samples per tree, from points equidistant around the crown, were collected at a height of 1.3 m (where possible) and immediately placed on ice for transport to the laboratory. If foliage mass on a selected tree was insufficient to collect five samples, as many 15 g samples as possible were obtained. Foliage samples were then placed in water and stored at 4°C for 1-15 days.

Total DNA Extractions and Digestion

Foliage samples for each tree were processed independently throughout the total DNA extraction procedure (David and Keathley in press). For initial screening purposes total DNA from each foliage sample of an individual was pooled and then digested for 1-3 hours with *Ava*I, *Bam*HI, *Cla*I or *Sma*I following manufacturers directions (BMB, Indianapolis, IN 46250) and stored at 4°C. Any deviations from strict blue spruce RFLP banding patterns or intensities were reinvestigated using unpooled total DNA.

Southern Analysis

Agarose gels, blotting procedures and creation of the COXII and ATPase α mt probes and the P16 and P3 cp probes, all follow David and Keathley (in press) except that the inserts for COXII and P3 were separated from their vectors using a FMC (Rockland, ME 04841) SpinBind DNA recovery kit. The inserts for probes ATPase α and P16 were not separated from their respective vectors after amplification and harvest.

Creation of the radioactive probes was accomplished with the aid of a BMB random primer labeling kit following standard directions for all probes. Each reaction used 3000Ci/mmol dCTP (DuPont NEN, Boston, MA 02118) along with approximately 20 ng of probe DNA per reaction.

Prehybridization, hybridization and wash conditions all follow those for David and Keathley (in press). Southern filters were allowed to expose Kodak XAR-5 film at -70°C with DuPont Cronex Lightning Plus intensifying screens for 12 hours to 7 days.

Cone Measurement

Cones were collected in the fall after seed dissemination by shooting them out of the tree with a high-powered rifle. They were then soaked in water for 3 days until closed and four cones were selected randomly for measurement. One cone scale from the middle of each cone

was randomly selected and measured without flattening. Cone length, cone scale width and cone scale size were recorded per Schaefer and Hanover (1985).

RESULTS

Inheritance of ATPase α and P3

Maternal inheritance of ATPase α was confirmed by probing total DNA digested with ClaI from two Serbian parents, one white spruce and 15 interspecific hybrids of *Picea glauca* and *P. omorika* from a total of five separate controlled crosses including reciprocals (David and Keathley in press). Paternal inheritance of P3 was confirmed using the parents and interspecific hybrids of controlled crosses between Engelmann and blue spruce (David Chapter 2) from a total of five separate controlled crosses (data not shown).

Efficacy of Probes

Suitability of COXII and P16 for use in distinguishing between Engelmann and blue spruce was determined previously (David Chapter 2). The same rangewide provenance of blue spruce and provenance of southwestern Colorado sources for Engelmann spruce were used to test ATPase α and P3 for their ability to differentiate between Engelmann and blue spruce. Total blue spruce DNA digested with AvaI and probed with ATPase α gave a diagnostic 4.5 kb RFLP. Total Engelmann

spruce DNA digested with *Ava*I produced a single 2.75 kb RFLP (data not shown). Total Engelmann and blue spruce DNA digested with *Sma*I and probed with P3 resulted in RFLP banding patterns that consistently differentiated blue from Engelmann spruce (data not shown).

Genotypes of the Individuals

Of the 38 trees sampled two seedlings, II5 and III5, and one mature tree, 574, from zones 2,3 and 1 respectively, resembled hybrids for their mt and cp backgrounds (Figure 1 and 2). Seedling III1 was heteroplasmic for blue and Engelmann markers in its mt and cp backgrounds (Figure 2). Seedling IV3 had a blue spruce mt background for COXII, a unique doublet blue spruce band, at 5.2 and 5.0 kb, for ATPase α , and an Engelmann spruce cp background for P3 and P16 (Figure 3). All other trees sampled generated blue spruce mt and cp backgrounds (Table 1).

Collectively, the E x B F₁ hybrids, and seedlings III1 and IV3 allow us to make a determination about the origin of the 3.5 kb RFLP that arises when blue spruce or hybrids are digested with *Bam*HI and probed with COXII. It is known from previous research (David Chapter 2) that the 3.5 kb RFLP is not of mt origin. Now it can be deduced that the 3.5 kb fragment is part of the blue spruce cp genome because it is only present when there is a blue spruce cp haplotype (Table 2).

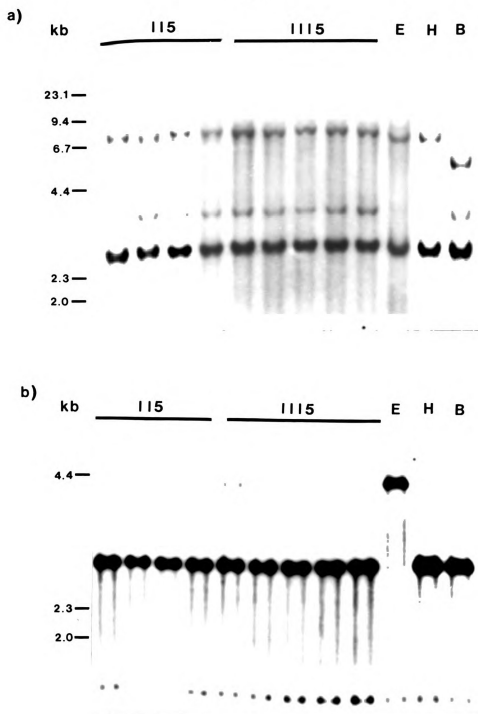
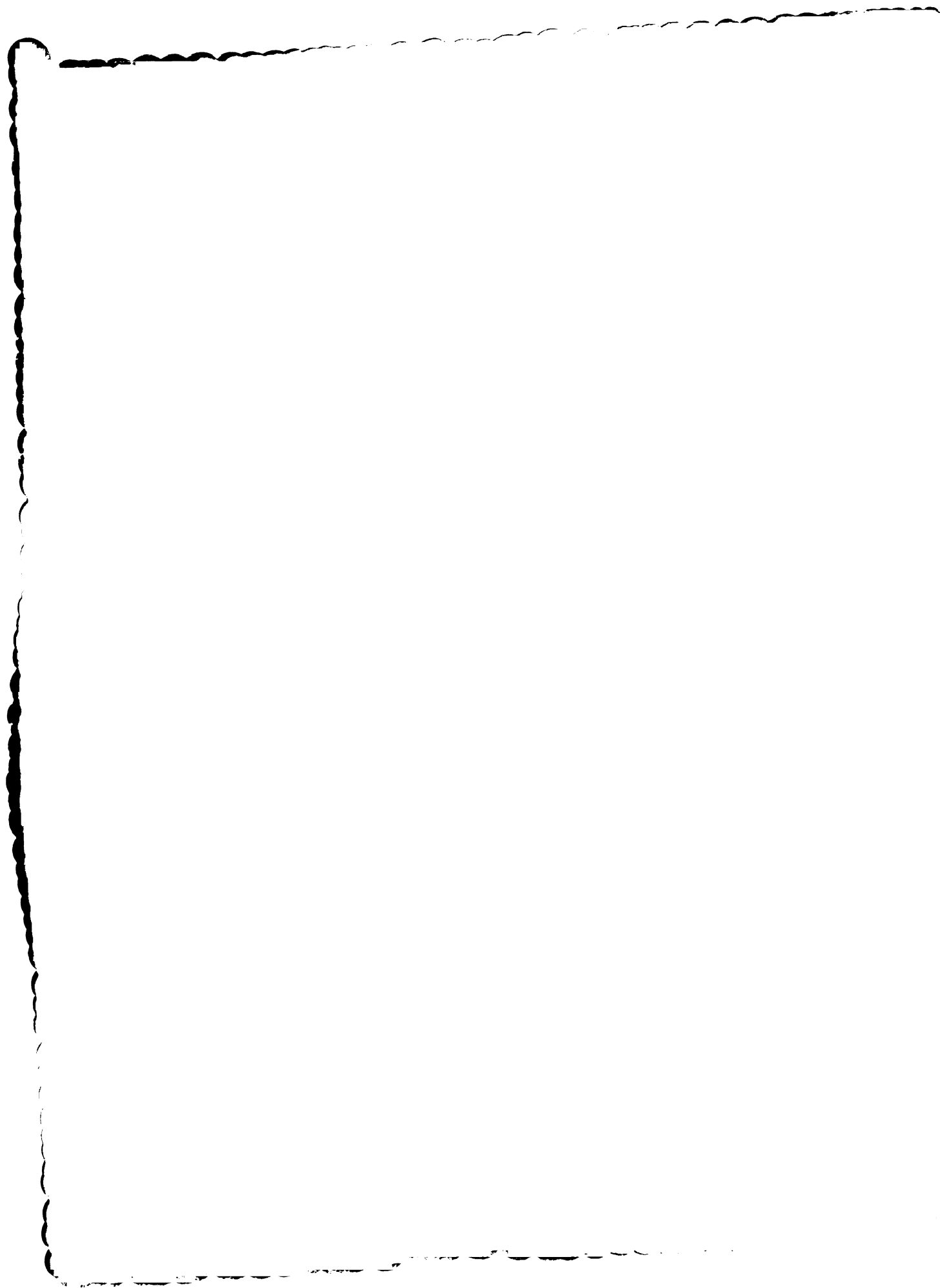


Figure 1. Total DNA from seedlings II5 and III5 a) digested with BamHI and probed with COXII and b) digested with ClaI and probed with P16. Multiple lanes for each individual represent separate foliage samples. E = Engelmann parent, H = E x B F_1 hybrid, B = blue parent.



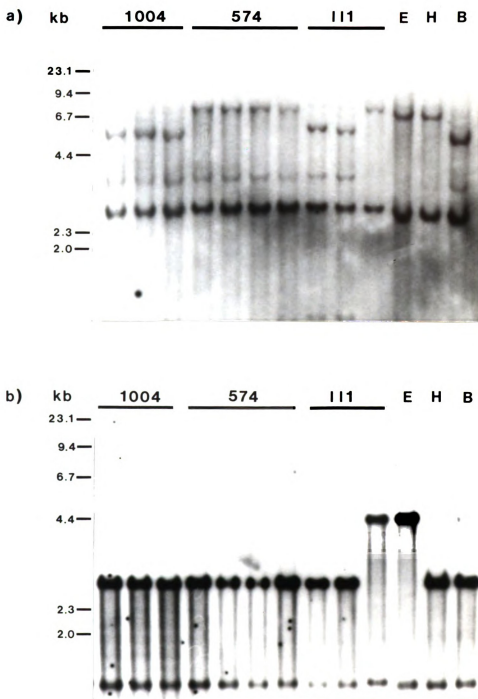


Figure 2. Total DNA from tree 574 and seedling 111 a) digested with BamHI and probed with COXII and b) digested with ClaI and probed with P16. Multiple lanes for each individual represent separate foliage samples. E = Engelmann parent, H = E x B F_1 hybrid, B = blue parent.

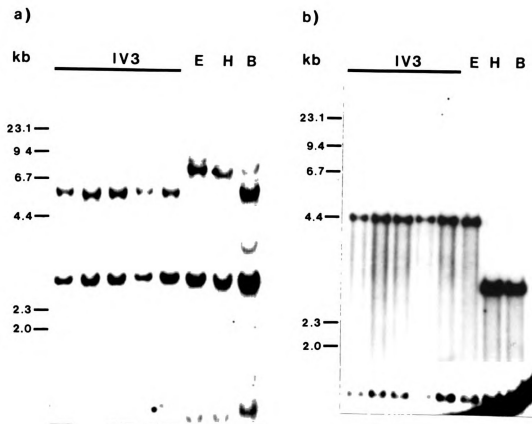


Figure 3. Total DNA from seedling IV3 a) digested with BamHI and probed with COXII and b) digested with ClaI and probed with P16. Multiple lanes for IV3 represent separate foliage samples. E = Engelmann parent, H = E x B F₁ hybrid, B = blue parent.

Table 1. Summary of organellar backgrounds as elucidated by the COXII and ATPase α mitochondrial (mt) probes and the P16 and P3 chloroplast (cp) probes for all trees sampled in Scotch Creek.

zone tree		mt	cp
1	I1	B	B
1	I2	B	B
1	I3	B	B
1	I4	B	B
1	I5	B	B
1	1004	B	B
1	52	B	B
1	1148	B	B
1	575	B	B
1	574	E	B
2	II1	B+E	B+E
2	II2	B	B
2	II3	B	B
2	II4	B	B
2	II5	E	B
2	1150	B	B
2	1149	B	B
2	1151	B	B
2	1152	B	B
2	1153	B	B
3	III1	B	B
3	III2	B	B
3	III3	B	B
3	III4	B	B
3	III5	E	B
3	1154	B	B
3	1155	B	B
3	1156	B	B
3	547	B	B
3	1157	B	B

Table 1 (continued).

zone tree		mt	cp
4	IV1	B	B
4	IV2	B	B
4	IV3	B/BB	E
4	649	B	B
4	645	B	B
4	644	B	B
4	1158	B	B
4	1159	B	B

Key:

B = blue spruce genotype

E = Engelmann spruce genotype

B+E = heteroplasmic among samples; one sample has Engelmann genotype for mt and cp

B/BB = blue spruce mt genotype for COXII, and blue spruce doublet bands, 5.2 and 5.0 kb for ATPase α

Table 2. Presence or absence of the BamHI/COXII 3.5 kb RFLP in different genetic backgrounds. The first letter of the pair indicates the mitochondrial background, the second, the chloroplast background.

Engelmann spruce	E x B hybrid ^a	blue spruce	seedling III ^b		seedling IV3
EE	EB	BB	BB	EE	BE
-	+	+	+	-	-

B = blue spruce background

E = Engelmann spruce background

^a E x B hybrids from David Chapter 2^b seedling III was heteroplasmic; see text for details

Cone Measurements

Cones were collected and measured from tree 574, the only mature tree to genetically resemble a hybrid. Results of the measurements showed a mixture of blue and Engelmann spruce traits. Cone length was within the range of blue spruce values but both cone scale width and cone scale size scored within the Engelmann spruce range (Figure 4).

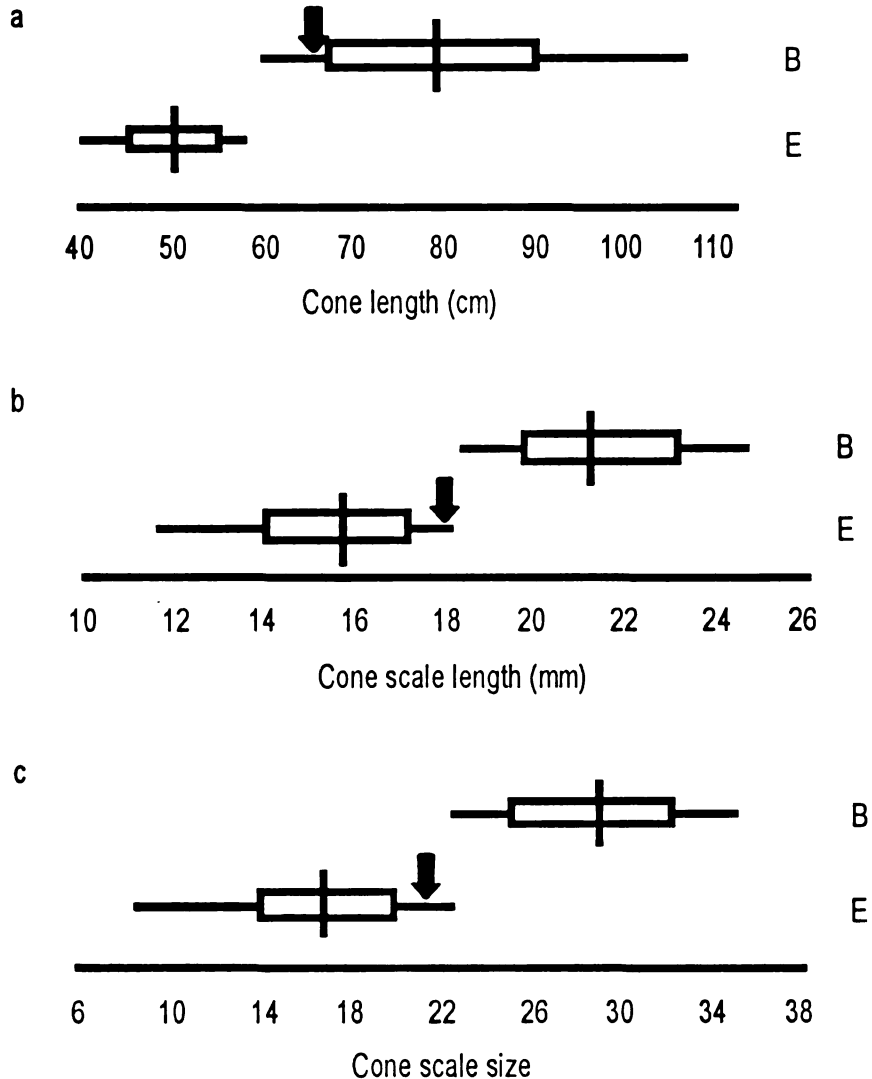


Figure 4 a-c. Tree 574 cone measurements (arrows) in relation to means (vertical lines), ranges (horizontal lines), and standard deviations (boxes) for 3 quantitative morphological traits from Scotch Creek reference populations of blue (B), and Engelmann (E) spruce (diagram after Schaefer and Hanover 1985).

DISCUSSION

Natural Hybridization

Genetic evidence of hybridization between blue and Engelmann spruce was found in one mature tree, 574, from zone 1, and seedlings II5 and III5 from zones 2 and 3 respectively. The presence of these hybrids in three different zones of the drainage indicates that natural hybridization is occurring on a widespread basis. Although theoretically possible, it is improbable that tree 574 acted as the female parent of either these two seedlings for the following reasons. First the geographical distance between zones is great. Tree 574 is well within zone 1, over 1 mile from the location of either seedling II5 or III5. Although studies have not been done in Scotch Creek to determine the average distance for spruce seed dissemination, work with mixed conifers (including blue spruce) in clearcut areas has shown that most seeds fell within 90 m of the upwind timber edge (Alexander 1974). This is well short of the distances needed to attribute seedlings II5 and III5 to tree 574. In addition, pollen as a vector for moving the hybrid mt genotype is discounted because of the maternal inheritance of mt in *Picea* (Sutton et al 1991; David and Keathley in press).

Haplotype Variation and Heterozygosity

Because samples collected from individual trees were not pooled we have gained rare insight into the nature of organelle genotypes in this sympatric region. In addition to blue and hybrid mt genotypes there was a novel mt genotype generated by seedling IV3. For the *AvaI*/*ATPase α* combination seedling IV3 generated a doublet band of 5.2 and 5.0 kb, which comigrated with the typical blue spruce 5.0 kb RFLP. Although rare, novel DNA variants have been found in the cp genome of natural hybrids between *Populus deltoides* and *P. nigra* (Rajora and Dancik 1995). Seedling IV3 represents the first known case of a novel mt DNA variant from a hybrid zone.

Seedling IV3 also generated an RFLP profile unlike blue spruce or Engelmann spruce for the *BamHI*/*COXII* combination. The profile resembled blue spruce except that all faint bands, including the 3.5 kb RFLP, (David Chapter 2) were missing. It is impossible to determine the exact nature of this seedling but one plausible explanation is that seedling IV3 is an advanced generation hybrid. This could occur in one of two ways, either an E x B F₁ hybrid was backcrossed as a female to an Engelmann spruce and a progeny crossed to a female blue spruce parent, or the E x B F₁ hybrid was backcrossed to a blue spruce female with a progeny crossed as a female to an Engelmann spruce. Either pedigree would create the blue spruce mt and Engelmann spruce cp background

seen in seedling IV3. The only other possibilities would be that seedling IV3 is a true B x E F₁ hybrid, or a true Engelmann spruce with two undiscovered mt haplotypes. Both of these possibilities are unlikely because the B x E cross has never been successfully accomplished (Fechner and Clark 1969; Ernst et al 1990) and the other explanation would require two new mt haplotypes, that were not identified in a provenance of southwestern Colorado Engelmann spruce, to be present in one individual.

Although not conclusive, evidence that points to the occurrence of introgression between Engelmann and blue spruce is accumulating in the literature. Isozyme evidence from Engelmann and blue spruce in the Dolores River valley (Scotch Creek empties into the Dolores River) implies bidirectional introgression may have occurred (Ernst et al 1990). This isozyme study identified two individuals, one blue spruce and one Engelmann spruce, that were heterozygous at the Got-3 and Pgi-2 loci respectively, with an allele that was otherwise present only in the other species. Neither of these trees possessed alleles of the opposite species at any of the other 12 loci investigated. A determination of whether these trees were pure species or backcrossed individuals was not possible due to the low number of loci analyzed (Ernst et al 1990). There is also indirect evidence for introgression in various populations in the southwestern United States. In a common garden study

Rehfeldt (1994) concluded that three southwest populations of Engelmann spruce may have been introgressed by blue spruce. Taken together these examples strongly suggest that introgression between Engelmann and blue spruce has occurred. Testing the total DNA of IV3 with a nuclear probe that could differentiate between the two spruces may provide a definitive answer.

There was also one example of heteroplasmy among the sampled trees. Seedling II1 was so diminutive that only three foliage samples could be collected. Two of these samples possessed blue spruce mt and cp backgrounds for all four enzyme/probe combinations. The third sample generated Engelmann spruce mt and cp backgrounds for the same four enzyme/probe combinations. This is the first report of a heteroplasmic individual from a hybrid zone possessing the RFLP profile of both organelles for both parents. This incidence of heteroplasmy is particularly interesting because of the differential inheritance of organelles in *Picea* where mt are inherited maternally and plastids paternally. Consequently, if seedling II1 was an F_1 hybrid then the only way this segregation pattern could have arisen is if both organelle inheritance mechanisms faltered and vegetative segregation placed similar species organelles together. Another possibility, albeit unlikely, is that mutation events occurred in only one section of the tree. This scenario requires at least four separate mutation

events in two different genomes, one for each of the four probes, and all of them to an Engelmann spruce genotype. A return trip to field check the collection site of seedling III1 indicated that there was no possibility of having inadvertently collected from more than one seedling.

Because the apical meristem was included in the sampling procedure, it is possible that had this seedling survived no trace of heteroplasmy would have been detectable once the lower branches self-pruned. Based on this example, heteroplasmy in hybrid zones may occur at a higher frequency than previously thought, especially if mature trees are the only ones sampled.

Differences in Morphological, Chemical and Genetic Evidence

Past research, although noting intermediate values for morphological and chemical traits, has failed to detect hybridization in the Scotch Creek drainage (Schaefer and Hanover 1985; 1986; 1990). Yet from the evidence in this paper it is apparent that hybridization has occurred. This suggests that the failure of earlier studies to detect hybridization was due to overlapping expression of morphological and chemical traits. Whether this overlap is a result of introgression or a combination of common ancestry and parallel evolution is unclear.

Introgressive hybridization (Anderson and Hubrecht 1938), or introgression, may explain the difference in

results between the earlier morphological and chemical analysis and our genetic analysis. In this scenario the original E x B F₁ hybrids were formed several generations ago and their progeny were repeatedly backcrossed as females to blue spruce (EB x BB). Crosses of this nature would maintain the organelle pattern seen in II5, III5 and 574, and each successive generation would more closely resemble the blue spruce parent. This would explain why hybridization had gone undetected by previous morphological and chemical analyses - the effect of any Engelmann nuclear genes had been diluted by the continued backcrossing to blue spruce. In contrast, the uniparentally inherited organelle genomes which are not recombined every generation still denote the original hybridization event. But cones collected from tree 574 do not support this theory. Measurements of these cones for three traits considered diagnostic for blue and Engelmann spruce in Scotch Creek (Schaefer and Hanover 1985) show tree 574 resembles Engelmann spruce for cone scale width and cone scale size but blue spruce for cone length. This expression of blue and Engelmann traits in the same tree is not expected of an advanced generation backcross individual.

The sheer number of initial hybrids needed to account for the 10.5% incidence of hybridization in our sample also makes introgression an unlikely explanation. Assuming that each F₁ hybrid has an equal opportunity as a male or female

to backcross to a blue spruce, and the direction of the cross does not confer a fitness advantage, then each successive generation halves the number of trees that maintain the hybrid organellar background.

This theory of advanced generation introgressants is also difficult to accept in light of the age structure of the forest in the Scotch Creek drainage. Quite simply this theory requires that older generations, or at least evidence of them, be present in the forest. The forest near Scotch Creek is relatively young with no overmature, dead or dying trees. Only in the upper elevations (> 2800 m) of slopes with a northern aspect are found what resemble unevenaged forests with large (> 0.9 m) Engelmann spruce, and occasional fallen trees (personal observation).

A more likely explanation for the differences between morphological, chemical and genetic analysis lies in overlapping character expression. Many traits show overlapping expression for Engelmann and blue spruce (Daubenmire 1972; Mitton and Andalora 1981; Schaefer and Hanover 1985), suggesting that there may not be a clear distinction between intermediate and parental types. A morphological analysis comparing 25 Engelmann, 25 blue and 25 known F_1 E $\times$ B hybrids for needle width, thickness, sharpness, and the maximum number of stomatal lines per needle, found that while the three class means were significantly different from each other for each trait the

range of character expression was so large that a clear distinction between them could not be made (Gruber 1990).

Conventional thought holds that hybrids will be intermediate in character expression between the two parental types, but current research into this theory of hybrid intermediacy has shown that hybrids actually express a blend of parental and intermediate morphological traits. Furthermore, whether or not the traits expressed are intermediate or parental varies by species and the trait selected for study (Rieseberg and Ellstrand 1993). In light of this evidence it is conceivable that because of overlapping character expression, and the possibility of the fallacy of hybrid intermediacy, the use of hybrid indices (Daubenmire 1972; Schaefer and Hanover 1985) and discriminate function analysis (Mitton and Andalora 1981) cannot adequately discriminate between the hybrid class and the parents for the traits selected.

Species Interaction in Scotch Creek

Any model for the interaction between blue and Engelmann spruce in Scotch Creek must reconcile the following: a high number of hybrid individuals, evidence that suggests introgression, the young age of the forest, and continued species integrity. The only explanation consistent with all of this information is the existence of a large disruptive force at periodic intervals that acts to

spatially separate refugial blue from Engelmann spruce in Scotch Creek and therefore temporarily halt hybridization and introgression.

Intense, periodic fires would be sufficient to accomplish this type of separation. Precedents for fires of this nature have already been established throughout the Central Rocky Mountains (Stahelin 1943; Veblen et al 1994). One of these burns, the Lime Creek fire of 1879, was so devastating that even 50 years later there were areas where no natural regeneration of Engelmann spruce had occurred (Stahelin 1943). Located approximately 29 km ENE of Scotch Creek, the Lime Creek burn site shares certain ecological characteristics with Scotch Creek. Open grasslands exist on predominately southern and western aspects, populations of pure spruce exist only at the highest altitudes, and spruce appear to be regenerating mainly under aspen (*Populus tremuloides* Michx.) a fire pioneer species (Stahelin 1943; David and Keathley personal observation). The similarities between Scotch Creek and Lime Creek are obvious and point to fire as an element for change in the drainages.

After a high altitude fire, grasses, especially a *Carex-Poa* association, are the first species to inhabit the site. Their density can prevent aspen or spruce seeds from reaching the soil and germinating, effectively maintaining the grasslands for decades or perhaps centuries (Stahelin 1943). As previously mentioned, the Scotch Creek forest is

relatively young with open, grassy areas, still available for colonization in all five zones and no evidence of dying, dead, or downed trees. The only exception to this is above 2800 m on the cooler, moister, more fire protected slopes with a northern aspect. Here larger Engelmann spruce (> 0.9 m dbh) exist along with medium sized trees, seedlings, and the occasional downed tree (David and Keathley personal observation).

Evidence from this research indicates a high number of hybrid individuals in the population (10.5%) despite a unilateral crossing incompatibility and a low success rate for artificial E x B crosses (Fechner and Clark 1969; Ernst et al 1990). This is not a contradiction because the presence or absence of a hybrid generation is not well correlated with the ease of hybridization but rather the availability of suitable habitat (Grant 1981). In this theory it is fire that provides the available habitat for new seedlings.

Despite the high level of hybridization and the potential for introgression mentioned earlier the two species still remain distinct. Although morphologically they are very similar, with overlapping expression for some traits, they are still discernable following simple guidelines (Jones and Bernard 1977). Perhaps the biggest conundrum is how the species remain distinct in the face of high levels of hybridization and evidence that suggests the

occurrence of introgression. The role of fire as an ecological force in the Scotch Creek drainage may provide an answer. An intense fire would destroy the majority of the spruce in the drainage. Only the Engelmann spruce on the moister sites with a northern aspect could be expected to survive. Blue spruce which traditionally inhabit the drier sites would be eliminated. Regeneration on these sites is a function of the number of seed trees remaining, the amount and location of aspen that survive, and the density of the grass that invades after the fire (Stahelin 1943). The Engelmann spruce that survived in a refugia on the moister slopes begin the process of recolonization by taking advantage of decreased grass density in rocky areas and under aspen canopies. The blue spruce, which have been all but eliminated by the fire, most likely recolonize the drainage from a population of blue spruce in the Dolores River valley and any scattered survivors in the Scotch Creek drainage. This process of recolonization brings the two species back into close proximity where they again have the potential for hybridization and introgression. The spatial and temporal interruption between the time of the fire and the next round of hybridization allows for the assimilation of introgressed genes into the gene pools of the individual species, thus maintaining species integrity despite introgression.

Extent of the Hybrid Zone

Previous literature (Ernst et al 1988; Schaefer and Hanover 1990) have considered zone 3, where Engelmann and blue are interspersed, to be the location of putative hybrids, and zones 2 and 4 to be composed primarily of blue and Engelmann spruce, respectively. The location of individuals with a hybrid profile from this study in zones 1, 2 and 3 increases the zone of hybridization.

Due to the small number of trees sampled, and the fact that only morphologically blue spruce trees were selected, the extent of the introgression zone is unknown. Because certain crosses, $B \times (E \times B)$ and $(E \times B) \times E$, disrupt the hybrid organellar backgrounds, nuclear markers would be necessary to truly determine the boundaries of any introgression zone.

Regions of sympatry other than Scotch Creek exist but the status of those populations with regard to natural hybridization and introgression is unknown. However, two populations of allopatric blue spruce geographically separated from Scotch Creek and each other near South Fork, and Almont, Colorado show no evidence of hybridization (data not shown). This suggests that natural hybridization and the results of introgression are not widespread and may be restricted to areas where the two species are currently sympatric.

SUMMARY

The use of organelle molecular markers to identify zones of introgression has been successful in other wind pollinated plants such as iris (Arnold et al 1991), spruce (Sutton et al 1991), pine (Dong and Wagner 1993) and poplar (Keim et al 1989). This research utilized organelle molecular markers to identify hybrid individuals despite a lack of morphological and chemical evidence for hybridization from previous work in the same drainage. This discrepancy is most likely the result of overlapping trait expression between the two species which could not be resolved through the use of hybrid indices or discriminate function analysis. A model for the interaction of blue and Engelmann spruce in the Scotch Creek drainage was presented that calls for fire to create a spatial and temporal break in the association of the two species. Recolonization efforts by Engelmann and blue spruce are slow due to heavy competition from grasses and a low fire survival rate which limits the number of available seed trees. This geographic and temporal distancing allows the species to maintain their integrity despite any introgression that may have occurred.

LITERATURE CITED

- Alexander, RR 1974. Silviculture of central and southern Rocky Mountain forests. USDA For. Ser. Res. Pap. RM-120. Rocky Mountain Forest and Range Experiment Station, Fort Collins, CO., 36p.
- Anderson, E and Hubrecht, L 1938. Hybridization in *Tradescantia*. III. The evidence for introgressive hybridization. *Amer. J. Bot.* 25:396-402.
- Arnold, ML, Buckner, CM and Robinson, JJ 1991. Pollen-mediated introgression and hybrid speciation in Louisiana irises. *Proc. Natl. Acad. Sci. USA* 88:1398-1402.
- Bousquet, J, Cheliak, WK, Wang, J, and Lalonde, M 1990. Genetic divergence and introgressive hybridization between *Alnus sinuata* and *A. crispa* (Betulaceae). *Pl. Syst. Evol.* 170:107-124.
- Daubenmire, R 1972. On the relation between *Picea pungens* and *Picea engelmannii* in the Rocky Mountains. *Can. J. Bot.* 50:733-742.
- Daubenmire, R 1974. Taxonomic and ecologic relationships between *Picea glauca* and *Picea engelmannii*. *Can. J. Bot.* 52:1545-1560.
- David, AJ and Keathley, DE 199x. Inheritance of mitochondrial DNA in interspecific crosses of *Picea glauca* and *P. omorika*. *Can. J. For. Res.* (in press).
- dePamphilis, CW and Wyatt, R 1989. Hybridization and introgression in Buckeyes (*Aesculus*: Hippocastanaceae): a review of the evidence and a hypothesis to explain long-distance gene flow. *Syst. Bot.* 14:593-611.
- Dong, J and Wagner, DB 1993. Taxonomic and population differentiation of mitochondrial diversity in *Pinus banksiana* and *Pinus contorta*. *Theor. Appl. Genet.* 86:573-578.
- Ernst, SG, Hanover, JW, Keathley, DE and Mao, IL 1988. Genetic variation and control of intraspecific crossability in blue and Engelmann spruce. *Sil. Genet.* 37:112-118.
- Ernst, SG, Hanover, JW and Keathley, DE 1990. Assessment of natural interspecific hybridization of blue and Engelmann spruce in southwestern Colorado. *Can. J. Bot.* 68:1489-1496.

- Fechner, GH and Clark, RW 1969. Preliminary observations on hybridization of Rocky Mountain spruces. Proc. 11th Meeting, Comm. Forest Tree Breeding in Canada. 11:237-247. Ste. Anne de Bellevue, Quebec, Aug. 8-10 1968.
- Grant, V 1981. Plant Speciation. Columbia University Press, NY.
- Gruber, KF 1990. Morphological trait analysis of synthetic *Picea engelmannii* (Parry) x *P. pungens* (Engelm.) hybrids. MS thesis, Department of Forestry, Michigan State University, East Lansing, Michigan.
- Habeck, JR and Weaver, TW 1969. A chemosystematic analysis of some hybrid spruce (*Picea*) populations in Montana. Can. J. Bot. 47:1565-1570.
- Jensen, RJ, Hokanson, SC, Isebrands, JG, and Hancock, JF 1993. Morphometric variation in oaks of the Apostle Islands in Wisconsin: evidence of hybridization between *Quercus rubra* L. and *Quercus ellipsoidales* Hill. Amer. J. Bot. 80:1358-1366.
- Jones, JR and Bernard, NT 1977. How to tell Engelmann from blue spruce in the southwest. USDA For. Ser. Gen. Tech. Rep. RM-34.
- Keim, P, Paige, KN, Whitham, TG and Lark, KG 1989. Genetic analysis of an interspecific hybrid swarm of *Populus*: occurrence of unidirectional introgression. Genetics 123:557-565.
- Lanner, RM and Phillips, AM, III 1992. Natural hybridization and introgression of pinyon pines in northwestern Arizona. Intl. J. Plant Sci. 153:250-257.
- Little, EL, Jr. 1953. A natural hybrid in Alaska. J. Forest. 51:745-747.
- Little, EL, Jr. and Pauley, SS 1958. A natural hybrid between white and black spruce in Minnesota. Amer. Mid. Nat. 60:202-211.
- Manley, SAM 1972. The occurrence of hybrid swarms of red and black spruces in central New Brunswick. Can. J. For. Res. 2:381-391.

- Mitton, JB and Andalora, R 1981. Genetic and morphological relationships between blue spruce, *Picea pungens*, and Engelmann spruce, *Picea engelmannii*, in the Colorado Front Range. *Can. J. Bot.* 59:2088-2094.
- Morgenstern, EK and Farrar, JL 1964. Introgressive hybridization in red and black spruce. Univ. Toronto, Fac. Forest., Tech. Rep. #4.
- Palmer, EJ 1948. Hybrid oaks of North America. *J. Arnold Arbor.* 29:1-48.
- Rajora, OP and Dancik, BP 1995. Chloroplast DNA variation in *Populus*. III. Novel chloroplast DNA variants in natural *Populus x canadensis* hybrids. *Theor. Appl. Genet.* 90:331-334.
- Rehfeldt, G 1994. Adaptation of *Picea engelmannii* populations to the heterogeneous environments of the Intermountain West. *Can. J. Bot.* 72:1197-1208.
- Riemenschneider, D and Mohn, CA 1975. Chromatographic analysis of an open-pollinated Rosendahl spruce progeny. *Can. J. For. Res.* 5:414-418.
- Rieseberg, LH and Ellstrand, NC 1993. What can molecular and morphological markers tell us about plant hybridization? *Crit. Rev. Plant Sci.* 12:213-241.
- Roche, L and Fowler, DP 1975. Genetics of Sitka spruce. USDA For. Ser. Res. Pap. WO-26.
- Schaefer, PR and Hanover, JW 1985. A morphological comparison of blue and Engelmann spruce in the Scotch Creek drainage, Colorado. *Sil. Genet.* 34:105-111.
- Schaefer, PR and Hanover, JW 1986. Taxonomic implications of monoterpene compounds of blue and Engelmann spruces. *For. Sci.* 32:725-734.
- Schaefer, PR and Hanover, JW 1990. An investigation of sympatric populations of blue and Engelmann spruces in the Scotch Creek drainage, Colorado. *Sil. Genet.* 39:72-81.
- Stahelin, R 1943. Factors influencing the natural restocking of high altitude burns by coniferous trees in the central Rocky Mountains. *Ecol.* 24:19-30.
- Stine, M and Keathley, DE 1990. Paternal inheritance of plastids in Engelmann spruce X blue spruce hybrids. *J. Hered.* 81:443-446.

- Stine, M, David, AJ, and Keathley, DE 1991. Inheritance of plastids and mitochondria in spruce. Plant Genetics Newsletter 8:2-4.
- Sutton, BCS, Flanagan, DJ, Gawley, JR, Newton, CH, Lester, DT and El-Kassaby, YA 1991. Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. Theor. Appl. Genet. 82:242-248.
- Taylor, RJ, Williams, S and Daubenmire, R 1975. Interspecific relationships and the question of introgression between *Picea engelmannii* and *Picea pungens*. Can. J. Bot. 53:2547-2555.
- Veblen, TT, Hadley, KS, Nel, EM, Kitzberger, T, Reid, M, and Villalba, R 1994. Disturbance regime and disturbance interactions in a Rocky Mountain subalpine forest. J. of Ecol. 82:125-135.
- Wheeler, NC and Guries, RP 1987. A quantitative measure of introgression between lodgepole and jack pines. Can. J. Bot. 65:1876-1885.
- Whittemore, AT and Schaal, BA 1991. Interspecific gene flow in sympatric oaks. Proc. Natl. Acad. Sci. USA 88:2540-2544.

SUMMARY AND CONCLUSIONS

In Chapter I Serbian parents, representative white spruce, and interspecific hybrids representing reciprocal crosses were used to demonstrate the maternal inheritance of mitochondrial DNA in *Picea*. Two different mitochondrial probes were utilized in conjunction with five different enzymes for a total of ten enzyme/probe combinations, nine of which were diagnostic. Maternal inheritance of mitochondria was indicated for each interspecific hybrid in every diagnostic enzyme/probe combination, insinuating that the mechanism that controls mitochondrial inheritance is consistently applied. There was no indication of paternal or nonparental bands in the interspecific hybrids.

These results along with the information on paternal plastid inheritance in *Picea* (Stine and Keathley 1990, Sutton et al 1991) indicate that in *Picea* three distinct genomic lineages exist, representing the mitochondrial, chloroplast and the nuclear genomes. The conservative nature of organelle inheritance in *Picea* means that mitochondria and chloroplasts are suitable for answering questions pertaining to pollen vs. seed gene flow, cytonuclear disequilibrium, genetic structure of populations, origin of populations, species interactions, and for use as a taxonomic tool to investigate species relatedness.

The work in Chapter II combined the knowledge of organelle inheritance in *Picea* with the knowledge of a unilateral crossing incompatibility between Engelmann and blue spruce (Fechner and Clark 1969, Ernst et al 1990) to investigate the feasibility of identifying Engelmann X blue spruce F_1 hybrids derived from controlled crosses. RFLP analysis showed that, as predicted, Engelmann X blue spruce F_1 hybrids could be differentiated from either parent based on the retention of their Engelmann spruce mitochondrial and blue spruce chloroplast backgrounds. In addition to the Engelmann spruce mitochondrial background each F_1 hybrid possessed one weak hybridizing fragment that was common to every blue spruce parent. This 3.5 kb RFLP was not a result of leakage because other blue spruce fragments, some of which were strong hybridizing fragments, were not inherited. This indicated that the 3.5 kb RFLP was not of mitochondrial origin and suggested cross-homology between the COXII mitochondrial probe and another genome. Subsequent work in Chapter III showed that the presence or absence this 3.5 kb RFLP was perfectly correlated with the presence or absence of a blue spruce chloroplast background in a given individual. Thus the 3.5 kb RFLP is probably the result of hybridization of the COXII mitochondrial probe to a portion of the blue spruce chloroplast genome. Because of the unique nature of this banding pattern it is theoretically possible to identify F_1 hybrids solely on the basis of the

BamHI/COXII enzyme/probe combination. The results of this chapter provide confidence in the ability of organelle markers to identify naturally occurring hybrids between Engelmann and blue spruce.

The results of Chapter III detail the analysis of a putative Engelmann/blue spruce hybrid zone in the Scotch Creek drainage of southwestern Colorado. The same enzyme/probe combinations that were used to identify Engelmann X blue F_1 hybrids from controlled crosses were utilized to identify three hybrid individuals, one mature tree and two seedlings, one heteroplasmic seedling, and a possible advanced generation introgressant. The high level of hybrids identified (10.5%) in the sample and the presence of a possible introgressant raise questions about the longterm stability of the integrity of Engelmann and blue spruce as individual species in the Scotch Creek drainage. If introgression occurs unabated between these two species there should be a mixing of traits resulting in a decrease in the ability to differentiate between them. Currently it is possible to differentiate between the two species in the Scotch Creek drainage, this suggests that little introgression is occurring, or that hybridization and introgression has only recently begun to occur. Ecological evidence from the drainage suggests that the latter is correct.

In the Scotch Creek drainage, unevenaged forests exist at higher elevations on cooler and moister slopes with northern aspects. These forests have large (> 0.9 m diameter) Engelmann spruce as well as dead and down trees (David and Keathley personal observation). In the area immediately around Scotch Creek and on slopes with a southern aspect is a young forest with open grassy areas and no dead or dying trees. This scenario is exactly the opposite of what is expected when trees colonize a drainage. If spruce were new to the drainage then the oldest portion of the forest would be found near the creek and the youngest portions on the slopes colonizing new areas. This inversion suggests a dramatic disturbance, possibly fire, that would create two refugial populations, one for Engelmann spruce in the drainage at high elevation on slopes with a northern aspect which are better protected from fire, and one for blue spruce outside the drainage in the Dolores River Valley because the sites that blue spruce inhabits in the drainage are the most fire prone.

This fire disturbance model has direct implications for the interactions between Engelmann and blue spruce in the Scotch Creek drainage. The spatial and temporal break in the association between these two species may provide a period in which genes from previous introgression events can be assimilated into the recipient population, thus

decreasing the longterm effect of introgression and allowing the integrity of the species to be maintained.

There are a number of different lines of investigation to validate the fire disturbance model. The most obvious one is the search for primary evidence of fire in the drainage. Charcoal in the creek bed and/or charred stumps would be the most obvious objects to look for. Other lines of investigation revolve around gene flow between Engelmann and blue spruce. In order to conclusively demonstrate that introgression has occurred it would be necessary to have nuclear evidence of blue and Engelmann genes in the same individual. This may be possible to accomplish with seedling IV3 whose organellar composition suggests that it is an advanced generation introgressant. Another opportunity to show the potential for introgression would be to investigate the viability of the existing Engelmann X blue F_1 hybrids and determine their ability to backcross to Engelmann and blue spruce. This may also provide information on the unilateral crossing incompatibility between Engelmann and blue spruce. The viability and potential backcross ability of the mature hybrid, tree 574, is also of interest. Cones collected from this tree for the purpose of measuring traits diagnostic of blue and Engelmann spruce, were empty of seed, but this was expected due to the lateness of the season and the actual presence of cones suggests that the tree is viable.

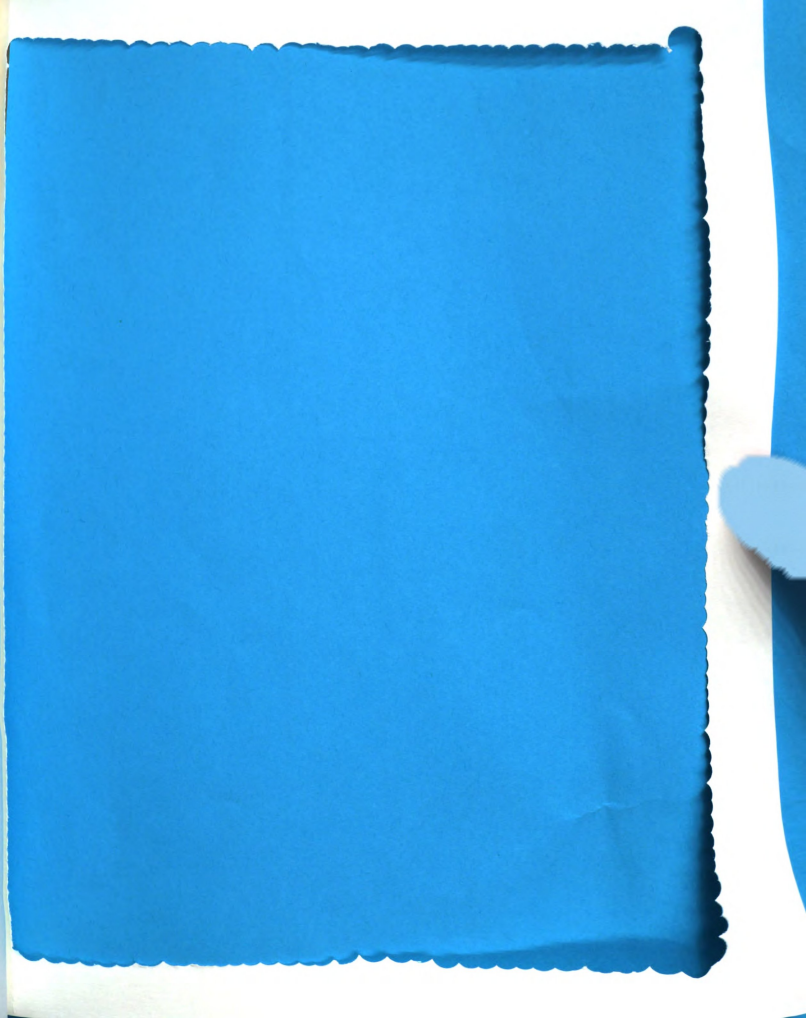
Evidence of introgression may also be obtained by comparing representative populations of Engelmann and blue spruce from Scotch Creek with disjunct populations where the two species are allopatric. Controlled crosses made between these individuals may indicate that reproductive character displacement has occurred if the crosses between allopatric populations of Engelmann and blue spruce have a higher success rate than crosses between Engelmann and blue spruce in Scotch Creek, or crosses between a Scotch Creek species and the reciprocal species in the allopatric population.

Finally, if primary evidence of fire can be found, and a date for the fire can be established, then any blue spruce in the drainage that predate the fire are a genetic link to the previous blue spruce population. If the time that has elapsed between these two populations is sufficient, and the refugia for blue spruce is outside the Scotch Creek drainage, then these 'prefire' trees may be genetically distinct from the blue spruce population presently in the drainage.

LITERATURE CITED

- Ernst, SG, Hanover, JW and Keathley, DE 1990. Assessment of natural interspecific hybridization of blue and Engelmann spruce in southwestern Colorado. Can. J. Bot. 68:1489-1496.
- Fechner, GH and Clark, RW 1969. Preliminary observations on hybridization of Rocky Mountain spruces. Proc. 11th Meeting, Comm. Forest Tree Breeding in Canada. 11:237-247. Ste. Anne de Bellevue, Quebec, Aug. 8-10 1968.
- Stine, M and Keathley, DE 1990. Paternal inheritance of plastids in Engelmann spruce X blue spruce hybrids. J. Hered. 81:443-446.
- Sutton, BCS, Flanagan, DJ, Gawley, JR, Newton, CH, Lester, DT and El-Kassaby, YA 1991. Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. Theor. Appl. Genet. 82:242-248.

APPENDIX



APPENDIX A

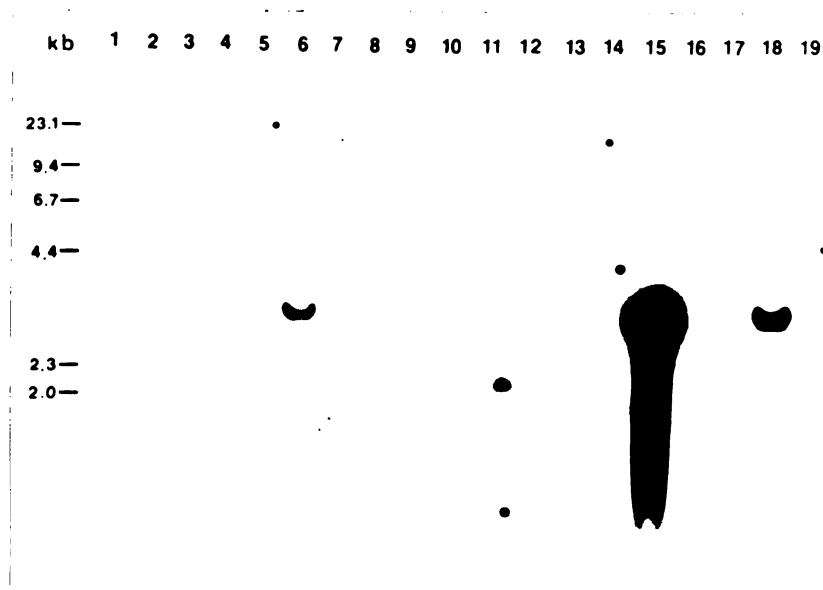


Figure 1. Hybridization of pBR322 to EcoRI digested total DNA extracted from Serbian, white and interspecific hybrids of Serbian and white spruce. Lanes 1 and 2 are Serbian parents. Lanes 3-9 are Serbian x White hybrids from two separate crosses. Lanes 10 and 11 are representative white spruce. Lanes 12-19 are White x Serbian hybrids from 3 separate crosses. Molecular weight markers are λ HindIII.

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