GENETIC EVIDENCE FOR THE PRESENCE OF CYTOPLASMIC DNA IN POLLEN AND MEGAGAMETOPHYTES AND MATERNAL INHERITANCE OF MITOCHONDRIAL DNA IN *PINUS*

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ABSTRACT

The inheritance of mitochondrial (mt) DNA was studied in two parents and 32 F_1 progeny from an inter-specific cross between *Pinus nigra* (Arnold) and *Pinus sylvestris* (L.). To provide new genetic information about elimination of the alternative cytoplasmic genomes, we have tested whether mt- and chloroplast (cp) DNA are present in pollen and megagametophytes of these two species. The occurrence of cp- and mtDNA was assessed by PCR amplification of the chloroplast *trnT-trnF* region and three intra-genic segments of the mitochondrial genes: *coxI, coxIII* and *orf25*. MtDNA polymorphisms distinguishing the two pines were detected with several restriction enzymes on the amplified *coxI* fragment. On the other hand, restriction fragment patterns for *coxIII* and *orf*25 were identical in all samples analysed. The detected *coxI* RFLPs were used to analyse the mtDNA transmission in the F_1 progeny. All the F_1 individuals tested had the coxI restriction fragment patterns specific for the maternal parent *P. nigra* which provides further genetic evidence for the maternal inheritance of mtDNA in the genus *Pinus*. The biparental mtDNA inheritance suggested by cytological studies was not observed. Both cp- and mtDNA were present in haploid megagametophytes and pollen. These results suggest that the elimination of alternative mt- and cpDNA in *Pinus* occurs through different mechanisms. Moreover, our results suggest that more than one copy of the *coxI* gene is present on the mitochondrial genome of the two pines studied.

Keywords: cytoplasmic genomes, mtDNA inheritance, PCR-RFLP, *Pinus nigra, Pinus sylvestris*

INTRODUCTION

Chloroplast and mitochondria contain extra nuclear genetic information that is important for population and evolutionary biology studies. Genetic studies on the chloroplast transmission in plants have been intensive and provided comprehensive knowledge on the subject (HAGEMANN & SCHRÖDER 1989; SEARS 1980). In contrast to the maternal inheritance in angiosperms, *cp*DNA was found to be primarily paternally transmitted in gymnosperms (NEALE *et al.* 1986, 1989, 1991; NEALE & SEDEROFF 1989; SZMIDT *et al.* 1987, 1988; WAGNER *et al.* 1989). Cytological studies have provided much information about the timing and mechanisms of elimination of the maternal cpDNA at fertilisation in gymnosperms (CHESNOY & THOMAS 1971; OWENS & MORRIS 1990; SINGH 1978). On the other hand, much less is known about the inheritance and organisation of *mt*DNA in gymnosperms. Furthermore, the genetic evidence for the presence of cytoplasmic DNA in *Pinaceae* pollen is lacking, and the cytoplasmic composition of the megagametophytes which are of monosporic origin is not clear (SINGH 1978). So far, *mt*DNA inheritance has been studied only in a few species from three families of gymnosperms, Cupressaceae, Taxodiaceae, and Pinaceae. (DEVERNO *et al.* 1993; NEALE *et al.* 1989, 1991; NEALE & SEDEROFF 1989; SUTTON *et al.* 1991; WAGNER *et al.* 1991a). Cytological observations suggest that *Pinaceae* pollen contains chloroplast and mitochondria and that both organelles are transmitted to the egg cell during fertilization (CHESNOY & THOMAS 1971; DAWKINS & OWENS 1993; OWENS & MORRIS 1990; SINGH 1978). These results indicate the potential for biparental inheritance of mtDNA in Pinaceae (OWENS & MORRIS 1990, 1991). However, contrary to this expectation, genetic studies revealed strictly uniparental inheritance of this genome in all gymnosperm species studied so far. Maternal and paternal inheritance of *mt*DNA was found in Pinaceae (DEVERNO *et al.* 1993; NEALE & SEDEROFF 1989; SUTTON *et al.* 1991; WAGNER *et al.* 1991a) but paternal inheritance in the Cupressaceae and Taxodiaceae families (NEALE *et al.* 1989, 1991). The mechanism responsible for the observed selective elimination of the alternative *mt*DNA is still unclear.

Conflicting evidence from genetic and cytological studies on the inheritance of cytoplasmic genomes has been reported (CORRIVEAU & COLEMAN 1988). Cyto-

Cytological methodology can, in some cases, produce artifacts such as chemical destruction of mtDNA (COLEMAN 1984), as well as the difficulties in distinguishing proplastids from mitochondria in an electron micrograph (CONNETT 1987). In addition, as pointed out by CORRIVEAU & COLEMAN (1988), the existence of proplastid-like organelles in a micrograph does not necessarily confirm the presence of plastid DNA. Finally, due to their complexity, cytological observations typically include limited number of individuals.

Therefore, inferences based on this type of studies should be validated by genetic analysis. To provide genetic information about the occurrence of *cp*- and *mt*DNA in haploid megagametophytes and pollen, we used specific primers for *cp*- and *mt*DNA regions to amplify corresponding sequences in pollen and megagametophytes of *Pinus nigra* (Arnold) and *Pinus sylvestris* (L.). In addition, we analysed the transmission of *mt*DNA in a controlled cross between these two species. For this purpose, *mt*DNA restriction fragment length polymorphism (RFLP) markers that distinguish between the two parental species were identified and used to analyse the origin of the *mt*DNA in the controlled F_1 progeny.

MATERIAL AND METHODS

Plant material and DNA isolation The plant material included two parental trees of *P. nigra* and *P. sylvestris*, and 32 F_1 progeny of *P. nigra* × *P. sylvestris* from the controlled cross between these two individuals. Needle samples from these plants were collected individually in the Hørsholm Arboretum, Denmark. Buds from ten more individuals of *P. sylvestris* were collected from a clonal archive in northern Sweden. Pollen from three individuals of *P. sylvestris* was collected by isolating small branches with male strobili in paper bags and slight tapping to release the pollen grains. The dry pollen grains were then gently filtered through a fine sieve to remove possible contamination with diploid material. Prior DNA isolation, pollen was kept overnight in a sterilized liquid sucrose medium (DAWKINS & OWENS 1993). Open-pollinated seeds from three individual trees of *P. nigra* were collected in the Kórnik Arboretum, Poland. Six megagametophytes from each of these three trees were used for DNA isolation.

Megagametophytes from individual seeds were separated from embryo and the surrounding seed coat with a needle under microscope. In *Pinus* seed, the embryo and megagametophyte have very discrete structure and are easy to separate. As demonstrated in our previous study (LU *et al.* 1995), this approach yields megagametophytes that are free of contamination with the surrounding diploid tissues. Genomic DNA from needles was extracted as described by (SZMIDT *et al.* 1986). DNAs from buds, pollen and megagametophytes were isolated with the CTAB procedure (DOYLE & DOYLE 1987). The final DNA pellets were suspended in TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0) and used for PCR amplification.

Primers

Three pairs of primers specific for mitochondrial genes: *coxI, coxIII* and *orf25*, and two pairs of primers for the chloroplast *trnT-trnF* region were used to amplify the corresponding mt- and cpDNA regions. The sequences of the primers employed in this study are listed in Table 1. The primer pair for *coxI* was synthesized using the sequences published by GLAUBITZ & CARLSON (1992). Primers for the *coxIII* and *orf25* regions were designed by aligning and selecting the conserved regions of the corresponding *coxIII* and *orf25* sequences from *Oenothera berteriana, Zea mays, Helianthus annuus, Arabidopsis thaliana, Oryza sativa, Triticum aestivum*, and *Triticum timopheevi*. The DNA sequences were retrieved from the EMBL database and had the following accession numbers: X04764, X53055, X57669, X67105, M74241, X54311, and X62094 respectively. The *coxIII* primers span the region from nucleotide position

Table 1 Primers used to amplify *mt*DNA and *cp*DNA regions in *Pinus nigra* and *P. sylvestris*

Gene	5'primer (5' → 3')	3'primer (5' → 3')	Size 3
CoxI	TTATTATCACTTCCGGTACT	AGCATCTGGATAATCTGG	712 bp
CoxIII	GGTAGATCCAAGTCCATGGC	CAGTACCATGCAGCTGCTTC	704 bp
orf25	ATGCTATTTGCTGCTATTCC	AGGACTATCAAGCCTTCTCG	512 bp
tmL-tmF2	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	950 bp
tmT-tmF2	CATTACAAATGCGATGCTCT	ATTTGAACTGGTGACACGAG	1,500 bp

1 primers published by GLAUBITZ and CARLSON (1992)
2 primers published by TABERLET *et al.* (1991)
3 expected size of PCR product relative to the reference sequence, see text =0C

33 to 734 relative to *H. annuus* sequence (QUAGLIARIELLO *et al.* 1990). The primers for *orf25* cover the region from nucleotide position 37 to 548 relative to *A. thaliana* sequence (BRANDT *et al.* 1992). The two pairs of cpDNA primers were described by TABERLET *et al.* (1991).

PCR amplification

PCR reaction mix contained 10–15 ng DNA, 0.4 μM each of the primers, 150 μM each of dNTP (Pharmacia), and 0.75 U Taq polymerase (Pharmacia) in a total volume of 25 μl. PCR amplification was carried out at 94 °C, 3 min for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing (53 °C for *coxI* and *trnT-trnF*, 60 °C for *coxIII*, 55 °C for *orf25* and *trnL-trnF*) for 1 min, extension at 72 °C for 1 min and 30 sec., and terminated by 10 min at 72 °C.

Digestion, electrophoresis and silver staining

To confirm successful amplification, the PCR products generated by each pair of the primers were first separated on 1.0% agarose gel and stained with ethydium bromide. The amplified mtDNA fragments from *P. nigra* and *P. sylvestris* were subsequently digested with several restriction enzymes (Table 2) and used for RFLP analysis. Three μl of the total 25 μl PCR product was used for each digestion. The digested mtDNA fragments were separated on 6% non-denaturing polyacrylamide gels in 1X TBE buffer in a vertical PROTEAN™-II (BioRad) apparatus. The gels were run at constant current of 50 mA for 4–5 hours. The restriction fragment patterns were visualized by a modified silver staining method (BASSAM *et al.* 1991; KLINKICHT & TAUTZ 1992). The procedure consists of three steps: (i) gel fixation in 10% EtOH, 0.5% HAc for two times of 7 min each; (ii) silver impregnation for 40 min in 0.15% $AgNO_3$; (iii) image development in a solution of 375 mM NaOH, 2 mM $Na(BH_4)$ and 0.4% formaldehyde. The last step takes about 5–10 min. The staining is performed on a shaker.

Table 2 List of restriction enzymes used to digest the PCR amplified mtDNA fragments and the genomic DNA

Fragment/DNA	Enzymes
coxI	*Alu*I,*Cfo*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Taq*I, *Sau*3A
coxIII	*Alu*I,*Cfo*I, *Dde*I, *Hin*fI, *Msp*I, *Rsa*I, *Scr*FI, *Taq*I, *Tru*91
orf25	*Alu*I,*Cfo*I, *Dde*I, *Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Scr*FI, *Taq*I, *Tru*91
genomic DNA	*Alu*I,*Ava*I, *Bam*HI, *Bgl*II, *Cfo*I, *Hind*III, *Hin*fI, *Kpn*I, *Msp*I, *Pst*I, *Rsa*I, *Taq*I, *Xba*I

Southern analysis

The amplified *coxI* fragment from *P. sylvestris* was isolated from low melting point agarose gel and used as a probe in Southern analysis of genomic DNA digests of *P. nigra* and *P. sylvestris*, digested individually with thirteen restriction enzymes (Table 2). The digestion products were separated on 1.0% agarose gel and vacuum transferred to nylon Hybond N membrane (Amersham). Probe was labelled with α-^{32}P-dCTP using the Oligolabelling kit (Pharmacia). DNA hybridization was carried out at 65 °C overnight as described previously (WANG & SZMIDT 1990).

RESULTS

PCR products in haploid gametophytes and diploid tissues

Two fragments of approximately 700 and 510 base pairs (bp) in length were amplified by the *coxIII* and *orf25* primers respectively (Fig. 1A). The fragment amplified by the *coxI* primers in the two *Pinus* species had the size similar to that observed in *Thuja plicata* (712bp) (GLAUBITZ & CARLSON 1992). The two pairs of *cp*DNA primers gave PCR products of approximately 950 bp and 1500 bp respectively (Fig. 1B).

All cp- and mtDNA fragments were amplified in haploid megagametophytes, pollen as well as in diploid buds and needles (Fig. 1A and B). The amplified cp- and mtDNA fragments found in haploid megagametophytes and pollen had the same size as the corresponding fragments observed in diploid buds and needles of the two species (Fig. 1A and B). No size variation between *P. nigra* and *P. sylvestris* was observed with respect to any of the amplified fragments.

MtDNA variation in *P. nigra*, *P. sylvestris*, and their F_1 progeny

To screen for polymorphism in the amplified mtDNA fragments, we selected mainly four-cutter restriction enzymes (Table 2). No variation between *P. nigra* and *P. sylvestris* was observed among *coxIII* and *orf25* restriction fragment patterns produced by any of the restriction enzymes employed in this study (data not shown). On the other hand, four of the eight enzymes used to digest the *coxI* fragment (*Alu*I, *Hin*fI, *Msp*I and *Rsa*I) detected differences between *P. nigra* and *P. sylvestris* (Table 3). Fragment patterns produced by *Hin*fI and *Msp*I are presented in Figure 2A and B. To

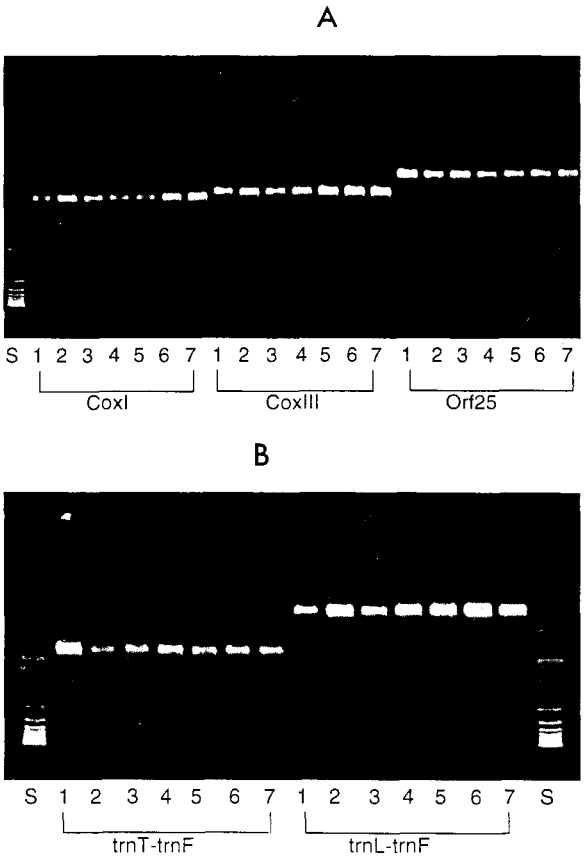

Figure 1 PCR amplified *mt*DNA (A) and *cp*DNA (B) segments in *P. nigra* and *P. sylvestris*. Lanes: S – 1 Kb standard, 1–2 – *P. nigra* female gametophyte, 3–4 – *P. sylvestris* pollen, 5–6 – needles of *P. nigra* × *P. sylvestris* progeny, 7 – *P. sylvestris* bud. 5 µl of the 25 µl PCR products were applied on the gel.

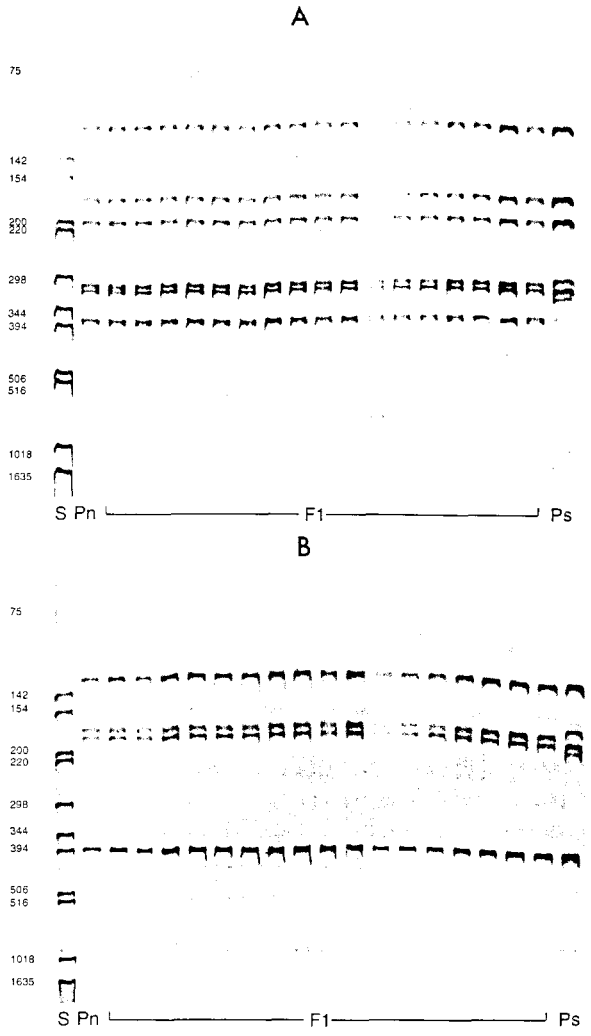

Figure 2 *CoxI* restriction fragment patterns generated by *Hinf*I(A) and *Msp*I(B). S – 1Kb standard (size in bp), Pn – *P. nigra*, F_1 – progeny of *P. nigra* ×*P. sylvestris*, Ps – *P. sylvestris*

confirm that the observed mtDNA polymorphism between the two species is truly species specific, DNA samples from 10 additional individual trees of *P. sylvestris* and from 18 megagametophytes collected from three individual trees of *P. nigra* were analysed in the same way as above. No intra-specific variation was observed with regard to the *coxI* restriction fragment patterns generated by the four enzymes in neither of the two species. The *coxI* restriction fragment patterns detected in haploid megagametophytes and pollen were identical with those found in diploid tissues of both species (data not shown).

The obtained *coxI* PCR -RFLP markers that distinguished between *P. nigra* and *P. sylvestris* were subsequently used to analyse the 32 F_1 progeny from the controlled cross. All the progeny showed the restriction fragment patterns specific for the maternal parent *P. nigra* (Fig. 2A and B). The markers specific for the paternal parent *P. sylvestris* were not observed in any of the progeny tested.

Patterns of *cox*I RFLPs

Surprisingly, the sum of sizes of individual *coxI* fragments generated by several restriction enzymes exceeded the size of an undigested *coxI* fragment (Table 3). A possible cause for this result could be the existence and amplification of more than one copy of the *cox*I and/or similar sequences that comigrated to the same position in the agarose gel. To verify this hypothesis, genomic DNA of *P. nigra* and *P. sylvestris* was digested with two groups of restriction enzymes and probed with the PCR amplified *coxI* fragment from *P. sylvestris* (Fig. 3). The first group included seven enzymes (*Ava*I, *BamH*I, *Bgl*II, *Cfo*I, *Hind*III, *Kpn*I and *Xba*I) that have no restriction site on the *coxI* fragment. This group of enzymes was selected by analysing the *cox*I sequence of *P. nigra* and *P. sylvestris* (LU, unpub

Table 3 Size (in bp) of the *coxI* restriction fragments in *P. nigra* (Pn) and *P. sylvestris* (Ps)

Enzyme	*Alu*I		*Hinf*I		*Msp*I		*Rsa*I	
Size	Pn	Ps	Pn	Ps	Pn	Ps	Pn	Ps
	465 440 290	475 435 290	385 330 315 190 170 115	340 330 310 190 170 115	385 185 170 115	385 195 185 160 115	515 225 210	515 240 225 220
Sum size	1195	1200	1505	1455	855	1040	950	1200

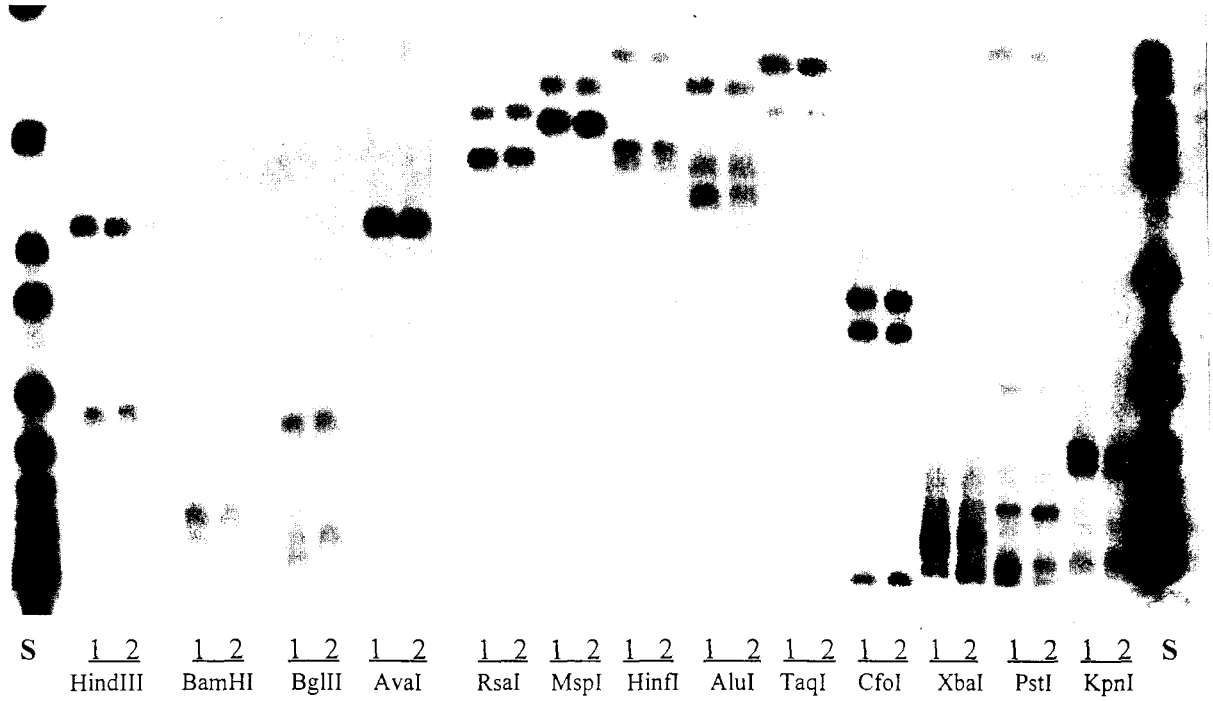

Figure 3 Hybridization of *cox*I to the genomic DNA digest of *P. nigra* and *P. sylvestris*. S – 1Kb standard, 1 – *P. nigra*, 2 – *P. sylvestris*

lished data) using the computer program DIGEST (version 1.0 by Ramin C. Nakisa). The second group comprised six enzymes that have restriction sites on the *coxI* fragment, including the four enzymes (*Alu*I, *Hinf*I, *Msp*I and *Rsa*I) that showed RFLPs on the amplified *coxI* fragment between the two pines.

Southern analysis of the genomic digests revealed fewer fragments as compared to the fragment patterns generated by digestion of the amplified *coxI* fragment with the corresponding enzyme (Figs. 2 and 3). Of the seven enzymes that do not cut the *coxI* fragment, six enzymes gave two or more hybridization signals. Only *Ava*I gave a single strong hybridization signal (Fig. 3). Differences in hybridization patterns between the two species were observed for two enzymes *Xba*I and *Bgl*II (Fig. 3). Hybridisation of the *coxI* probe to the *Xba*I digest revealed three fragments (6.5, 7.0, 10kb) in *P. nigra* while only two fragments (6.5, 11kb) in *P. sylvestris*. In the *Bgl*II digest, the *coxI* hybridized to two large fragments in both species of which one differed between the two species (Fig. 3). The remaining enzymes, including those that revealed RFLPs on the amplified *coxI* fragment, gave multiple hybridization signals but did not detect differences on the DNA hybridization patterns between *P. nigra* and *P. sylvestris* (Fig. 3).

DISCUSSION

MtDNA inheritance in Pinaceae

The present genetic knowledge about the transmission of mtDNA in Pinaceae is based on a limited number of species and individuals. The present study provides additional information on this subject. All F_1 progeny analysed in this study possessed only maternal mtDNA pattern. This finding is concordant with results of genetic studies of other *Pinus* species (NEALE & SEDEROFF 1989; WAGNER *et al.* 1991a). Similar mtDNA transmission has been demonstrated in two additional genera of the Pinaceae family: *Picea* and *Larix* (DEVERNO *et al.* 1993; SUTTON *et al.* 1991).

Cytological studies suggest that male gametes and a large amount of body-cell cytoplasm containing paternal plastids and mitochondria are released into a receptive vacuole at fertilization (DAWKINS & OWENS 1993; OWENS & MORRIS 1991; SINGH 1978). The neocytoplasm surrounding the newly formed zygote still contains both maternal and paternal mitochondria (CHESNOY & THOMAS 1971; OWENS & MORRIS 1991). However, OWENS & MORRIS (1991) estimated that only about 10% of mitochondria were of paternal origin, based on ultrastructural studies. These observations clearly suggest that the most likely mode of mtDNA transmission in Pinaceae is either maternal or biparental. Taking into account the low proportion of paternal mitochondria present in the neocytoplasm, strictly paternal mtDNA inheritance appears as the least likely outcome. The maternal mtDNA transmission found in this study and in most other members of the Pinaceae family (DEVERNO *et al.* 1993; NEALE & SEDEROFF 1989; SUTTON *et al.* 1991) is concordant with this expectation. So far, however, there is no genetic evidence for biparental inheritance of mtDNA in gymnosperms. Surprisingly, the only example for the departure from the maternal inheritance of mtDNA in Pinaceae was found in crosses between *Pinus banksiana* and *P. contorta* where 7% (6 out of 84) F_1 progeny contained mtDNA identical with the paternal parent (WAGNER *et al.* 1991a). Unequivocal explanation of such paternal departure is difficult as it may also result from genome rearrangement and/or seed contamination. The absence of biparental mtDNA transmission suggests the existence of precise mechanism(s) aiding elimination of the alternative mtDNA similar to those suggested by CONNETT (1987). More studies are necessary to establish the nature of these mechanisms.

Presence of cp- and mtDNA in *Pinus* pollen and megagametophytes and its applications

In many angiosperms the maternal inheritance of cpDNA is due to the absence of cpDNA in pollen (CORRIVEAU & COLEMAN 1988; CORRIVEAU *et al.* 1990). However, cytological observations indicate that Pinaceae pollen contains chloroplasts and mitochondria (DAWKINS & OWENS 1993; OWENS & MORRIS 1991; SINGH 1978). Our present results provide first genetic evidence for the presence of both cp- and mtDNA in the *Pinus* pollen and exclude the possibility that the maternal inheritance of mtDNA in Pinaceae could be due to the absence of this genome in pollen.

Most previous studies have focused on the organelle transmission during fertilisation. Hence, little is known about the fate and composition of the organelles in the haploid megagametophyte tissue surrounding the embryo (SINGH 1978). The presence of both cp- and mtDNA in megagametophytes revealed in this study implies that the elimination of the maternal cpDNA that leads to the paternal inheritance of this genome in *Pinus*, does not occur in the surrounding megagametophyte cells. Megagametophyte and embryo tissues often serve as material for genetic analysis in Pinaceae. In embryo, the cpDNA and mtDNA are of paternal and maternal origin respectively. In megagametophyte, however, both cp- and mtDNA are maternal. This situation has practical applications in genetic analysis of controlled crosses and hybrid identification in Pinaceae. Namely, by analysing cpDNA variation in embryo and the corresponding megagametophyte, it is possible to determine the female and male parent of individual seeds if the parents are distinguishable with cpDNA markers. Similar information can be obtained through simultaneous analysis of mt- and cpDNA in diploid progeny. However, this second approach is currently limited by the paucity of suitable mtDNA markers in Pinaceae. On the other hand, species specific cpDNA markers are often observed for Pinaceae species (*e.g.*, SIGURGEIRSSON & SZMIDT 1993; STRAUSS & DOERKSEN 1990; WANG & SZMIDT 1993) which makes the present approach more feasible. Furthermore, the use of the seed material makes it particularly useful for *e.g.*, examination of the genetic efficiency of hybrid seed orchards and polycrosses. Hybridization and introgression among closely related conifer species are often observed in natural populations and in artificial plantations (ENNOS & QIAN 1994; SZMIDT *et al.* 1988; WAGNER *et al.* 1991b; WANG & SZMIDT 1994). The present approach can be used to determine the proportion of hybrid embryos and the maternal and paternal contributions to the embryo population.

CoxI gene polymorphism

The sums of sizes of the fragments produced by digestion of *coxI* fragment with *Alu*I, *Hinf*I, *Msp*I and *Rsa*I were larger than the size of the undigested *coxI* fragment. Furthermore, the restriction patterns generated by digestion of the amplified *coxI* fragment revealed more fragments than were observed on the Southern blots employing the same enzymes. Finally, polymorphisms on the *coxI* fragment between the two *Pinus* species were observed with four restriction enzymes *Alu*I, *Hinf*I, *Msp*I, and *Rsa*I. The same enzymes, however, did not show visible differences between the two species on the genomic DNA hybridization patterns.

At least three different factors could have contributed to these differences. First, the large sum size of the digested *coxI* fragment could be due to the presence of more than one copy of *coxI* or of similar sequence. Results from the present Southern analysis strongly support this suggestion. Six of the seven restriction enzymes that have no restriction site on the *coxI* fragment generated more than one hybridization signals on the genomic DNA blots of the two species. Under the high stringency of DNA hybridization used in the present study, such multiple signals are expected if there are more copies of *coxI* and/or similar sequences in the genome. In addition, all restriction fragments generated by digestion of the amplified *coxI* fragment were maternally transmitted to the F_1 progeny which supports their mitochondrial origin. Second, the large number of fragments observed after separation of the *coxI* digests could also be due to the formation of heteroduplex molecules among multiple *coxI* sequences which acted as templates during PCR amplification. Formation of heteroduplex molecules is especially likely in the presence of multiple copies of similar sequences that serve as templates in PCR. In such situation, heteroduplex molecules can form through repeated denaturation and renaturation steps (ROSSITER & CASKEY 1994). When such a mixture of PCR products is digested with restriction enzymes, additional fragments could be generated. Such additional fragments, however, will not arise in the Southern analysis of the genomic DNA. To our knowledge, multiple copies of coxI have not been reported in other plant species. However, recent RFLP analysis employing the *coxI* fragment as a probe has suggested that the *coxI* gene is repeated at least four times in the mtDNA of *Pinus radiata, P. muricata* and *P. attenuata* (STRAUSS *et al.* 1993). The exact copy number of the *coxI* gene in *P. nigra* and *P. sylvestris* could not be determined in the present study. Cloning and sequence analysis of the *cox*I gene is currently under way in our laboratory and should provide more information on this subject.

Finally, the lack of detectable RFLPs between the two species on Southern blots with the four enzymes which detected RFLPs on the *coxI* digests could be due to unequal resolution power of agarose and polyacrylamide gels. In the present study, the digests of *coxI* fragment were separated on 6% polyacrylamide gel. On the other hand, 1% agarose was used for separation of genomic DNA digests. The resolution of the agarose gel is much poorer than that of the polyacrylamide gel, especially when the size differences among fragments are very small. In fact, most of the fragments generated by the restriction of the *coxI* fragment were smaller than 500 bp, and the differences between polymorphic fragments distinguishing the two species were also very small (less than 20bp in most cases). It is therefore likely that some of the fragments detectable on the polyacrylamide gels were not separated on agarose gel and thus would not show polymorphism on the Southern autoradiogram.

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