Inheritance of Chloroplast and Mitochondrial DNA in Interspecific Hybrid between *Pinus thunbergii* and *P.massoniana*

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Summary : Inheritance of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) was investigated in interspecific hybrid of *Pinus*. Total DNAs of sixteen hybrid plants between *P. thunbergii* and *P. massoniana* were digested with *Eco*RI and hybridized with tobacco cpDNA, pTB 8. Fifteen plants had the same Southern hybridization patterns as the male parent species, *P. massoniana* but one plant showed a faint pattern. The DNAs of the hybrid were also digested with *Apa*I and hybridized with mtDNA, *cox* I. Fifteen plants had the same Southern hybridization patterns as the female parent species, *P. thungergii* but one hybird showed a faint pattern. It was concluded that cpDNA is inherited paternally and mtDNA is inherited maternally in this hybrid. There were more inter- and intra-specific polymorphisms in mtDNA than in cpDNA. The partial sequences of *cox* I gene in *P. thunbergii* were clarified.

1 Introduction

Paternal inheritance of anomalous leaf color was firstly reported in *Cryptomeria japonica* using crossed progeny by Ohba *et al.* (1971). As DNA analysis techniques have been developed, paternal inheritane of chloroplast DNA (cpDNA) was clarified firstly in *Pseudotsuga menziesii* (Neale *et al.* 1986). Then several reports were released concerning inheritance of organelle DNA in coniferous species and cpDNA is showed to be inherited paternally in *Larix*, (Szmidt *et al.* 1987), *Picea* (Stine *et al.* 1989, Stine and Keathley 1990, Sutton *et al.* 1991), *Pinus* (Wagner *et al.* 1987, 1989, Neale and Sederoff 1989, Dong *et al.* 1992), *Sequoia* (Neale *et al.* 1989), *Calocedrus* (Neale *et al.* 1991), *Chamaecyparis* (Kondo *et al.* 1998). On the other hand, mitochondrial DNA (mtDNA) inherited maternally in *Larix* (DeVerno *et al.* 1993), *Picea* (Sutton *et al.* 1991), *Pseudotsuga* (Marshall and Neale 1992) and *Pinus* (Neale and Sederoff 1989, Wagner *et al.* 1991) and inherited paternally in *Sequoia* (Neale *et al.* 1989), *Calocedrus* (Neale *et al.* 1991) and *Chamaecyparis* (Kondo *et al.* 1998). Most reports concerning the inheritance of organelle DNA dealed with Pinaceae.

We investigated inheritance of cpDNA and mtDNA using interspecific hybrid between *P. thunbergii* and *P. massoniana*. This hybrid is resistant to pine wood nematode which has prevailed most parts of Japan except Hokkaido. The leaf color of the female parent, *P. thunbergii* was dark green and that of the male parent, *P. massoniana*

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was yellowish green. As the leaf color of the hybrid was yellowish green, it was reported that the leaf color was inherited paternally in this hybrid (Sasaki *et al.* 1981). It might be helpful for this phenomenon to understand the inheritance mode of organelle DNA in this hybrid.

2 Materials and methods

2.1 Plant materials

Two interspecific hybrid families between two plus trees of *P. thunbergii* and *P. massoniana* were used; Kitasouma 2 × P. massoniana, and Mizunami 4 × *P. massoniana*. The numbers of the progeny were 7 and 9, respectively. They were grown in Forest Tree Breeding Center situated in Ibaraki Prefecture. As the male parent plants were not clear or absent in these interspecific crossing, different individuals were used as the male parent species in this experiment. Six individuals of *P. massoniana* were used to examine intraspecific polymorphisms.

2.2 DNA isolation and Southern blot hybridization

Five g of leaves were frozen in liquid nitrogen and ground with Iwatani grinder (IFM-150). The ground powder was added 20 ml of ice-cold acetone and filtered through filterpaper (Toyo Rosi No. 2) using aspirator. Then total DNA was isolated according to the CTAB procedure (Murray and Thompson 1980).

DNAs were digested with restriction enzymes, and approximately 4 μg of each sample was fractionated on 0.8 % agarose gel. DNAs were blotted to nylon membranes and hybridized with probes using DIG system (Boehringer Mannheim) according to the manufacture's instruction. pTB 8 clone of tobacco was used for cpDNA probe and cox I gene amplified by PCR was used for mtDNA probe. Primers were designed based on the nucleotide sequence data from Kemmerer *et al.* (1989). Two oligonucleotide primers, 5'-CGATGGCTGTTCTCCACTAA -3' (forward) and 5'- ATCTGGATAATCTGGAATGC -3' (reverse) were used for *cox* I (Kondo *et al.* 1998).

2.3 PCR amplification and sequencing of *cox* I gene

PCR amplification was performed as follows: reaction mixtures (100 μl) contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.1 % Triton X-100, 0.01 % gelatin, 0.1 mM dNTP, 100 pmol of each primer, 600 ng of template DNAs of Kitasouma 2, and 5 unit of Taq polymerase (Boehringer Mannheim). Amplification was carried out for 1 min of 94 ℃, followed by 35 cycles of 1 min of 94 ℃, 1 min at 55 ℃, and 2 min at 72 ℃, with final 1 min incubation at 72 ℃ with an air thermo-cycler 1605 (Idaho Technology). The amplified DNAs were fractionated on 0.85 % agarose gel and approximately 1300 bp product was dissected and purified by Genepure (Nippon Gene Co.). This DNA was used as a template for the sequencing reaction which was performed using dye terminator sequencing kit (Applied Biosystems) and then analysed by a 373S automaic sequencer (Applied Biosystems). As the template was too long to sequence at one time, other primers were designed using Oligo software (NBI/Genovus, Inc.).

3 Results

3.1 Paternal inheritance of cpDNAs

The DNAs of both parent species were digested with 16 restriction enzymes, *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hin*fI, *Kpn*I, *Msp*I, *Pst*I, *Pvu*II, *Sca*I, *Sma*I, *Xba*I and *Xho*I, and hybridized with each of two tobacco cpDNA probes, pTB 8 and pTB 15. Only in the combination of EcoRI and pTB 8 there was a differnece between parent species in Southern hybridization patterns and no polymorphisms were detected among 6 individuals of *P. massoniana* in this combination. The DNA of each hybrid individual was digested with *Eco*RI and hybridized with

pTB 8. All 15 hybrid individuals showed the same Southern hybridization patterns as the male parent species, *P. massoniana* but one individual of Mizunami 4 × *P. massoniana* showed a faint pattern (Fig. 1 A, B).

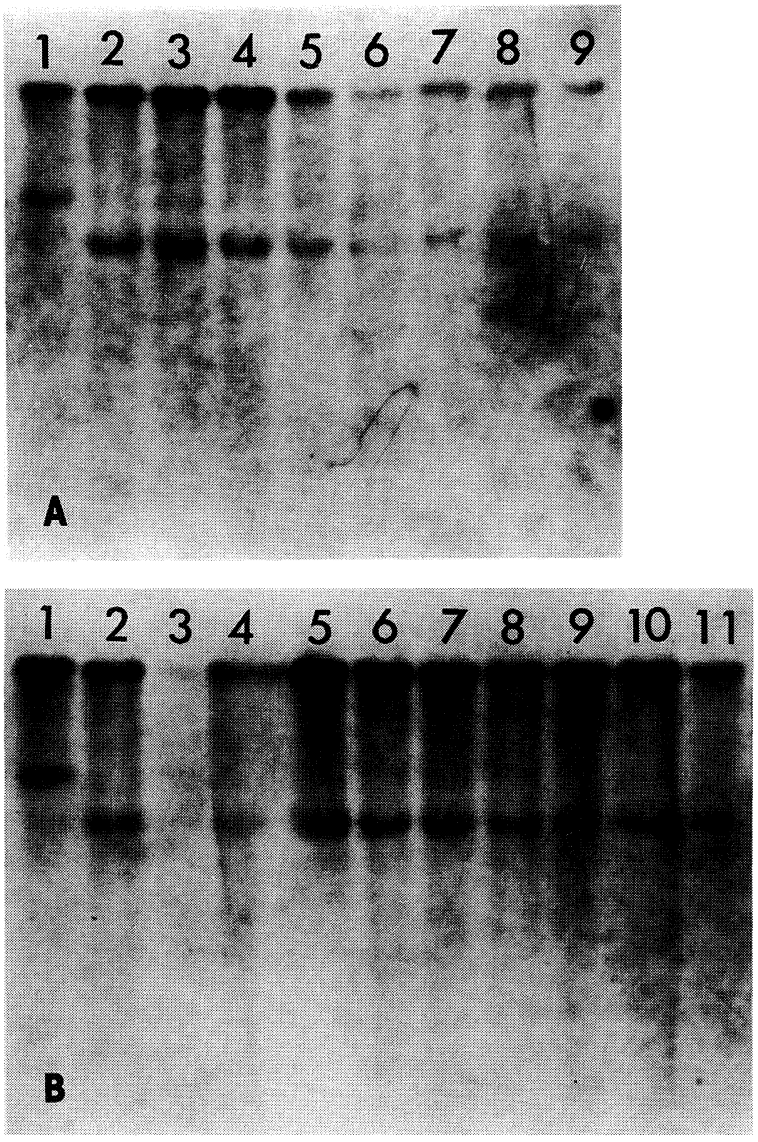

Fig. 1. Southern hybridization pattern of cpDNA in *Pinus*.

A. *Eco*RI digests of Kitasouma 2 (*P. thunbergii*) × *P. massoniana*. Lane 1, Female parent, Kitasouma 2; Lanes 2-8, Hybrid progeny; Lane 9, Male parent, *P. massoniana*.
B. *Eco*RI digests of Mizunami 4 (*P. thunbergii*) × *P. massoniana*. Lane 1, Female parent, Kitasouma 2; Lanes 2-10, Hybrid progeny; Lane 9, Male parent, *P. massoniana*.

3.2 Maternal inheritance of mtDNA

The DNAs of both parent species were digested with 15 restriction enzymes, *Apa*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hinf*III, *Kpn*I, *Msp*I, *Pst*I, *Pvu*II, *Sal*I, *Xba*I and *Xho*I, and hybridized with mtDNA probe, *cox* I. There were clear differences between parent species in Southern hybridization patterns in two restriction enzymes, *Apa*I, *Bgl*II, and no polymorphisms were detected among 6 in-

dividuals of *P. massoniana* in these combinations. There were also some differences between parent species in 6 restriction enzymes, *Dra*I, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, and *Xho*I. The DNA of each hybrid individual was digested with *Apa*I and then hybridized with *cox* I. All 15 hybrid individuals showed the same Southern hybridization patterns as female parent, *P. thunbergii* but one individual of Mizunami 4 × *P. massoniana* showed a faint pattern (Fig. 2 A, B). This individual was same one which had faint pattern in the case of cpDNA.

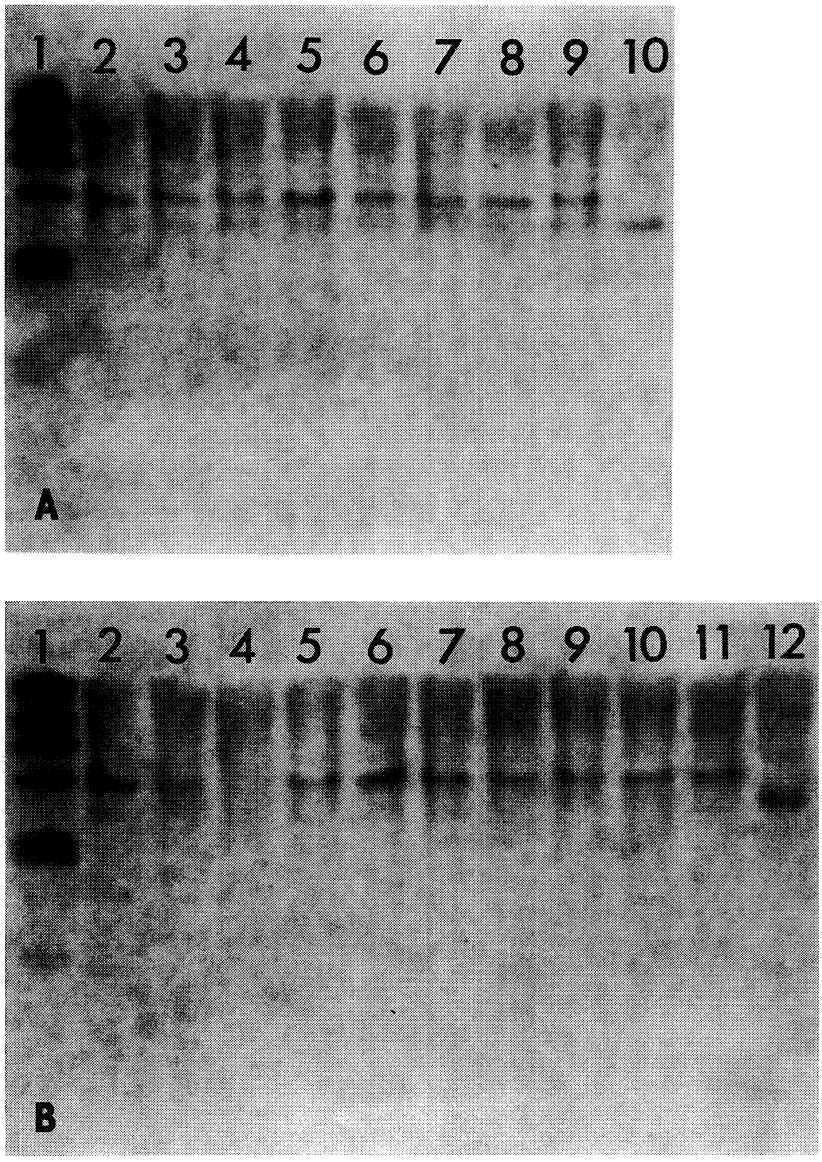

Fig.2. Southern hybridization pattern of mtDNA in *Pinus*.

A. *Apa*I digests of Kitasouma 2 (*P. thunbergii*) × *P. massoniana*. Lane 1, *Hind* III/Lambda DNA size marker; Lane 2, Female parent, Kitasouma 2; Lanes 3-9, Hybrid progeny; Lane 10, Male parent, *P. massoniana*.
B. *Apa*I digests of Mizunami 4 (*P. thunbergii*) × *P. massoniana*. Lane 1, *Hind*III/Lambda DNA size marker; Lane 2, Female parent, Kitasouma 2; Lanes 3-11, Hybrid progeny; Lane 12, Male parent, *P. massoniana*.

3.3 Sequence of *cox* I

The partial sequences of *cox* I were 1325 bp long and shown in Fig. 3. This sequence had 90 % identity with soybean cytochrome oxidase subunit I gene, pea mitochondrial *cox* I gene for cytochrome oxidase subunit, *Lycopersicon esculentum* mitochondrial *cox* I gene for cytochrome oxidase subunit I, *Solanum tuberosum* mitochondrial *cox* I gene, *Raphanus sativus* mitochondrial *cox* I gene for cytochrome c oxidase subunit I, *Oenothera* mitochonodrial *cox* I gene for cyotochorome on the database GenBank by BLAST.

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5'                                                  50
CGTGGCTGTTCTCCACTAACCACAAGGATATAGGGACTCCACATTCAATC
                                                   100
TTCGGTGCCATTGCTGGAGTAATGGGCACATGCTTCTCAGTACCAATTCG
                                                   150
TATGGAATTAGCACAACCCGGCGATCAAATTCTTGGTGGGAATCATCAAC
                                                   200
CTCATAATGTGTTAATAACGGCTCACGCTTCTCCAATGATCCCTTTTATG
                                                   250
GTTATGCCGGCGGTGATAGGTGGATCTGGTAATTGGTCCGTTCCGATTCC
                                                   300
TATAGGTGCACCTGACATGGCATTTCCACGATTGAATAATATTCCATCCC
                                                   350
GGTTGTTGCCACCTTCGCTGTTGCTCCCATTAAGCCCAGCCTCGGTAGAA
                                                   400
GTGGGTAGCGGCACTGGGTGGACGGTCTATCCGCCCCTAAGTGGTATTAC
                                                   450
CAGTCATTCCGGAGGAGCTGCTGATCCAGCGATTTCTAGTCCTCATCTAT
                                                   500
CAGGTGTTTCATCCATTTCAGGTTCTATCAATCTCATAACTACTATCCCC
                                                   550
AACATGCGCGGGCCTGGAATGACTATGCATAGATCACCCCTATTTGTGCG
                                                   600
GTCCGTTCCAGTGACAGCATTCCTACTCTTATCATCACTTCCGGTACCGG
                                                   650
CAGGGGCAATTACCATGTTATCAACTGATCGAAGCTTTAATACAACCTTT
                                                   700
TTCGATCCTGCTGGAGGGGGAGACCCGATATTATACCAGCATCTCTTTCG
                                                   750
GTTCTCCGGTCATCCAGAGGTGTATATTCCCATTCTGCCCGGATTCGGTA
                                                   800
TCATTAGTCATATCGTATCGACTTTTTCGGGAAAACCGGTATTCGGGTAT
                                                   850
CCAGGCATGGTTTATGCCATGATCAGTATTGGTGTTCCTGGATTTCCTGT
                                                   900
TCGGGCTCATCATATGTTTACTGTGGGCTCAGACGTTGATACGCGTGCTC
                                                   950
ACTCTACCGCAGCTACCATGATCATAGCTGTCCCCACTGGAATCAAAATC
                                                  1000
TTTAGTCGGATCGCTACCATGTGGGGAGGTTCGATACGATACAAAACACC
                                                  1050
CATGTTACTTGCTGCAGGGTCCATCTTTCCGTCCACCATAGGAGGACTCA
                                                  1100
CTGGAATAGTCCTGGCAAATCCTGGGCTAGACATTGCTCTGCATGATACT
                                                  1150
CATTATGTGGTTGCACATTCCCATTATGTACTTTCTATGGGAGCCGTTTC
                                                  1200
TGCTTCATCTGCAGGATTTCACTTCCGGGTGGGTAAAATCCCTGGTCGAA
                                                  1250
CATACCCTGAAACTTTAGGTCAAATCCATCTTCGGATCACTCTTTTCGGG
                                                  1300
GTGAATTCGACCTTCTTTCCCATGCATTTCTTGGGGCTTTCGGGTATGCC
                        1325 bp
ACGTCGCATTCCAGATTATCCAGAT
                        3'
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Fig.3. Partial sequence of *cox* I gene in *Pinus thunbergii*.

4. Discussion

There were several reports concerning the inheritance of organelle DNA in coniferous species and paternal inheritance of cpDNA is consistent. In the hybrid between *P. thunbergii* and *P. massoniana*, we observed same mode of inheritance, paternal inheritance of cpDNA. mtDNA was inherited maternally in *Pinus* in two reports. Wagner *et al.* (1991) detected paternal leakage of mtDNA in 6 out of 84 progeny in *Pinus* . We didn't detect such leakage but perfect maternal inheritance of mtDNA. Paternal inheritance of leaf color was reported by Sasaki *et al.* (1981) using the hybrid between the same parent species, *P. thunbergii* and *P. massoniana*. As they did not use reciprocal crossing families, it has been still ambiguous about the inheritance mode of leaf color. There was the possibility that this phenomenon was affected by the dominant nuclear gene, which controlled the leaf color of the male parent, *P. massoniana*. Electron microscopy observations also suggested maternal inheritance of mtDNA in *Pinus* (Willemse1974, Chesnoy and Thomas 1971) .

cpDNA of the parent species was digested with 16 restriction enzymes and there was a difference between the parent species only in one restriction enzyme. In mtDNA, there were differences between the parent species in 8 out of 15 restriction enzymes. In *Chamaecyparis*, we also observed higher degree of interspecific polymorphism in mtDNA than in cpDNA (Kondo *et al.* 1998). Neale and Sederoff (1989) found no intraspecific polymorphism of cpDNA in *Pinus taeda* using the combinations of 6 restriction enzymes and 6 probes of petunia but found polymorphism in mtDNA. We checked 3 individuals of *Pinus thunbergii* and 6 individuals of *P. massoniana* and found same trend of intraspecific polymorphism. Although there was no intraspecific polymorphism in cpDNA, polymorphism was found in mtDNA of both species. As there are more polymorphisms in mtDNA, intraspecific polymorphism will be available to clarify the inheritance of mtDNA in other coniferous species.

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References

Chesnoy, L. and Thomas, M. J.: Electron microscopy studies on gametogenesis and fertilization in gymnosperms. Phytomorphology 21, 50-63 (1971)

DeVerno, L. L., Charest, P. J. and Bonen, L.: Inheritance of mitochondrial DNA in the conifer *Larix*. Theor Appl Genet 86, 383-388 (1993)

Dong, J., Wagner,D. B., Yanchuk, A. D., Carlson, M. R., Magnussen, S., Wang, X.-R. and Szmidt, A. E.: Paternal chloroplast DNA inheritance in *Pinus contorta* and *Pinus banksiana*: Independence of parental species or cross direction. J. Hered. 83, 419-422 (1992)

Kemmerer, E. C., Kao,T., Deng, G. and Wu, R.: Isolation and nucleotide sequence of the pea cytochrome oxidase subunit I gene. Plant Mo. Biol. 13, 121-124 (1989)

Kondo, T., Tsumura,Y., Kawahara, T. and Okamura, M.: Paternal inheritance of chloroplast and mitochondrial DNA in interspecific hybrids of *Chamaecyparis* spp. Breeding Science 48, 177-179 (1998)

Marshall, K. A. and Neale, D. B.: The inheritance of mitochondrial DNA in Douglas-fir (*Pseudotsuga menziesii*). Can. J. For. Res. 22, 73-75 (1992)

Murray, M. G. and Thompson, W. F.: Rapid isolation of

high molecular weight plant DNA. Nucleic Acid. Res. 8, 4321-4325 (1980)

Neale D. B., Marshall, K. A. and Harry, D. E.: Inheritance of chloroplast and mitochondrial DNA in incense-cedar (*Calocedrus decurrens*). Can. J. For. Res. 21, 717-720 (1991)

Neale, D.B., Marshall, K.A. and Sederoff, R. R.: Chloroplast and mitochondrial DNA are paternally inherited in Sequoia sempervirens D. Don Endl.. Proc. Natl. Acad. Sci. USA 86, 9347-9349 (1989)

Neale, D.B. and Sederoff, R. R.: Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor Appl Genet 77, 212-216 (1989)

Neale, D.B., Wheeler, N. C. and Allard, R. W.: Paternal inheritance of chloroplast DNA in Douglas-fir. Can. J. For. Res. 16, 1152-1154 (1986)

Ohba, K., Iwakawa, M., Okada, Y. and Murai, M.: Paternal transmission of a plastid anomaly in some reciprocal crosses of Sugi, Cryptomeria japonica D. Don. Silvae Genet. 20, 101-107 (1971)

Sasaki, M., Kawamura, K. and Furukoshi, T.: Back crosses and three-way crosses among two-needle pine species (II) Investigation on the morphological characters, winter buds and needle coloration of back crossed one year seedlings. Trans. 92nd Mtg. Jpn. For. Soc. 275-276 (1981)

Stine, M. and Keathley, D. E.: Paternal inheritance of plastids in Engelmann spruce †~ blue spruce hybrids. J. Hered. 81, 443-446 (1990)

Stine,M., Sears, B. B. and Keathley, D. E.: Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. Theor Appl Genet 78, 768-774 (1989)

Sutton, B. C. S., Flanagan, D. J., Gawley, J. R., Newton, C. H., Lester, D. T. and El-Kassaby, Y. A.: Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. Theor Appl Genet 82, 242-248 (1991)

Szmidt, A. E., Alden, T. and Hallgren, J-E.: Paternal inheritance of chloroplast DNA in *Larix*. Plant Mol. Biol. 9, 59-64 (1987)

Tsumura, Y., Yoshimura, K., Tomaru, K. N. and Ohba, K.: Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. Theor Appl Genet 91, 1222-1236 (1995)

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

Wagner, D. B., Furnier, G. R., Saghai-Maroof, M. A., Williams, S. M., Dancik, B. P. and Allard, R. W.: Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA 84, 2097-2100 (1987)

Wagner, D.B., Govindaraju, D. R., Yeatman, C. W. and Pitel, J. A.: Paternal chloroplast DNA inheritance in a diallel cross of jack pine (*Pinus banksiana* Lamb.) . J. Hered. 80, 483-485 (1989)

Willemse, M. T. M.: Megagametogenesis and formation of neocytoplasm in Pinus sylvestris L.. In "Fertilization in higher plants" Linskens, H. F. (ed.) , North-Holland Publishing Company, Amsterdam, 97-102 (1974)

クロマツとタイワンアカマツとの種間雑種における葉緑体およびミトコンドリアDNAの遺伝様式

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要旨：クロマツとタイワンアカマツとの種間雑種における葉緑体DNAおよびミトコンドリアDNAの遺伝様式を調べた。雑種15個体から全DNAを単離し、葉緑体DNAについては、制限酵素*Eco*RIで切断後、タバコの葉緑体DNAの断片pTB8をプローブにしてサザンハイブリダイゼーションを行ったところ、14個体はすべて父親であるタイワンアカマツと同じバンドパターンを示したが、1個体は明瞭なバンドを示さなかった。ミトコンドリアDNAについては、制限酵素*Apa*Iで切断後、PCRで増殖した*cox*Iの遺伝子をプローブにしてサザンハイブリダイゼーションを行ったところ、14個体はすべて母親であるクロマツと同じバンドパターンを示したが、1個体は明瞭なバンドが出なかった。以上のことから、この雑種においては、葉緑体DNAは父性遺伝し、ミトコンドリアDNAは母性遺伝していると考えられた。また、PCRで増殖したクロマツの*cox*I遺伝子の一部の塩基配列を明らかにした。

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