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Inheritance of mitochondrial DNA in the conifer *Larix*

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Summary. Restriction fragment length polymorphisms between *Larix leptolepis* and *Larix decidua* were identified in heterologous hybridization experiments, using wheat mitochondrial DNA probes specific for *atp9*, *cox*I, *nad3/rps12*, and *orf25*. Analysis of eight individuals of each reciprocal hybrid of these two species revealed that mitochondrial DNA was maternally inherited. Furthermore, sequences homologous to wheat *orf25* were also identified in *Larix gmelini*, *Larix siberica*, *Larix olgensis*, and *Larix laricina*, as well as *Ginkgo biloba*, *Picea mariana*, *Picea glauca* and *Pinus contorta*.

Key words: Mitochondria – Inheritance – RFLP – *Larix* – Conifers

Introduction

Genetic information contained within the mitochondria and chloroplasts of land plants is distinct from that of the nuclear genome. In higher plants, the mode of inheritance of these organelles is non-Mendelian and predominantly maternal in origin. There is evidence that mitochondrial DNA (mtDNA) is maternally inherited in some conifers (Neale and Sederoff 1989; Sutton et al. 1991) while in others it appears to be paternal in origin (Neale et al. 1989). In contrast to most angiosperms (Sears 1988), transmission of chloroplast DNA (cpDNA) in gymnosperms is primarily paternal (Ohba et al. 1971; Neale et al. 1986; Wagner et al. 1987; Szmidt et al. 1988; Neale and Sederoff 1989; Neale et al. 1989; Stine et al. 1989; Stine and Keathley 1990; Sutton et al. 1991), with the possible exception of biparental transmission in *Larix* Mill. hybrids (Szmidt et al. 1987) and in *Pinus monticola* Doug. ex D. Don (White 1990). Maternal inheritance of one organellar genome, and paternal inheritance of the other organellar genome within the same plant appears to be unique to certain conifers. This indicates that these tree species must have special mechanisms for organelle exclusion and/or degradation. Ultrastructural observations of *Pseudotsuga*, *Pinus*, and *Larix* (Camefort 1968; Chesnoy and Thomas 1971; Owens and Morris 1990) provide physical explanations for the phenomena of paternal inheritance of chloroplasts and maternal inheritance of mitochondria in conifers. Egg cell plastids are sequestered into inclusions, followed by disruption of the original plastid structure and subsequent destruction of maternal chloroplast DNA. However, the egg cell mitochondria aggregate, migrate to the perinuclear zone, and may become altered prior to fertilization. These maternal mitochondria become incorporated into the cytoplasm of the new embryo (Owens and Morris 1990).

Further support for this ultrastructural evidence has been demonstrated for various gymnosperms using the technique of restriction fragment length polymorphism (RFLP) analysis. By this method cpDNA has been shown to be predominantly paternally transmitted in *Pseudotsuga menziesii* (Mirb.) Franco (Neale et al. 1986), *Larix* Mill. hybrids (Szmidt et al. 1987), *Pinus* L. hybrids (Wagner et al. 1987), *Picea* A. Dietr. hybrids (Szmidt et al. 1988; Stine et al. 1989; Stine and Keathley 1990), *Sequoia sempervirens* D Don Endl. (Neale et al. 1989), and *Pinus taeda* L. (Neale and Sederoff 1989), *P. banksiana* Lamb. (Wagner et al. 1987), *P. monticola* Doug. ex D. Don (White 1990), and *Calocedrus decurrens* [Torr.] Florin (Neale et al. 1991). Analysis of RFLPs has been used to follow mtDNA inheritance in only a few gymnosperms. As expected, mitochondrial DNA is ma-

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ternally inherited in *P. taeda* L. and hybrids of *Pinus strobus* × *P. griffithii* McClelland as demonstrated by RFLP analysis of intraspecific crosses (Neale and Sederoff 1989; Sutton et al. 1991). However, recent analyses of mtDNA inheritance in *S. sempervirens* (Family Taxodiaceae) and *C. decurrens* (Family Cupressaceae) suggest that mitochondria are paternally inherited in these conifers (Neale et al. 1989, 1991). Because members of all Orders of gymnosperms appear to have maternal inheritance of mtDNA, the question arises as to whether paternal inheritance of mtDNA may be limited to members of the Families Cupressaceae and Taxodiaceae in the Order Coniferales, or if members of other Families exhibit this phenomenon.

This paper reports on a study examining the inheritance of mitochondrial DNA in *Larix* species (Family Pinaceae). RFLP markers were identified between the mitochondrial genomes of *Larix decidua* (European larch) and *L. leptolepis* (Japanese larch). These RFLPs were then used to identify the origin of the mitochondrial DNA in reciprocal hybrid crosses of these two larch species. Inheritance was inferred from RFLP segregation in the hybrid trees and the results of these analyses are discussed.

Materials and methods

Plant materials

The larch trees used in this study are part of Experiment # 252-C, a demonstration of larch species and hybrids, located at the Petawawa National Forestry Institute, Chalk River, Ontario, Canada. The trees were sown in 1977, transplanted in 1978, and planted at the current location in 1980 at a spacing of 5 m × 5 m. Nine trees of each of the four following seedlots were planted: *L. leptolepis* Lot #J.8951-79050; *L. decidua* Lot #J.7462-748524; Hybrid *L. decidua* × *L. leptolepis* Lot #J.9982-748526; Hybrid *L. leptolepis* × *L. decidua* Lot #J.9981-748525. The seedlot of *L. decidua* originated from Jagesborg, Denmark (improved Sudeten larch); *L. leptolepis* from Central Honshu, Japan, Nagaro Provenance; and both open-pollinated hybrids were from Germany. Seedlings of the following PNFI Seed Bank seedlots were used for DNA isolations; *Larix gmelini* Lot #8380678; *L. laricina* Lot #7930280; *L. olgensis* Lot #8480985; *L. siberica* Lot #8580240; *Pinus contorta* Lot #7060480; *Picea glauca* Lot #6730800; and *Picea mariana* Lot #8630180. Total genomic DNA was also isolated from leaves of a single *Ginkgo biloba* tree from the Central Experimental Farm, Agriculture Canada, Ottawa, Ontaria, Canada

Plant DNA isolation and analysis

A modification of the procedure of Murray and Thompson (1980) was used to isolate high-molecular-weight DNA from mature needles and seedling tissue of *Larix* and other gymnosperms, as previously described (DeVerno et al. 1988). DNA samples were digested with the restriction endonucleases *Bam*HI, *Cfo*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, and *Msp*I according to instructions supplied by the manufacturer (BRL, Gaithersburg, Md.). Five micrograms of each restricted DNA sample were separated by electrophoresis in 0.7% horizontal agarose gels in TAE buffer (0.4 M Tris-acetate, 1 mM EDTA, pH 8.0) at 0.5 V/cm for 16 h. DNA was transferred to Biotrans (ICN, Irvine, Calif.) nylon membranes using the LKB Vacugene Vacuum Blotting Unit Model 2016 (Pharmacia LKB Biotechnology, Uppsala, Sweden). DNA was covalently bound to the membrane by exposure to ultraviolet radiation at an energy of 120 mJoules/cm^2 using the FB-UVXL-1000 Cross Linker (Fisher Scientific). pUC plasmid clones containing the *orf25* (Bonen et al. 1990), *nad3/rps12*, (Gualberto et al. 1988), *nad5* (deSouza et al. 1991; Bonen et al., unpublished data), *coxI* (Bonen et al. 1987), *cob* (Boer et al. 1985), *atpA* and *atp9* (Bonhomme et al. 1989) coding sequences of wheat mitochondria were digested with the appropriate restriction endonucleases, and inserts were separated from vector sequences by agarose-gel electrophoresis. DNA fragments were purified with Geneclean II (Bio 101 Inc., La Jolla, Calif.) and labelled with ^{32}P α-dCTP (Amersham, Oakville, Ont.) according to the manufacturer's instructions using the Random Primer Labelling System (BRL, Gaithersburg, Md.) Hybridizations were conducted at 60 °C with gentle shaking overnight, in a solution containing 5 × Denhardts solution (0.1% Ficoll, 0.1% polyvinylpyrolidine, 0.1% BSA), 5 × SSPE (0.9 M NaCl, 0.05 M sodium phosphate pH 8.3, 0.05 M EDTA), and 0.2% SDS. Two hybridization washes of 20 min each were conducted in 2 × SSPE with 0.5% SDS at 60 °C. Hybridized blots were exposed to Kodak XAR X-ray film in the presence of DuPont Cronex Lightning Plus intensifying screens at −70 °C for 24–72 h.

Results

Hybridization of total genomic DNA of *L. decidua* and *L. leptolepis* with wheat mitochondrial gene probes produced distinct hybridization signals that were used to detect RFLPs between the mitochondrial genomes of these two species. Various restriction digests of total DNA of *L. decidua* and *L. leptolepis* were hybridized with a number of wheat mitochondrial gene probes to identify RFLPs that were distinctive to each tree species (Table 1, Fig. 1). Digestion of total genomic DNA of *L. leptolepis* and *L. decidua* tree needles with *Kpn*I, followed by hybridization with an 800-bp *Bam*HI/*Mlu*I fragment containing *orf25*, produced a 2.4-kb band unique to the *L. leptolepis* mitochondrial genome, and a 2.8-kb band unique to the *L. decidua* mitochondrial genome (Fig. 1). When eight trees of each reciprocal cross of these two *Larix* species were analyzed by restriction with *Kpn*I and hybridized with *orf25* all hybrid trees showed hybridization signals identical to the maternal species (Fig. 2). In addition, RFLPs were also detected between *L. leptolepis* and *L. decidua* using the restriction endonuclease *Msp*I and the *orf25* probe (Fig. 1). Similarly, total genomic DNA of *Larix* digested with *Dra*I and hybridized with the *nad3/rps12* probe from wheat mitochondria produced a 13-kb band unique to the *L. leptolepis* mitochondrial genome and a 10-kb band unique to the *L. decidua* mitochondrial genome (Fig. 3). When the same eight trees of each reciprocal cross of these two *Larix* species were analyzed by restriction with *Dra*I and hybridized with the *nad3/rps12* probe, mito-

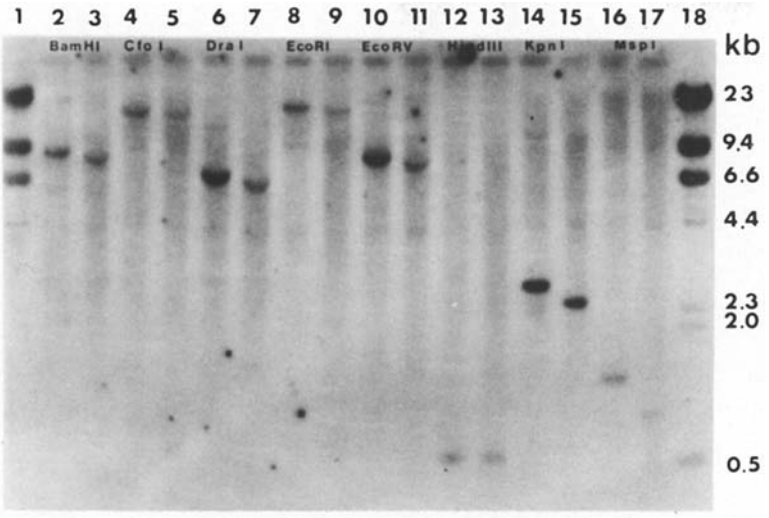

Fig. 1. Identification of RFLPs between *L. leptolepis* and *L. decidua* by hybridization of total genomic DNA with the wheat mitochondrial gene probe *orf25*. *Lanes 1 and 18*, lambda DNA *Hin*dIII digest molecular weight marker. Even-numbered lanes contain DNA from *L. decidua*. Odd-numbered lanes contain DNA from *L. leptolepis*. *Lanes 2 and 3*, *Bam*HI; *lanes 4 and 5*, *Cfo*I; *lanes 6 and 7*, *Dra*I; *lanes 8 and 9*, *Eco*RI; *lanes 10 and 11*, *Eco*RV; *lanes 12 and 13*, *Hin*dIII; *lanes 14 and 15*, *Kpn*I; *lanes 16 and 17*, *Msp*I

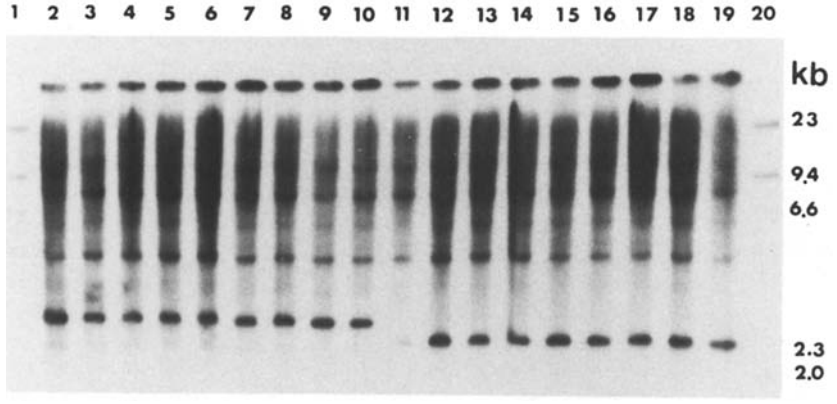

Fig. 2. Inheritance of mitochondrial DNA sequences homologous to *orf25* in reciprocal hybrid crosses of *L. leptolepis* and *L. decidua*. *Lanes 1 and 20*, lambda DNA *Hin*dIII digest molecular weight marker; *lane 2*, *L. decidua* digested with *Kpn*I; *lanes 3–10*, *L. decidua* (female) × *L. leptolepis* (male) digested with *Kpn*I; *lanes 11–18*, *L. leptolepis* (female) × *L. decidua* (male) digested with *Kpn*I; *lane 19*, *L. leptolepis* digested with *Kpn*I

Table 1. Identification of RFLPs to distinguish *Larix leptolepis* and *L. decidua*

Probe/endonuclease	*Dra*I	*Eco*RV	*Kpn*I	*Bam*HI	*Cfo*I	*Eco*RI	*Hin*dIII	*Msp*I
atp9	+	–	–	–	+	+	–	NT
atpA	–	–	–	–	–	–	–	–
cob	–	–	–	NT	NT	NT	NT	NT
coxI	+	–	–	NT	NT	NT	NT	NT
nad3/rps12	+	–	–	–	–	NT	–	NT
nad5	–	–	–	NT	NT	NT	NT	NT
orf25	–	–	+	–	–	–	–	+

+, presence of RFLP; –, no RFLP; NT, not tested

chondrial inheritance was seen to be maternal, except in one tree which had a fragment that appears similar to one present at low levels in the maternal species (Fig. 3). The restriction endonuclease *Dra*I also revealed RFLPs between these two species with the probes *coxI* and *atp9*. In contrast, digestion of total genomic DNA of *L. leptolepis* and *L. decidua* tree needles with any of the restriction endonucleases *Bam*HI, *Cfo*I, *Eco*RI, or *Eco*RV did not reveal RFLPs between these two tree species with any of the mitochondrial probes examined.

Total genomic DNA of *L. olgensis*, *L. gmelini*, *L. siberica*, and *L. laricina*, *Pinus contorta*, *P. glauca*, *P. mariana*, and *G. biloba* was hybridized with the wheat mitochondrial probe *orf25* to determine if homologous sequences were present in other gymnosperms. Sequences homologous to *orf25* were detected in all the

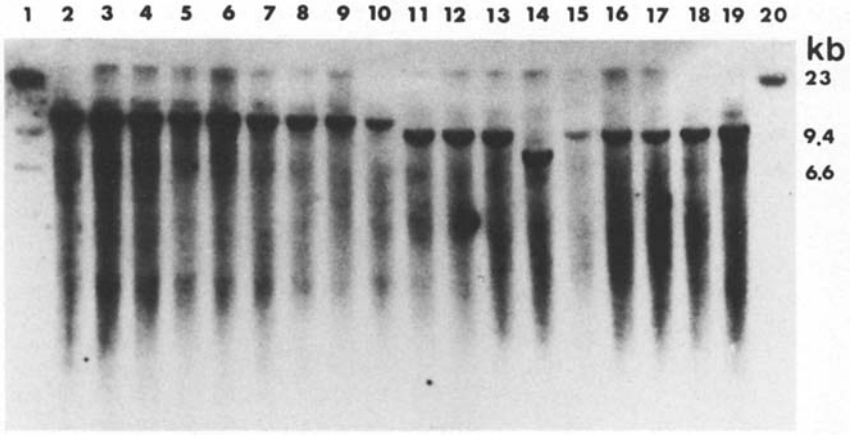

Fig. 3. Inheritance of hybrid *Larix* mitochondrial DNA sequences homologous to *nad3/rps12*. *Lanes 1 and 20*, lambda DNA *Hin*dIII digest molecular weight marker; *lane 2*, *L. leptolepis* digested with *Dra*I; *lanes 3–10*, *L. leptolepis* (female) × *L. decidua* (male) digested with *Dra*I; *lanes 11–18*, *L. decidua* (female) × *L. leptolepis* (male) digested with *Dra*I; *lane 19*, *L. decidua* digested with *Dra*I

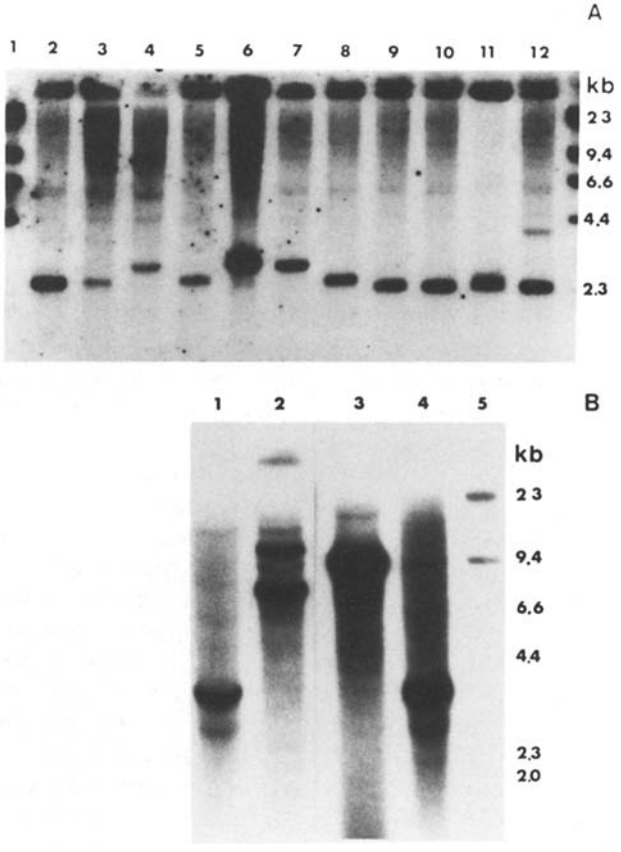

Fig. 4 A, B. Southern-blot analysis of DNA from several *Larix* species and other gymnosperms hybridized with *orf25*. **A** Various *Larix* species total DNA digested with *Kpn*I and hybridized with *orf25*. *Lane 1*, lambda DNA *Hin*dIII digest molecular weight marker; *lanes 2 and 3*, *L. leptolepis* × *L. decidua*; *lane 4*, *L. decidua* × *L. leptolepis*; *lane 5*, *L. leptolepis*; *lanes 6 and 7*, *L. decidua*; *lane 8*, *L. siberica*; *lanes 9 and 10*, *L. olgensis*; *lane 11*, *L. laricina*; *lane 12*, *L. gmelini*. **B** Gymnosperm species total genomic DNA digested with *Dra*I and hybridized with *orf25*. *Lane 1*, *P. glauca*; *lane 2*, *P. contorta*; *lane 3*, *G. biloba*; *lane 4*, *P. mariana*; *lane 5*, lambda DNA *Hin*dIII digest molecular weight marker

Larix species examined (Fig. 4A) and in other gymnosperms (Fig. 4B), on either a single restriction fragment or on two fragments of unequal intensity. The hybridization patterns of *P. mariana* (black spruce) and *P. glauca* (white spruce) appear to be identical with respect to fragment sizes and relative intensities. This indicates that sequences homologous to *orf25* are present in both angiosperms and gymnosperms.

Discussion

Plant mitochondrial protein-coding sequences are highly conserved, suggesting that heterologous probes can be used successfully to identify the respective coding sequences in diverse plant species. Recent studies of conifer mitochondrial genome inheritance have used maize mitochondrial DNA fragments as probes to detect RFLPs. The cytochrome oxidase II probe from maize mitochondria has been used to detect homologous sequences in pine, spruce, and coast redwood (Neale 1989; Sutton et al. 1991; Wagner et al. 1991). Inheritance of mitochondrial DNA in *C. decurrens* was determined with the maize *coxI* probe (Neale et al. 1991). In addition, sequences homologous to *atpA* have been examined in spruce (Sutton et al. 1991), and 18S and 5S rRNA sequences have been detected in pine (Neale and Sederoff 1989). In the present study we have used seven wheat mitochondrial protein-coding sequences, five of which had not previously been examined in conifers. They all provided distinct signals when hybridized with *Larix* total genomic DNA, confirming the presence of sequences homologous to the mitochondrial genes *atpA*, *atp9*, *nad3/rps12*, *nad5*, *coxI*, *cob*, and *orf25*.

Examination of several species of *Larix* demonstrated that sequences homologous to wheat mitochondrial *orf25* are usually present as a single hybridizing fragment

in *Larix*, suggesting that this DNA sequence is represented in the mitochondrial genome by a single copy. This open reading frame has also been identified as a single-copy gene in wheat (Bonen et al. 1990), maize, tobacco (Stamper et al. 1987), and liverwort (Oda et al. 1992). Although *orf25* sequences have not been identified by sequence comparison in either animal or fungal mitochondrial genomes, it is present in diverse angiosperm plant species such as maize, tobacco, bean, wheat, pea, and rice (Dewey et al. 1986; Stamper et al. 1987; Bonen et al. 1990). Properties such as transcription and sequence conservation suggest that *orf25* encodes a functional plant mitochondrial gene. This gene also hybridized to DNA from the conifers *P. glauca*, *P. mariana*, and *P. contorta*, in addition to the ancient gymnosperm *G. biloba*, which is believed to be the common ancestor of all conifers. This result suggests that *orf25* could be ubiquitous amongst higher plants, as it is common to both angiosperms and gymnosperms. Wheat mitochondrial protein-coding sequence *atp9* has subsequently been used to investigate the stability of the mitochondrial genome of *Larix* during in-vitro somatic embryogenic culture and in the corresponding regenerated trees (DeVerno et al., in preparation).

RFLPs detected between *L. leptolepis* and *L. decidua* were used to monitor inheritance of mitochondrial DNA sequences in reciprocal hybrids of these two species. Although maternal transmission of mitochondrial DNA in plants is predominant, it is not universal among gymnosperms. The various modes of mitochondrial inheritance exhibited by different gymnosperms can be explained by the characteristics of the male gametes and the ultrastructural changes occurring during fertilization. In members of the Orders Gingkoales and Cycadales, the oldest living gymnosperms, a single spermatozoid nucleus enters the egg cell during fertilization, with the male cytoplasm being discarded just outside the egg cell near the point of nuclear entry (Whatley 1982). This ultrastructural evidence suggests that only the maternal organelles would be transmitted to the embryo. Members of the Order Coniferales are believed to be descendants of these ancient gymnosperms, and modifications of the structure and behavior of the gametophytes appears to have altered the pattern of organelle inheritance.

Gymnosperms have been divided into two distinct groups based on ultrastructural features of the male gametes, the behaviour of the pollen tube, and the nature of the pollen tube components that enter the egg (Whatley 1982). The first group is composed of members of the Families Cupressaceae and Taxodiaceae which have fertilization mechanisms and male gamete characteristics consistent with paternal transmission of both organelles. Molecular techniques have identified paternal inheritance of mitochondrial and chloroplast DNA in the conifers *S. sempervirens* of the Family Taxodiaceae (Neale et al. 1989) and *C. decurrens* of the Family Cupressaceae (Neale et al. 1991), which is consistent with the ultrastructural evidence.

Ultrastructural observations of the gametophytes and fertilization mechanisms suggest that members of the Pinaceae, Cephalotaxaceae, Podocarpaceae, and Taxaceae belong in a separate group, and would be expected to exhibit maternal mitochondrial transmission and paternal chloroplast transmission to progeny (Camefort 1968; Chesnoy and Thomas 1971). Mitochondrial DNA sequences of hybrid crosses of the conifer *Larix* have been shown in this study to be of maternal origin, whereas chloroplast DNA is predominantly paternal in origin (Szmidt et al. 1987). Because *Larix* is a member of the Family Pinaceae, these results are consistent with earlier ultrastructural evidence. This phenomenon makes it possible to determine both the maternal and paternal contributions in hybrid and introgressed *Larix* populations. In addition, phylogenies based on data of maternal and paternal origin from an individual tree will be more precise than a single lineage analysis. Molecular techniques have been used to support ultrastructural evidence for plastid and mitochondrial inheritance in only three of the six conifer families, with most of the species studied belonging to the Family Pinaceae. Further studies are required to determine if organelle inheritance in members of all conifer families is consistent with the modes predicted by the ultrastructural evidence.

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