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Incomplete paternal inheritance of chloroplast DNA recognized in *Chamaecyparis obtusa* using an intraspecific polymorphism of the *trn*D-*trn*Y intergenic spacer region

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Abstract Using a fluorescence-based PCR-SSCP (single-strand conformation polymorphism), we verified imperfectibility in the paternal inheritance of chloroplast DNA (cpDNA) in *Chamaecyparis obtusa* (Cupressaceae) controlled crosses. An intraspecific sequence polymorphism of the intergenic spacer region between the *trn*D and *trn*Y genes was utilized as a molecular marker. Of 361 progenies, in which the cpDNA haplotypes of their female and male parents were different, 352 (97.5%) possessed the same haplotypes as their male parents, and nine (2.5%) the same haplotypes as their female parents. The parentage of the nine progenies with female parental types was diagnosed using DNA fingerprinting based on fluorescence-based RAPD profiles. Their parentage showed convincing evidence of the low frequency of maternal inheritance. Moreover, heteroplasmy was observed in the open-pollinated seeds collected in a seed orchard. The confirmation of maternal plastid transmission in the full-sib families and the observation of heteroplasmy in seeds reveal that the paternal inheritance of cpDNA is not an exclusive phenomenon and that the mode of its inheritance is biparental in *C. obtusa*.

Keywords Biparental inheritance · Paternal inheritance · Heteroplasmy · Chloroplast DNA · *Chamaecyparis* · PCR-SSCP

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Introduction

Cytoplasmic organelles such as the chloroplast and mitochondria possess characteristic genomes. Organelle inheritance in most plants is purely maternal, though there are some exceptions in which organelle DNA is inherited biparentally or paternally (Reboud and Zeyl 1994). Ohba et al. (1971) were the first to delineate the mode of organelle DNA inheritance in conifers while investigating a chromatophore mutation that manifested itself as an anomalous needle color of *Cryptomeria japonica*.

The recent remarkable development of molecular biological techniques has made it possible to easily obtain genetic information that has been impossible to obtain by means of morphological observations. Consequently, various DNA markers have been developed for genome analyses. Neale et al. (1986) proved the paternal inheritance of *Pseudotsuga menziesii* chloroplast DNA (cpDNA) using an RFLP (restriction fragment length polymorphism) analysis. The inheritance mode of coniferous cpDNA has been investigated using molecular markers such as RFLP (Dong et al. 1992; Neale et al. 1986, 1989, 1991; Neale and Sederoff 1989; Stine et al. 1989; Stine and Keathley 1990; Szmit et al. 1987; Wagner et al. 1989) and SSR (simple-sequence repeat; Cato and Richardson 1996; Vendramin and Ziegenhagen 1997). As a result of these studies, the paternal inheritance of cpDNA in coniferous species has been generally accepted. Less frequent non-paternal inheritance and heteroplasmy, however, were observed in some studies, and there is still some question about the completeness of paternal inheritance in conifers.

In the study reported here, we surveyed intraspecific variation in several non-coding regions of cpDNA in *Chamaecyparis obtusa* Seib. et Zucc (Cupressaceae). By utilizing a sequence polymorphism found in the intergenic spacer region between the *trn*D(GUC) and *trn*Y(GUA) genes as a PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) marker, the imperfectibility in the paternal inheritance of *Chamaecyparis* cpDNA was verified using several artificial-crossed full-sib families.

Materials and methods

Plant materials

Four plus tree clones (Aira 46, Oita 7, Higashiusuki 3, and Aso 1) of *Chamaecyparis obtusa* were selected for investigating an intraspecific variation of cpDNA. Estimation of the frequency of two haplotypes observed in the *trn*D(GUC)-*trn*Y(GUA) spacer (CS4) was carried out using a total of 105 clones from two seed orchards and one clone bank (Table 2). The 2-year-old offspring of four full-sib families in the Kyushu Breeding Office, Forest Tree Breeding Center, were utilized for confirming the paternal inheritance of cpDNA. Eighty epicotyls from open-pollinated and germinated seeds of Kajikazawa 5 in the Tomisawa seed orchard of Yamanashi Prefecture were also used for this purpose.

DNA isolation

With respect to the needles of plus tree clones or F_1 progenies, the genomic DNAs were extracted using a modified CTAB procedure (Murray and Thompson 1980; Shiraishi and Watanabe 1995). The DNAs were purified with GENECLEAN III (BIO 101) and used as template DNA for PCR. For the epicotyls of the open-pollinated seeds from Kajikazawa 5, after the epicotyls were about 1 cm in length, they were separated from the seed and the genomic DNAs extracted with ISOPLANT DNA Extraction (Wako). The crude DNAs were utilized directly for PCR.

Polymerase chain reaction

Four non-coding regions were amplified using the four primer pairs (B49317/A49855, B49873/A50272, CS4U/CS4L, and CS5 U/CS5L) shown in Table 1. Primers designed by Taberlet et al. (1991) were used to amplify the two regions [*trn*L(UAA) intron and *trn*L(UAA) – *trn*F(GAA) spacer], and the primers were newly designed for amplification of two remaining intergenic spacer regions, the *trn*D(GUC) – *trn*Y(GUA) and *trn*P(UGG) – *trn*W(CCA) spacers. A 10-µl aliquot of reaction mixture contained 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 200 µ*M* each of dNTPs, 0.25 µ*M* each of primers, 0.5 U/µl AmpliTaq DNA polymerase, Stoffel Fragment, 3.0 m*M* $MgCl_2$, and 0.1 ng/µl template DNA. PCR amplification was carried out using a Perkin Elmer model 9600 thermal cycler with the following profile: a 1-min denaturation at 95°C; 30 cycles of a 30-s denaturation at 94°C, 30-s annealing at 55°C, 90-s extension at 72°C; and a final extension at 72°C for 60 s.

Cold-SSCP analysis

Cold-SSCP analysis (Orita et al. 1989; Hongyo et al. 1993) was carried out according to the methods of Narazaki et al. (1996). PCR products were mixed with 11 µl of 1× TBE and 1 µl of 1 *M* methylmercury hydroxide and then denatured by heating at 94°C for 5 min. The denatured samples were immediately cooled on ice and applied (20 µl/lane) to a 10% non-denatured polyacrylamide gel (1× TBE; 29:1 acrylamide: bis-acrylamide cross-linking). Electrophoresis was performed in 0.5× TBE at 250 V for 16 h. The gel temperature was kept at 10°C. After electrophoresis, the gel was stained with ethidium bromide, and SSCPs were detected using a UV (302-nm) transilluminator.

Fluorescence-based SSCP analysis

The CS4 spacer region, in which an intraspecific variation was observed, was amplified using Cy5-labeled primers (cyCS4U, cyCS4L in Table 1). Fluorescence-based SSCP analysis was carried out according to the methods of Maeda and Shiraishi (1997).

Fluorescence-based random amplified polymorphic DNA (RAPD) analysis

RAPD analysis was performed using 24 arbitrary decamer primers provided by Operon Technologies. DNA amplification with a RAPD primer was carried out using the above-mentioned PCR composition, except with a different primer concentration and the addition of fluorescence dUTP. The primer concentration was changed to 0.5 µ*M*. PCR products were labeled by a PRISM dUTP set (Perkin-Elmer). Final concentrations of fluorescence-labeled dUTP were 0.2 µ*M* (R110 and R6G) and 0.8 µ*M* (TAMRA), respectively. The thermal cycler was programmed as follows: a 60-s denaturation at 95°C; 45 cycles of a 30-s denaturation at 95°C, a 30-s annealing at 37°C, a 90-s extension at 72°C; and a final ex-

Table 1 PCR primers used in amplifying four regions of cpDNA

DNA region	Primer	
	Name	Sequence (5′ to 3′)
*trn*L intron	B49317	CGAAATCGGTAGACGCTACG[a]
	A49855	GGGGATAGAGGGACTTGAAC[a]
*trn*L-*trn*F spacer	B49873	GGTTCAAGTCCCTCTATCCC[a]
	A50272	ATTTGAACTGGTGACACGAG[a]
*trn*D-*trn*Y spacer (CS4)	CS4U	TGACAGGGCGGTACTCTAAC
	CS4L	CGATGCCCGAGTGGTTAATG
	cyCS4U	Cy5-TGACAGGGCGGTACTCTAAC[b]
	cyCS4L	Cy5-CGATGCCCGAGTGGTTAATG[b]
	sCS4U	tgtaaaacgacggccagtTGACAGGGCGGTACTCTAAC[c]
	sCS4L	caggaaacagctatgaccCGATGCCCGAGTGGTTAATG[c]
*trn*P-*trn*W spacer (CS5)	CS5U	TTGGTAGCGTGTTTGTTTTGGG
	CS5L	TACGGCATCAGGTTTTGGAGAC

[a] Taberlet et al. (1991)
[b] Cy5 is a fluorescent dye provided from Amersham-Pharmacia
[c] Sequences indicated by lowercase letters are the sequences of the Perkin-Elmer –21M13 and M13Rev sequencing primers

tension at 72°C for 7 min. PCR products were diluted with 20 times their volume of sterilized ultra-pure water. One microliter of this solution was mixed with 1 µl of loading buffer (5% blue dextran and 25 m*M* EDTA). This mixture (1 µ l) was loaded onto a 5% non-denatured polyacrylamide gel (1× TBE; 29:1 acrylamide to bis-acrylamide cross-linking). Electrophoresis was performed in 1× TBE using an ABI DNA Sequencer 373XL (Perkin-Elmer) with the GeneScan ver. 2.1, and the fragment pattern was analyzed by GENOTYPER ver. 1.1.1 software.

Sequencing

The sequences of the CS4 spacer region were determined by direct sequencing of PCR products (Shiraishi and Watanabe 1995). Different haplotypes were amplified using a primer pair of sCS4U and sCS4L (Table 1). PCR amplifications were carried out using a Perkin-Elmer model 9600 thermal cycler in a total of 50 µl containing 2 m*M* TRIS-HCl (pH 8.0), 100 m*M* KCl, 0.01 m*M* EDTA, 0.1 m*M* DTT, 0.05% Tween 20, 0.05% Nonidet P-40, 5% Glycerol, 200 µ*M* each of dNTPs, 0.25 µ*M* each of primers, 0.25 U/µl TaKaRa ExTaq (TAKARA), 2 m*M* $MgCl_2$, and 0.1 ng/µl template DNA. The thermal cycler was programmed according to the same profile as described above. PCR products were separated in a 2% agarose gel, and then a target DNA fragment was recovered from the gel using QIAEX II Gel Extraction (QIAGEN). With the DNA as a template, a sequencing reaction was carried out using an AutoSequencer Core Kit (TOYOBO) and analyzed using an ALFred DNA Sequencer (Pharmacia Bioteq).

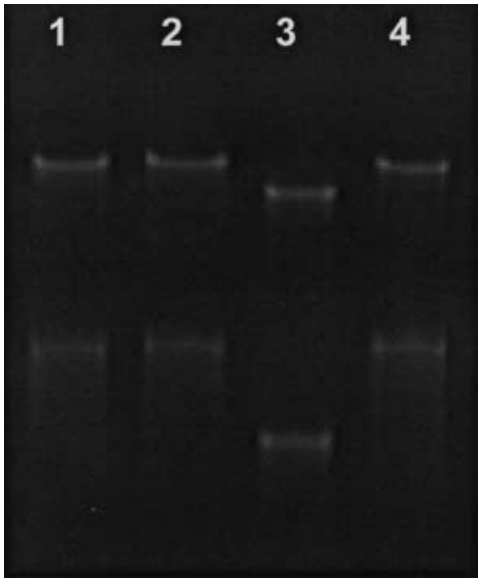

Fig. 1 Electrophoretic profiles of the CS4 region in Cold-SSCP analysis. *Lane 1* Aira 46, *lane 2* Oita 7, *lane 3* Higashiusuki 3, *lane 4* Aso 11

Results

Intraspecific sequence polymorphism of cpDNA

The SSCP of four plus tree clones (Aira 46, Ooita 7, Higashiusuki 3 and Aso 11) was investigated for four non-coding regions of cpDNA, as shown in Table 1, using a Cold-SSCP method (Hongyo et al. 1993). As a result, an SSCP polymorphism was observed in the intergenic spacer region (CS4) between the *trn*D and *trn*Y genes (Fig. 1). Three clones, namely, Aira 46, Oita 7, and Aso 11, showed the same SSCP profile, whereas the SSCP of Higashiusuki 3 revealed a remarkably different migration pattern compared with the other three clones. Based on this result, the SSCP type observed in Higashiusuki 3 was denoted as a mutant type, while the other was denoted as a wild type. The sequences of both SSCP types in the region were determined in order to clarify their differentiation from one another at the sequence level (Fig. 2). The length of the sequences was 286 bp with no length variation. The length of the only spacer region was 198 bp. Only one base substitution (transition from C to T) was recognized at the 41st base between the wild and mutant types.

A rapid analysis using fluorescence-based PCR-SSCP

The Cold-SSCP method is not suitable for analyzing numerous samples, since this method takes a long time and a large number of samples cannot be treated at one time. A new SSCP method was developed using a fluorescence-labeled primer. The SSCP chromatograms of the PCR product amplified with three primer combinations (cyCS4U/cyCS4L, cyCS4U/CS4L, and CS4U/cyCS4L) are shown in Fig. 3. Since the A-strand of the PCR product was fluorescence-labeled with cyCS4U and the B-strand with cyCS4L, both strands were labeled with fluorescent dye in the PCR product using cyCS4U/cyCS4L

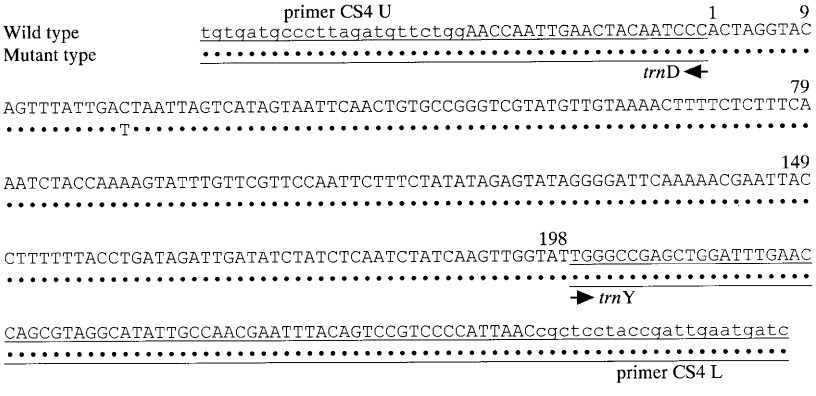

Fig. 2 Sequences of the chloroplast intergenic spacer region (CS4) between the *trn*D and *trn*Y genes. The *dots* indicate the postions where the mutant type has the same nucleotides as the wild type. *Underlining* indicates the coding region of the *trn*D and *trn*Y genes. *Small letters* denote the annealing sites of primers CS4U and CS4L

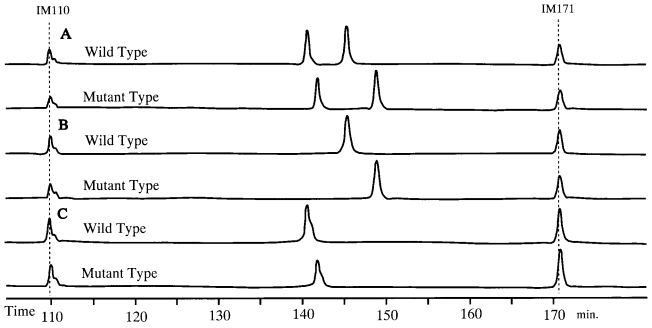

Fig. 3A–C SSCP chromatograms of the cpDNA CS4 region. **A** SSCPs using cyCS4U/cyCS4L primers, **B** SSCPs using cyCS4U/CS4L primers, **C** SSCPs using CS4U/cyCS4L primers. *IM110*, *IM171* Internal standard marker

(Fig. 3A). The chromatograms were standardized using two inner markers (M110 and M171), and the peaks representing A- and B-single strands were correctly distinguished between the wild and mutant types. A comparison of the SSCPs with primer pairs of cyCS4U/CS4L (Fig. 3B) and CS4U/cyCS4L (Fig. 3C) revealed that a mobility shift of the A-strand was larger than that of the B-strand. The SSCP analysis with cyCS4U and CS4L produced a reliable cpDNA haplotyping.

Chloroplast DNA haplotype frequency

The frequency of haplotypes in the spacer region of cpDNA was estimated using plus tree clones preserved in two seed orchards and a clone bank of three research institutes (Table 2). Of a total of 105 clones investigated, 97 (92%) were of the wild type and eight (8%) were of the mutant type. The frequency of the mutant type was extremely low, especially in the Tomisawa seed orchard where this type was only one of 33 clones.

Inheritance of the cpDNA in artificially pollinated families

The paternal inheritance of cpDNA in *C. obtusa* was verified using the variation of the intergenic spacer region between the *trn*D and *trn*Y genes as a genetic marker. Four full-sib families, namely, Aira 32×Aira 6, Aira 32×Satsuma 8, Isa 1×Aira 47, and Isa 1×Aira 6, were utilized for this purpose. The segregations into the two haplotypes of these families are shown in Table 3. In Aira 32×Aira 6 (family A), both of whose parents were wild types, the haplotypes of all progenies were wild types. In the progenies of Aira 32×Satsuma 8 (family B), produced with a combination of a female parent of the wild type and a male parent of the mutant type, 180 progenies showed their male parental types (mutant types) and four their female parental types (wild types). Most of the progenies of Isa 1×Aira 47 (family C) and Isa 1×Aira 6 (family D), in which the female parent was a mutant type and the male parent was a wild type, were the same wild type as their male parent. One progeny of family C and four progenies of family D were mutant types, that is, the same as their female parent. In total, of the three families (families B, C, and D) in which the haplotypes of the female and male parents were different, 352 (97.5%) of 361 progenies were the same type as their male parent and 9 progenies (2.5%) were the same as their female parent.

Table 2 Frequencies of the two cpDNA haplotypes detected in *Chamaecyparis obtusa*

Seed orchard/clone bank	Haplotype		
	Total	Wild type	Mutant type
Seed orchard of Kyushu Breeding Station, National Forest Tree Breeding Center	43	41 (0.95)[a]	2 (0.05)
Clone bank of Oita Prefectural Forest Experiment Station	29	24 (0.83)	5 (0.17)
Tomisawa seed orchard of Yamanashi Forestry and Forest Products Research Institute	33	32 (0.97)	1 (0.03)
Total	105	97 (0.92)	8 (0.08)

[a] Frequencies are in parentheses

Table 3 Segregations of cpDNA haplotypes in four families (*W* wild type, *M* mutant type)

Family; Cross combination (♀×♂)	Haplotype		
	Total	Wild type	Mutant type
Family A; Aira 32 (W)×Aira 6 (W)	28	28 (100.0%)	0 (0.0%)
Family B; Aira 32 (W)×Satsuma 8 (M)	184	4 (2.2%)	180 (97.8%)
Family C; Isa 1 (M)×Aira 47 (W)	50	49 (98.0%)	1 (2.0%)
Family D; Isa 1 (M)×Aira 6 (W)	127	123 (96.9%)	4 (3.1%)

Parentage diagnosis of the progenies showed the female parental type

To reveal whether the nine progenies of female parental types were pseudo-progenies caused by a contamination during the process of artificial pollination and raising of the seedlings, we analyzed the parentage of these progenies using a fluorescence-based RAPD analysis. For every progeny possessing the cpDNA haplotype of a female parental type, the DNA fingerprints of the progeny and both its parents were compared using the chromatograms of the RAPD analysis. The RAPD chromatograms with OPH-05 primer are shown in Fig. 4. If the parentage of these progenies is real, the peak detected in a progeny must also appear in one/both of its parents.

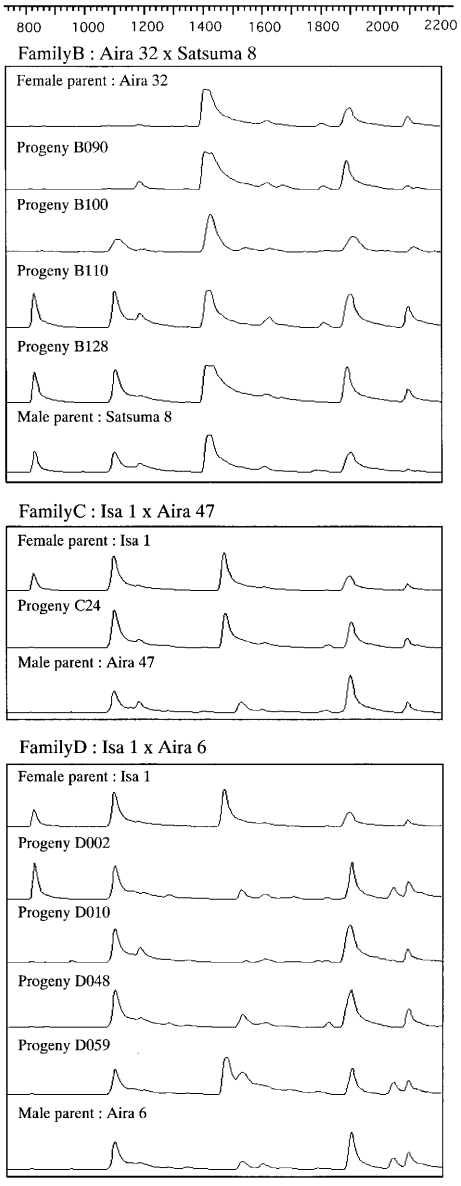

Fig. 4 DNA fingerprint analysis of the progenies showing female parental chloroplast haplotypes using RAPD with the OPH-05 primer

Progeny C24 possessed a total of 241 peaks in the RAPD analysis with 24 primers. All of these peaks were detected in one/both of its parents, Isa 1 and Aira 47. This followed the essential rule for parentage diagnosis in DNA fingerprinting. In the remaining eight progenies, 212–234 peaks were observed. It was verified that all peaks of every progeny were inherited from one or both of its parents. As a result of the parentage diagnosis, it was safe to believe that all of the progenies possessing female parental haplotypes were true offspring.

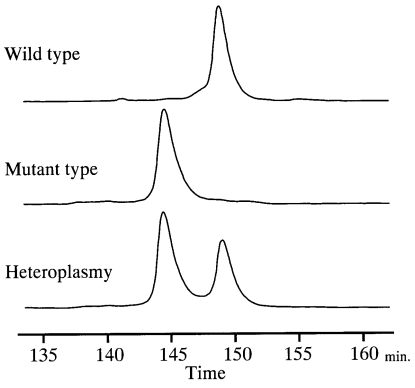

Fig. 5 Three haplotype chromatograms of the CS4 region in open-pollinated seeds of Kajikazawa 5

Chloroplast DNA haplotypes of open-pollinated seeds from Kajikazawa 5

Kajikazawa 5 is one of the 33 plus tree clones composing the Tomisawa seed orchard, and it is a solitary mutant-type clone in this orchard (Table 2). The epicotyls of germinated seeds from this clone were used for fluorescence-based SSCP analysis. As a result of the analysis of 80 epicotyls, the three SSCP profiles shown in Fig. 5 were observed. Seven epicotyls were the same mutant type as their mother tree, and 70 were wild types. The profile with double peaks originated from wild and mutant types and was recognized in the remaining three epicotyls. These individuals were heteroplasmies that possessed cpDNA from both the female and male parents.

Discussion

Intraspecific sequence polymorphism of cpDNA in *C. obtusa*

An intraspecific sequence polymorphism was observed in the intergenic spacer region between the *trn*D and *trn*Y genes. The two haplotypes (wild type and mutant type) were caused by only one single-base substitution in the 198-bp spacer sequence. This point mutation could easily be distinguished by fluorescence-based PCR-SSCP, and this made it possible to analyze numerous samples speedily. The sequence polymorphisms of cpDNA have been reported for many coniferous species, but most of these reports have been on DNA length variations, such as a minisatellite (Hipkins et al. 1995) and microsatellite DNA (simple sequence repeats, SSR). The complete sequence of cpDNA has already been determined in *Pinus thunbergii* (Wakasugi et al. 1994). On the basis of this sequence information, many SSR markers of cpDNA have been developed (Cato and Richardson 1996; Powell et al. 1995; Vendramin et al. 1996). Several of these markers can be utilized not only in *Pinus* species but also in *Abies* and *Picea* of the Pinaceae (Vendra-

min and Ziegenhagen 1997). Some of the cpDNA SSR regions have also been confirmed to be PCR-amplified in other coniferous families that are distantly related to *Pinus* phylogenetically (Cato and Richardson 1996), but no intraspecific polymorphism of these has been reported so far. The newly detected cpDNA variation reported here can be utilized as a practical genetic marker in the genetics and actual breeding of *C. obtusa*.

Imperfectibility of paternal inheritance in *C. obtusa*

Ohba et al. (1971) found that a chromatophore mutation of the needle was inherited from the male parent in *Cryptomeria japonica* and thus predicted the paternal inheritance of cpDNA. Neale et al. (1986) directly proved the paternal inheritance of cpDNA based on RFLP analysis of artificially pollinated progeny of *Pseudotsuga menziesii*. Since then, this phenomenon has been verified in many coniferous species using DNA molecular markers (Cato and Richardson 1996; Dong et al. 1992; Neale et al. 1989, 1991; Neale and Sederoff 1989; Stine et al. 1989; Stine and Keathley 1990; Szmit et al. 1987; Vendramin and Ziegenhagen 1997; Wagner et al. 1989). Nowadays, it is generally accepted that cpDNA is paternally inherited in conifers (Reboud and Zeyl 1994). In *Chamaecyparis*, the paternal inheritance of cpDNA has been investigated using interspecific hybrids (Kondo et al. 1998).

In this study, paternal inheritance in the full-sib families, which were raised by intraspecific crosses, was verified using a cpDNA polymorphism as a genetic marker. As a result, 352 of 361 progenies in families B, C, and D were found to possess the same haplotype as their male parents (Table 3). This indicates that cpDNA is generally under paternal inheritance in *C. obtusa*. The remaining nine progenies, however, were of the female parental types, and 2.5% of the progenies showed maternal inheritance.

It is possible that a contamination of the pollen/seed/seedlings of these families could have caused this result. However, our parentage diagnosis of the nine progenies showing maternal inheritance refuted the possibility of contamination. Furthermore, the fluorescence-based SSCP analysis of open-pollinated seeds collected from Kajikazawa 5 revealed the three cpDNA haplotypes (Fig. 5). One was the same mutant type as Kajikazawa 5; another was the same wild type as the trees surrounding Kajikazawa 5. The mutant types observed in the open-pollinated seeds might have been caused by the self-pollination of Kajikazawa 5, while the wild types might have been delivered from the surrounding trees by allogamy. The third haplotype was the heteroplasmy that possessed both peaks of the wild and mutant types. The relative frequency of heteroplasmy was 3.8%. Heteroplasmy has previously been observed in seedlings of *Pinus monticola* (White 1990), in a natural population of *Pinus leucodermis* (Powell et al. 1995), and in the F_1 progeny of *Abies alba* (Vendramin and Ziegenhagen 1997). In conifers, paternal chloroplast transmission to offspring results from the transformation of maternal plastids into large inclusions in the egg cell, which are subsequently excluded from the cytoplasm of the developing proembryo (Chesnoy and Thomas 1971; Owens and Morris 1990, 1991). In some proembryos, however, an incomplete exclusion of the maternal plastids may occasionally allow the coexistence of paternal and maternal plastids in a proembryo, and this heteroplasmic condition may be maintained during the seeds' development. The heteroplasmic seeds from Kajikazawa 5 might have originated from a maternal plastid leakage in some egg cells and the resultant presence of both maternal and paternal plastids in the embryo.

Our parentage diagnosis of the progenies which showed a female parental haplotype and our observation of heteroplasmy in open-pollinated seeds revealed that the paternal inheritance of cpDNA is not an exclusive phenomenon in *C. obtusa*. The incomplete genetic mechanism might produce the heteroplasmic condition of embryos at a low frequency. In the heteroplasmic embryos, either male or female parental plastids would be selected by a random drift during the process of somatic divisions, and the heteroplasmic embryos might finally be occupied with either male or female parental plastids. The fact that the heteroplasmy observed in the seed stage could not be found in 361 two-year-old seedlings might support this hypothesis.

In previous studies on the inheritance of cpDNA in coniferous species, the maximum sample size of the progenies per family was 57 (Cato and Richardson 1996), while most of the studies have dealt with a smaller number of samples. This has made it difficult to detect maternal inheritance, which exists at a low frequency in progenies. Progenies that showed the same cpDNA haplotype as their female parents have been found in several studies (Cato and Richardson 1996; Dong et al. 1992; Neale et al. 1991; Szmit et al. 1987). These studies suggest the possibility of biparental inheritance of cpDNA in conifers. Cato and Richardson (1996) attempted to verify the parentage diagnosis of samples that showed non-paternal inheritance by using two nuclear SSR markers. Some of these samples seemed to show biparental inheritance, but the possibility of contamination could not be excluded completely. Some of the samples possessing the maternal cpDNA type observed in those studies might have truly originated in the maternal transmission of cpDNA. Moreover, White (1990) found many heteroplasmies in seedlings raised from natural *Pinus monticola* seeds. Heteroplasmy has also been recognized in *Pinus leucodermis* and *Abies alba* at a low frequency (Powell et al. 1995; Vendramin and Ziegenhagen 1997). These facts might indicate that the biparental inheritance of cpDNA is a common occurrence in other coniferous species.

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