conventional classification systems by LIU (1971) and FARJON (1989, 1990) suggests the necessity of reconstructing the currently accepted systematics. An accumulation of more DNA information in addition to our results will clarify the systematics and phylogenetic relationships of genus *Abies*.

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Determination of the Selfing Rate in a Hinoki (*Chamaecyparis obtusa*) Seed Orchard by Using a Chloroplast PCR-SSCP Marker

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(Received 14th January 1999)

Abstract

The selfing rate in a Hinoki (*Chamaecyparis obtusa*) seed orchard, representing the most common coniferous species in Japan, was determined by using a highly specific PCR-SSCP (Polymerase Chain Reaction – Single-Strand Conformation Polymorphism) marker which recognizes the spacer region between the genes *trn*D and *trn*Y of the chloroplast DNA. One-thousand and three open-pollinated offsprings were analyzed from plus tree clone YKZ5. This clone has a single point mutation in comparison with 32 wild type clones which are also present in the orchard. Among the offsprings tested, the mutant chloroplast haplotype was detected in 23 samples. Based on the paternal inheritance of the chloroplast DNA marker, the mean selfing rate of clone YKZ5 was determined to 2.3% in good agreement with the theoretical value expected. Data demonstrate that the newly-developed PCR-SSCP marker derived from chloroplast DNA provides a powerful tool for accurate and effective analyzing of gene flow within a Hinoki seed orchard.

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Key words: PCR-SSCP, chloroplast DNA; selfing rate, seed orchard, *Chamaecyparis obtusa*.

Introduction

Hinoki (*Chamaecyparis obtusa*) is one of the most common conifers in Japan. The species is widely used in reforestation with exception of the island Hokkaido, representing the most northern Japanese island. Superior genotypos (plus trees) have been selected and grown in single orchards. The seeds harvested from such orchards yield now 40% of the plant stock used in Japan (National Forest Tree Breeding Center, 1997).

Researchers recently characterized the genetic information of Hinoki trees by using isoenzyme markers. Based on these markers, the genetic variation in both natural and artificial forests (SHIRAISHI *et al.,* 1986; SEIDO *et al.,* 1987, UCHIDA *et al.*, 1991), the genetic variability of plus trees (UCHIDA *et al.*, 1993), and the breeding structure of natural populations have been reported (SEIDO, 1990). Seed orchards planted by plus trees are designed and managed in order to produce a large amount of seeds for reforestation. If cross-fertilization, however, is limited in seed orchards, and if self-fertilization occurs at a high level,

the quantity and genetic quality of seed crops can be severely reduced possibly due to the existence of inbreeding depression (WOESSNER and FRANKLIN, 1973; FURUKOSHI, 1978). It is, therefore, absolutely required to assess the degree of self-fertilization and cross-fertilization to guarantee genetically valuable seed producing processes (SPROULE and DANCIK, 1996).

Currently, many forest geneticists have transferred their genetic analysis techniques from classical isoenzyme markers to modern molecular DNA markers. In particular, there is a tendency to use PCR-based SSCP (Polymerase Chain Reaction – Single-Strand Conformation Polymorphism) markers for the monitoring of single point mutations, because it is a simple, fast, and sensitive method (BODENES *et al.*, 1996; MAEDA and SHIRAISHI, 1997; NARAZAKI *et al.*, 1996; WATANO *et al.*, 1996), The SSCP technique is based on the principle that single-stranded DNA molecules possess a specific sequence-based secondary structure under non-denaturing conditions (ORITA *et al.,* 1989) . Molecules differing by as little as a single base substitution may form different conformations which can result in different mobilities on a neutral polyacrylamide gel (BODENES *et al.,* 1996). HAYASHI (1991) combined this method with PCR, and developed a simple analysis method called PCR-SSCP.

We recently detected an intraspecific variation in the spacer region between genes *trn*D and *trn*Y of the chloroplast DNA in Hinoki plus trees by using the PCR-SSCP technique and confirmed that the variation found was due to a single base substitution (SHIRAISHI et al., unpublished). In this paper, we report on the accurate determination of the selfing rate among the trees of a Hinoki seed orchard using the above-mentioned chloroplast DNA marker.

Materials and Methods

A Hinoki seed orchard (*Chamaceyparis obtusa*), located in the Yamanashi prefecture (Nanbu town, about 130 km south-western of Tokyo; latitude 35° 29,' longitude 138° 15'), was selected for this study. It was established between 1968 and 1970. Their area covers 4.56 ha, harbouring a total number of 33 plus tree clones now. The clone arrangement was designed in order to avoid closely planted ramets of the same clone, and the trees were then planted randomly with an original spacing of 3.5 m x 3.5 m. Trees were cutted at 3 m to 4m height for easy cone collection during every second year. At present, there are approx. 400 trees per ha. The mean height of all trees is 5.8 m and the mean diameter of breath height is 21 cm. The mean tree number per clone is 16.

The chloroplast haplotype analysis of the 33 orchard clones previously revealed that 32 clones represent a wild type, whereas clone Kajikazawa 5 (YKZ5) is the only mutant chloroplast haplotype detected. For the present study, we selected five ramets of clone YKZ5 in the orchard. After an open pollination of the mother trees happened from the end of March until April in 1995, seeds were harvested from each available tree at the end of the vegetation period. Seeds were then germinated under the following aseptic conditions: temperature 25±2 °C, 16 hours photoperiod and a light intensity of 5000 lx. After three weeks, the epicotyls were taken from the seedlings and applicated for the isolation of DNA.

Total DNA was extracted from each plantlet by the ISOPLANT procedure originally described by JHINGAN (1992). All chemicals were provided by WAKO Pure Chemical Industries, Ltd. DNA was further purified with the GENECLEAN^{R} III Kit (BIO 101) according to the protocol of the manufacturer.

The chloroplast CS4 spacer region between genes *trn*D and *trn*Y was amplified by using primer pair cyCS4U and CS4L (5'-TGACAGGGCGGTACTCTAAC-3', and 5'-CGATGCCCGAG-TGGTTAATG-3'). The 5' end of primer cyCS4U was labelled with a fluorescence dye(cy5:Amershampharmacia). PCR was carried out in a final volume of 10 μl, containing 0.1 ng/μ 1 template DNA, 0.25 μ M of each primer, 1 x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KC1), 200 μ M dNTP Mixture, 0.5 U of *ArnpliTaq* DNA polymerase, Stoffel Fragment (Perkin-Elmer Cetus), and 3 mM $MgC1_2$. For DNA amplification, PCR reactions were conducted in an automatic thermocycler (type: System model 9600; Perkin Elmer Cetus) with the following program: 60 s at 9 °C then 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C, followed by 60 s final elongation at 72 °C.

For SSCP analysis, PCR products were diluted in the same volume of sterilized pure water. One μl of this solution was mixed with 4 μl loading buffer (96% formamide, 20 mM EDTA, 0.05% bromophenol blue) and subsequently concentrated for 10 min using a vacuum evaporator (1400 rpm, exhaust 50 l/min). After heating (94 °C, 5 min), the sample was rapidly cooled on ice and immediately loaded onto a 50% non-denaturating LONG Ranger gel (chemicals provided by FMC Bio Products) containing lxTBE buffer. Conditions of electrophoresis were: 1200 Volt, 56 mA, 20 °C, 35 W, 180 min. Electrophoresis was performed by an ALFred DNA sequencer and fragments were automatically analyzed by the Fragment Manager software version 1.2.

Results and Discussion

Using the PCR-SSCP marker CS4, specified within Materials and Methods, chloroplast haplotypes were analyzed from 1,003 open-pollinated offsprings of the orchard clone YKZ5. The results obtained from such experiments are exemplarily shown in *figure 1.* All data of the five different investigated ramets are summarized in *table 1.*

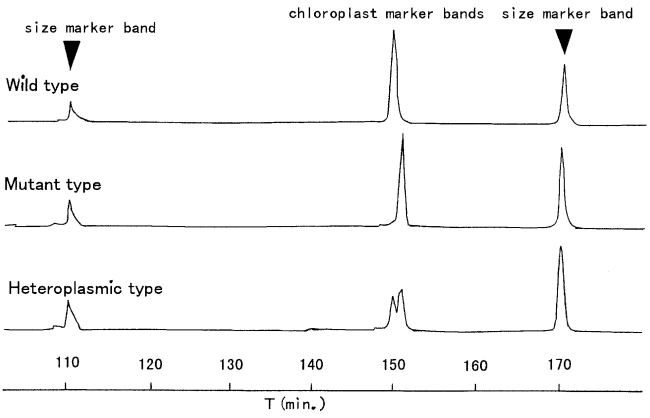

Fig. 1. – Three chloroplast haplotypes identified in a Hinoki seed orchard by using the PCR-SSCP marker CS4. After amplification, fluorescently labelled PCR products were separated (running time T indicated in minutes) under non-denaturing conditions. Detected marker bands are shown, indicating the most frequent wild type DNA, the mutant haplotype as well as a heteroplasmic haplotype. The symbol ▼ shows the internal size marker bands derived from standard DNA fragments.

In fact, a tremendous amount of 969 DNA samples (96.6%) showed the wild type PCR-SSCP marker bands after electrophoresis. These specific marker bands were not found in the chloroplast genetic information of the mother trees representing clone YKZ5. Their occurrence in the offsprings of clone

Table 1. – Estimation of selfing rate of clone YKZ 5 in a Hinoki orchard.

Ramet No.	Height	Number of seedlings analyzed	Chloroplast haplotype identified			Fertilization rate measured		Selfing Rate calculated
			Wild type	Mutant type	Heteroplasmic type	Cross fertilization	Self fertilization	
1	4.6m	202	185	12	5	190	12	5.9%
2	6.45	201	196	2	3	199	2	1.0%
3	6.3	199	195	3	1	196	3	1.5 %
4	5.6	202	199	3	0	199	3	1.5 %
5	5.7	199	194	3	2	196	3	1.5 %
Total		1003	969(96.6%)	23(2.3%)	11(1.1%)	980(97.7%)	23(2.3%)	mean 2.3%

YKZ5 strongly indicates, however, that a very effective pollen flow has occurred during the fertilization. This pollen flow was obviously produced by the surrounding 32 other plus tree clones which are characterized by the presence of the wild type SSCP marker. The data confirm considerable existence of cross-fertilization among all five ramets tested. Furthermore, eleven DNA samples (1.1%) yielded both the wild type and the mutant SSCP marker bands, suggesting the presence of maternal chloroplast DNA as well as the presence of paternal chloroplast DNA. This demonstrates that paternal inheritance of chloroplast DNA in Hinoki is not perfect and heteroplasmic haplotypos were found in small frequence.

On the other hand, for all five ramets investigated 12, 2, 3, 3, and 3 mutant chloroplast haplotypes were found, respectively. Taken together, the mutant type was only present in 23 samples derived from a total amount of 1,003 open pollinations. Because the mutant-type SSCP marker is highly specific for the chloroplast genetic information of clone YKZ5 its presence among the offspring of YKZ5 indicates 2.3% selfing rate. Interestingly, the rate of self-fertilization among the trees of ramet No. 1 is much more higher in comparison with the other four ramets tested (5.9% versus 1% and 1.5%, see *Table 1*). Note that the average tree height of this ramet is the lowest among all five ramets.

It has been reported that the chloroplast DNA of distinct coniferous tree species shows paternal inheritance (OHBA et al., 1971; NEALE et al., 1986), KONDO and co-workers (1998) as well as SHIRAISHI et al. (unpublishod) verified paternal inheritance of chloroplast DNA within the genus *Chamaceyparis.* The chloroplast haplotype present within each Hinoki progeny is, therefore, derived from the chloroplast haplotype of the male germ cell. Because the chloroplast haplotype representing clone YKZ5 is the only mutant type found among 33 orchard clones, we were able to estimate its rate of self-fertilizution by using a molecular PCR-SSCP marker, recognizing the spacer region between genes *trn*D and *trn*Y. Using the chloroplast genetic marker technique described, we determined 2.3% mean self-fertilization rate of clone YKZ5 among 1,003 open-pollinated offsprings tested. This is in good agreement with a theoretical value of 3.0% expected from an open-pollinated orchard consisting of 33 clones (1/33 = 0.03). Random mating has been proviously found in Hinoki tree natural populations (SEIDO, 1990). Based on nuclear peroxidase isoenzyme markers, the self-fertilization rate among 14 clones in a Hinoki seed orchard has been recently determined by TAJIMA (1979). The results were spanning from 0% to 30% with a mean rate of 16.8%. MÜLLER-STARK (1979) found a self-fertilization ratio as high of 12% to 14% in a 12 to 15-year-old Scots pine seed orchard, RUDIN and LINDGREN (1977) reported that the frequency of plants originating from selfing following open pollination was indicated to be in the rage of 2% to 5% in Swedish Scots pine seed orchards. ADAMS and JOLY (1980) estimated 1.2% self-fertilizution rate among 513 seeds, representing five clones. In contrast, our data clearly suggest that chloroplast DNA markers enable a more accurate calculation of the selfing rate within coniferous tree species because their paternal inheritance directly monitors the gene flow via pollen movement. We are encouraged now to design new chloroplast SSCP markers for the other plus tree clones which are present within the seed orchard.

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Buchbesprechung

Ökosystemforschung im Schwarzwald. Auswirkungen von atmogenen Einträgen und Restabilisierungsmaßnahmen auf den Wasser- und Stoffhaushalt von Fichtenwäldern. Verbundprojekt ARINUS. Herausgegeben von S. RASPE, K. H. FEGER und H. W. ZÖTTL. 1998. ECOMED Verlagsgesellschaft, Landsberg. ISBN 3-609-65900-9. 533 Seiten mit Abbildungen und Tabellen. Paperback DM 78,–/öS 569,–/sFr 75,–.

In den Jahren 1986 bis 1996 wurden von der Projektträgerschaft des Landes Baden-Württemberg PEF (Projekt Europäisches Forschungszentrum für Maßnahmen der Luftreinhaltung) Forschungsaktivitäten an zwei Versuchsstandorten im zentralen Südschwarzwald und am Ostrand des mittleren Schwarzwaldes zur Untersuchung der standörtlichen Grundlagen der betroffenen Wälder, ihrer Umsatzdynamik und der Wirkungsweise von Gegenmaßnahmen in Form von Düngung/Kalkung gebündelt. Aus Einzelvorhaben des Instituts für Bodenkunde und Waldernährung der Universität Freiburg (ARINUS = Auswirkungen von Restabilisierungsmaßnahmen und Immissionen auf den N- und S-Haushalt der Öko- und Hydrosphäre von Schwarzwaldstandorten) entwickelte sich durch das Einbinden weiterer Arbeitsgruppen verschiedener Institutionen ein interdisziplinärer Forschungsverbund. In der Abschlusspublikation präsentieren 34 Autoren eine arbeitsgruppenübergreifende Darstellung und Bewertung der Ergebnisse, die zeigen, dass es sich bei den im Schwarzwald beobachteten Schäden häufig nicht um direkte Immissionswirkungen handelt sondern um komplexe Störungen im Stoffhaushalt, die sich besonders in der Nährstoffversorgung äußern.

Das Buch enthält eine Einführung in das Verbundprojekt, die Untersuchungsgebiete sowie die Konzeption und den experimentellen Rahmen. Die Ergebnisse folgen in 7 Hauptkapiteln: Wasser- und Energiehaushalt; Stoffhaushalt auf Ökosystem- und Einzugsgebietsebene; Umsätze in Boden und Rhiosphäre; Untersuchungen zum N-Haushalt; Untersuchungen zum S-Haushalt; Ca- und Mg-Aufnahme und -Transport in Fichten; Zuwachsreaktion auf Witterung und Düngung. Die Hauptkapitel sind in 2 bis 7 Unterkapitel gegliedert und schließen jeweils mit einer zusammenfassenden Diskussion. Eine Synthese, in der die Einzelbefunde zusammengefasst und interpretiert werden, sowie ein 33-seitiges Literaturverzeichnis runden diesen interdisziplinären Abschlussbericht ab. Durch die Veröffentlichung bei der ECOMED Verlagsgesellschaft in der etablierten Reihe „Umweltforschung in Baden-Württemberg" sind die Ergebnisse auch über Baden-Württemberg hinaus für Wissenschaft, Verwaltung und Praxis verfügbar. Das modular aufgebaute Buch spricht insbesondere Mitarbeiter in den Bereichen Ökologie, Biologie, Forst- und Geowissenschaften sowie Boden und Wasserschutz an.

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Herausgeberin: Bundesforschungsanstalt für Forst- und Holzwirtschaft: Schriftleitung: Institut für Forstgenetik und Forstpflanzenzüchtung, Siekerlandstrasse 2, D-22927 Grosshansdorf — Verlag: J. D. Sauerländer's Verlag, Finkenhofstrasse 21, D-60322 Frankfurt a. M. — Anzeigenverwaltung: J. D. Sauerländer's Verlag, Frankfurt am Main. — Satz und Druck: Graphische Kunstanstalt Wilhelm Herr, D-35390 Giessen
Printed in Germany.

ISSN 0037-5349