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Pollen Dispersal in a Hinoki (*Chamaecyparis obtusa*) Seed Orchard Detected Using a Chloroplast DNA Marker

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Pollen dispersal was estimated in two test plots in a hinoki (*Chamaecyparis obtusa*) seed orchard using a chloroplast DNA marker, the spacer region between the *trnD* and *trnY* genes, and SSCP (single strand conformation polymorphism). In Plot 1, 2,020 seeds from 40 trees within 30 m of the marker tree were analyzed using the PCR-SSCP method. In Plot 2, 1,850 seeds from 37 trees were analyzed in the same manner. The results revealed that the maximum pollen dispersal distance in the two plots exceeded 25 m. Pollen dispersal appeared to be inversely proportional to the distance from the marker tree. The effective pollen dispersal was suggested to be less than about 20 m in a mature hinoki seed orchard. Adjacent trees had an excessive influence when the pollen density was increased by artificial flower stimulation. Therefore, it was suggested that seed production better resembles ideal random mating when carried out as naturally as possible. In conclusion, the SSCP chloroplast DNA marker was a useful tool for amassing basic information on pollen management in seed orchards of coniferous species.

Key words: *Chamaecyparis obtusa,* chloroplast DNA, pollen dispersal, seed orchard, SSCP

Hinoki, *Chamaecyparis obtusa,* is one of the most important trees in Japan. Its high quality as a building material has created a great demand. Current reforestation efforts have put hinoki at the top of the planting list, and it currently comprises 40% of all planting stock from seed (National Forest Tree Breeding Center, 1997).

The seed stock currently used is harvested from commercial orchards. Due to the great importance of the seeds, it is necessary to understand the mechanisms of hinoki mating to establish appropriate genetic techniques for future management of the orchards.

A seed orchard consists of a limited number of clones and the mating system assumes panmixis. Under ideal conditions, component clones produce a large amount of pollen, contamination with pollen from external sources is prevented, and the pollen from clones is evenly distributed for fertilization. Therefore, it is important to understand the pattern of pollen dispersal for the genetic management of seed production.

The dispersion distance and distribution of pollen in conifers have been studied using various methods. These include examining the ratio of full embryo seeds in an isolated tree (Johnson *et al.*, 1945), plastid mutations inherited paternally (Furukoshi, 1979), as dominant genes (Langner, 1959), or as recessive homozygotes (Squillace and Kraus, 1963), pollen density (Kanazashi *et al.*, 1984), and isozyme markers (Shen *et al.*, 1981; El-Kassaby *et al.*, 1986; Yazdani and Lindgren, 1992; Burczyk and Prat, 1997). However, pollen dispersal in seed orchards has not been studied well.

We searched for a genetic marker to indicate paternity in hinoki, and recently detected intraspecific variation in the noncoding region (CS4 region) between the chloroplast genes *trnD* and *trnY* in hinoki trees, using the PCR-SSCP technique. This variation was due to a single base substitution (Shiraishi *et al.*, 2000). Chloroplast haplotype analysis of the 33 clones in the seed orchard investigated revealed that 32 clones were the wild type, and only one mutant chloroplast haplotype, Kajikazawa 5 (YKZ5), was detected (Shiraishi *et al.*, 2000).

This paper estimates the effective range of pollen dispersal and the density of pollen distribution in a hinoki seed orchard using YKZ5 as a marker tree.

Materials and Methods

The hinoki seed orchard studied is located in Nanbu Town, in Yamanashi Prefecture, Japan, about 180 km southwest of Tokyo (35°29′ north latitude and 138°15′ east longitude). The trees were planted in 1968 and 1970. The seed orchard consists of three gardens covering 4.56 ha, and contains 33 clones. The clones were arranged in such a way that ramets of the same clone were not close to each other. Originally, the trees were planted randomly with a spacing of 3.5 × 3.5 m (800 trees/ha). In 1983, thinning was carried out, reducing the density to 400 trees/ha, with 5-m spacing. The average number of trees/clone is 16. For ease of cone collection, the trees are cut to a height of 3–4 m every other year. The average height of the trees was 5.8 m and the diameter was 21 cm.

A chloroplast haplotype analysis of the 33 orchard clones previously revealed that 32 clones were the wild type, and the clone Kajikazawa 5 (YKZ5) was the only mutant chloroplast haplotype detected (Shiraishi *et al.*, 2000). This study used YKZ5 as a marker clone. There are 14 ramets of YKZ5 in the first garden and 18 in the second. Figure 1 shows their positions. Test plots were set around YKZ5 trees, such that neighboring trees were not the same clones. Plot 1 was set in the first garden and Plot 2 in the second. We examined 40 trees around the marker tree in Plot 1 and 37 trees around the marker tree in Plot 2. Assuming a low fertilization rate on the windward side of marker trees, more trees were selected on the lee side. Consequently, the distances of selected trees from

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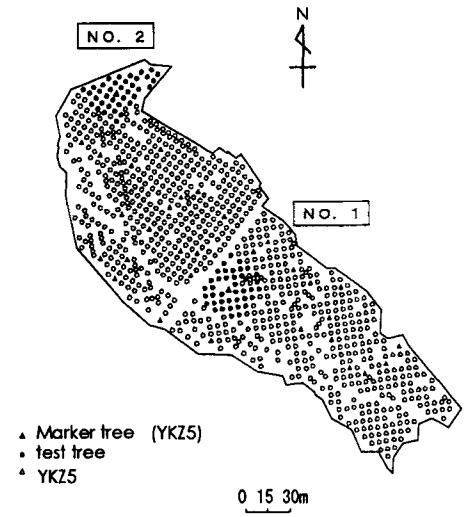

Fig. 1 Study area used in the *Chamaecyparis obtusa* seed orchard.

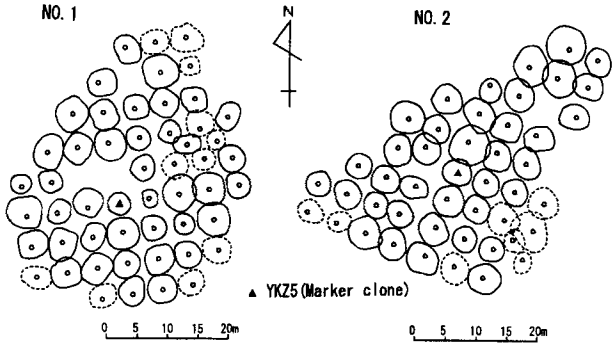

Fig. 2 Crown size of test trees on each test plot. ▲ indicates marker clone YKZ 5 which is only mutant type found among 33 orchard clones in the studied orchard. Circles of solid line denote crowns of test trees. Circles of dotted line indicate crowns of untested trees.

the marker ranged from 5 to 26 m in Plot 1 and from 5 to 29 m in Plot 2 (Fig. 1).

The previous year, all the trees in the first garden were treated with 5 mg gibberellin mixed with CMC (sodium carboxymethyl cellulose) to promote flowering. Five lower to middle branches on each tree were selected and the gibberellin mixture was applied to each branch to a depth of 2 cm. The trees in the second garden were untreated. The average tree height and diameter were 4.8 m and 21.6 cm in Plot 1 and 5.6 m and 25 cm in Plot 2, respectively. Figure 2 shows the locations of the crowns in each plot. The crowns almost fill both plots, although there are larger gaps in Plot 1. Cones were harvested randomly from each plot separately in early October. Seeds were extracted from each cone and germinated under aseptic conditions at 25 ± 2°C, with a 16-h photoperiod and a light intensity of 5,000 lux. In all, 3,870 seeds were examined, 2,020 from Plot 1 and 1,850 from Plot 2.

DNA was extracted from radical 5 to 10 mm long using ISOPLANT (Wako Pure Chemical Industries, Ltd.), using a procedure described by Jhingan (1992).

The chloroplast DNA spacer region (CS4) was amplified by PCR using the extracted DNA as the template. To speed up SSCP analysis of the CS4 region, two primers were used: CS4U (5'-TGACAGGGCGGTACTCTAAC-3') and CS4L2 (5'-GGCACAGTTGAATTACTATGCTA-3'). The 5' end of primer CS4U was labeled with fluorescent dye (cy5; Amersham Pharmacia). PCR was carried out in a final volume of 10 μL containing 0.1 ng/μL template DNA; 0.25 μM each primer, 1 × reaction buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 200 μM dNTP mixture, 0.5 U of AmpliTaq DNA polymerase, Stoffel Fragment (Perkin Elmer Cetus), and 3 mM $MgCl_2$. PCR reactions were conducted in an automatic thermocycler (Model 9600; Perkin Elmer Cetus) using the following program: 60 s. denaturation at 95°C; 25 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, 60 s extension at 62°C; and a final extension at 72°C for 60 s.

For SSCP analysis, the PCR products were diluted with three volumes of sterilized water. One μL of dilute solution was mixed with 4 μL of loading buffer (96% formamide, 20 mM EDTA, 0.05% Trazine, and Inner marker) and subsequently concentrated for 10 min using a vacuum evaporator (1,400 rpm, exhaust 50 L/min). The sample was heated for 5 min at 94°C, rapidly cooled on ice, and immediately loaded onto a 5% non-denaturing LONG Ranger gel (FMC Bio Products) containing 0.6 × TBE buffer. Electrophoresis was performed using an ALFRED DNA sequencer at 1,200 V, 50 mA, 20°C, 35 W for 70 min and the fragments were automatically analyzed using Fragment Manager software, version 1.2.

Results

Chloroplast DNA is cytoplasmically inherited; moreover, it is paternally inherited in a number of conifers (Neale *et al.*, 1986, 1989a, b). The mutation used as a DNA molecular marker in this study is usually paternally inherited in chloroplast DNA (Shiraishi *et al.*, 2000). Since YKZ5 is the only mutant type in the seed orchard, any seed containing mutant chloroplast DNA haploid must have been fertilized by pollen from YKZ5. Therefore, the range and density distribution of pollen dispersal from YKZ5 can be determined by investigating the incidence of the mutant in seed samples from trees around the marker trees.

We designed a new primer to amplify the 89 base pair (bp) CS4 region to improve the efficiency of the PCR-SSCP analysis. SSCP analyses using the new (CS4U, CS4L2) and old primer pairs (CS4U, CS4L, Seido *et al.*, 2000) are compared in Fig. 3. The PCR product using the new primers is much shorter than the product using the old primers. Therefore, the time required for the analysis was shortened from about 160 min to about 60 min. In addition, since the mobility differences of the wild type and mutant were large using the new set of primers, the identification of haplotypes was more straightforward. Therefore, the new primer pair greatly improved the efficiency and reliability of the analysis.

The positions of the trees in each plot are shown in Fig. 4. Concentric circles 5 m apart, centered on the marker tree, indi-

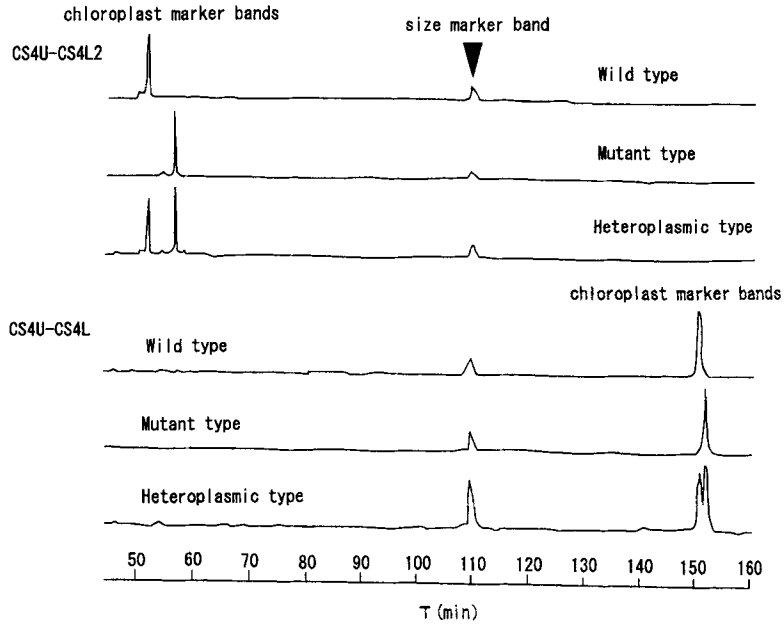

Fig. 3 Comparison of PCR-SSCP haplotypes of chloroplast DNA in *Chamaecyparis obtusa* by two primers of CS4.

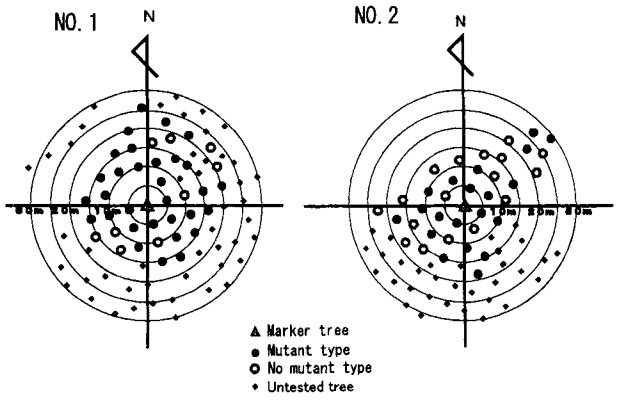

Fig. 4 Pollen dispersal from a marker tree in each test plot.

cate the distance from the marker tree. Trees with as few as one mutant seed in each analysis sample (*ca.* 50 seeds) are shown with solid black dots. Open circles indicate trees from which no mutant haplotypes were detected.

In Plot 1, the tree furthest (26 m) from the marker tree was fertilized with pollen from the marker tree. Similarly, in Plot 2, the tree 29 m from the marker tree was fertilized by the marker tree.

During the pollen dispersal season, the prevailing winds blow toward the northeast. However, trees with the mutant haplotype were not restricted to the lee side. Plot 1 contained the greatest percentage of trees (77.5%) confirmed fertilized with marker pollen. The percentage in Plot 2 was 54.1% (Table 1). The frequency of seeds from individual trees carrying the mutation ranged from 0 to 32%, averaging 5.1% in Plot 1. In contrast, the frequency in Plot 2 was lower, averaging 1.7% with a range from 0 to 6%.

In three trees located 5 m from the marker tree in Plot 1, the frequencies of mutant seed was 10, 24, and 32%, averaging 22%. This indicates that the marker tree has a great influence on mating with adjacent trees. In Plot 2, however, the frequencies in four seed trees within 5 m of the marker tree were extremely low, 0, 2, 4, and 6%, averaging 3%. The average frequency of mutant seeds from trees within each concentric circle was compared. In Plot 1, the cumulative contribution rates were 57, 75, 84, and $>90\%$ for trees within 5, 10, 15, and 20m from the marker tree, respectively. In contrast, in Plot 2, since the frequency of mutant type seed was low as a whole, the differences with distance were small, and the cumulative contribution rate increased gradually, exceeding 80% by 25 m. Although there was a large difference in the frequency of the mutant-type seed between the two plots, the frequency tended to decrease with distance in both plots.

Fertilization of each clone by the marker trees was examined (Table 2). Five out of 33 clones (15%) did not show evidence of fertilization by the marker trees. Fertilization with YKZ5 pollen was not evident in 1 out of 24 clones in Plot 1, and in 4 out of 24 clones in Plot 2. Of these 5 clones, one tree was within 5 to 10 m of a marker tree, 3 trees were within 10 to 15 m, and one tree was within 20 to 25 m. The clone, closest to the marker tree, Kajikazawa 1, was located 10 m upwind of YKZ5.

Discussion

Using a plastid mutation as a marker, Yamate *et al.* (1979) determined that in *Cryptomeria* (a common conifer in Japan) seed orchards beginning to close, the effective pollen dispersal distance is 9 m, with a maximum of 10 to 15 m. Using the same plastid mutation, Furukoshi (1978) reported that the effective distance of pollen dispersal in a model *Cryptomeria* seed orchard was less than 10 m and concluded that the effective pollen dispersal distance in *Cryptomeria* seed

Table 1 Appearance of mutant type and the contribution in each plot.

Plot	Distance from marker tree (m)	No. tested	Mutant appear/ No. tested (%)	Mean mutant appear/tree (%)	Range Min–Max (%)	Contribution (%)	Cumulative contribution (%)
No.1	0–5	3	100.0	22	10–32	57.1	57.1
	5–10	7	71.4	6.9	0–26	17.9	75.0
	10–15	16	87.5	3.3	0–10	8.6	83.6
	15–20	9	66.7	3.3	0–12	8.6	92.2
	20–25	4	50.0	1.0	0–2	2.6	94.8
	25–30	1	100.0	2.0	2	5.2	100.0
	Mean	40	77.5	5.1			
No.2	0–5	4	75.0	3	0–6	30.3	30.3
	5–10	8	75.0	2	0–2	20.2	50.5
	10–15	12	50.0	1.5	0–2	15.2	65.7
	15–20	6	33.3	1.1	0–2	11.1	76.8
	20–25	4	25.0	1.0	0–4	10.1	86.9
	25–30	3	66.7	1.0	0–2	13.1	100.0
	Mean	37	54.1	1.7			

Table 2 Fertilization of each orchard clone by the marker tree.

No.	Clone	0 m	−5 m	−10 m	−15 m	−20 m	−25 m	−30 m
1	YKZ1			□			□	
2	YKZ2				●□■■			
3	YKZ3			■				
4	YKZ4				○●□	●		
5	YKZ5	●■						
6	YKZ6		●		□■			
7	YKZ7		■				■	
8	YKZ8						□	
9	YOT3				●□			
10	QNIN1			●■	●			
11	OMT1					●		
12	NSI1					●		
13	QNNJ1			■	●	●		
14	QNNJ2			■	●●			
15	QNAG1		●			●		
16	QNAG4				●■	□		
17	QNAG8					●		
18	QNAG10			●□			□	
19	QNSK3				●		○	
20	IKJ1				○			
21	QNTM5			●	●			
22	KTN1			●		○		●
23	KTN3				■	□		
24	KTN7		●	■		○		□
25	KTN8				□			
26	QOTK2			●				
27	QOTK4		□	○		■□		
28	QOTK5							■
29	KKT5				●●		●	■
30	KMH2		■		●■	○	●	
31	KMH3				□			
32	QNOT102			○■	●	■	○	
33	QOTK9		■			□		

●, the mutant type appeared in offspring of individual trees in plot 1; ○, the mutant type did not appear in offspring of individual trees in plot 1; ■, the mutant type appeared in offspring of individual trees in plot 2; □, the mutant type did not appear in offspring of individual trees in plot 2.

orchards is within 10 m. In addition, Kanazashi *et al.* (1984) reported that in a *Cryptomeria* seed orchard containing only a few trees and few male flowers, at a collection site, 15% of pollen was from the marker tree, 54% from adjacent trees, and 30% from trees at distances of 65 to 150 m. Shen *et al.* (1981) analyzed the relationships between distance from the marker tree, flowering synchrony, wind direction, and pollen dispersal in a Scots pine seed orchard using isozyme markers. They showed that the contribution rate to adjacent trees was 31.4% and that the marker tree made a significant contribution.

Less pollen was distributed to trees 10 to 20 m away, and little was contributed to trees 40 m away or upwind (Shen *et al.*, 1981).

In still air, hinoki pollen falls at 6 cm/s (Seido, unpublished). This means that it takes about 90 s for pollen to reach the ground from a height of 5 m with no wind. The average wind velocity measured in this seed orchard during the dispersion period was 2.04 m/s, with a maximum average of 4.6 m/s (Seido, 1983). Therefore, the wind should carry pollen over long distances (Iwanami, 1981; Kanazashi *et al.*, 1984). In our study, dispersion to the edge of both study plots was confirmed, so it is likely that pollen travelled even further downwind. The percentage of trees with mutant seeds was high: 77.5% in Plot 1 and 54% in Plot 2. The difference in the percentages between the two plots is related to the amount of pollen produced by the marker trees. Male flower production was 3.5 times higher in Plot 1, where the trees were treated with flowering stimulant, than in Plot 2, where there was no treatment.

The prevailing winds blow toward the northeast in the seed orchard. However, we did not obtain results biased toward downwind pollen dispersion. It is likely that wind speed and direction are variable, with a tendency for turbulent flow (Di-Giovanni and Kevan, 1991; Webber and Painter, 1996). The mutant seed rate in each tree adjacent to the marker tree averaged 22% in Plot 1 and only 3% in Plot 2. The selfing rate of the marker tree was 5.9% in Plot 1 and 1.5% in Plot 2 (Seido *et al.*, 2000). The extent of the contribution to adjacent trees depends on the pollen production level. Nonetheless, when there is a significant amount of pollen, pollen disperses in high density to nearby trees and in lower density to distant trees.

There are differences in the flowering periods of different clones. There is a two-week difference in female flowers and a 10-day difference in male flowers. However, mating between all clones is possible (Seido, 1980). Therefore, it appears that the failure of the marker tree to fertilize 5 clones was not the result of differences in the flowering period, but due to the pollen dispersal density. In support of this, in Plot 2 trees were not treated with flowering stimulant and the marked tree failed to fertilize more clones. A relationship between mutant seed frequency and distance was not established in the study, although the distance influenced the frequency of fertilization by the marker. Both the marker pollen density and the total pollen density should influence the fertilization of each tree (Kanazashi *et al.*, 1984). Under natural flowering conditions in Plot 2, the rate of mutant type seed in each concentric circle was low and the contribution rate was also small.

The influence of other ramets of the same marker clone was not completely removed. We only considered pollen dispersal from the marker tree in this analysis, and assumed that the contribution of other ramets was negligible for the following reasons. In Plot 1, the closest ramet of the same clone was 11 m downwind from the marker tree, and there was another ramet 12 m northwest of the marker tree. The tree adjacent to the latter ramet had only a 2% frequency of mutant seed, which shows the low influence of this ramet. On the other hand, in Plot 2 the closest ramet was 40 m upwind from the marker tree. The influence of this ramet is considered zero because the mutant seed rate in the nearest tree in Plot 2, which was 20 m away, was 0%.

This seed orchard consists of 33 clones. Assuming panmixis, with all the clones contributing equally as pollen parents, the theoretical contribution rate as a pollen parent becomes 1/33 or 3.3%. In this study, the effective pollen dispersal distance was defined by the point at which fertilization by the marker tree exceeded this expected contribution rate (3.3%). The effective pollen dispersal distance in Plots 1 and 2 was 20 and 22.5 m, respectively. Naturally, tree height influences pollen dispersal (Shen *et al.*, 1981; Webber and Painter, 1996). These effective pollen dispersal distances are four times the height of the marker tree. The effective distance also depends on the tree height of the pollen supplier. However, since the seed trees are pruned to 3 or 4 m for easy cone collection in hinoki seed orchards, it might be appropriate to say that the effective pollen dispersal distance is 20 m or less in mature seed orchards.

In seed orchards where random mating is required, the contribution rate of each clone should be as equal as possible. Adjacent trees may have an excessive influence when the pollen density is increased by artificial flower stimulation. Therefore, seed production more closely resembles ideal random mating when carried out as naturally as possible.

This study clearly observed the dispersal of pollen using a chloroplast DNA marker. Genetic analysis of seed orchards has been performed using isozymes primarily (Shen *et al.*, 1981; El-Kassaby *et al.*, 1986). Recently, advances in molecular biology have led to the development of DNA techniques, which are not affected by the environment or the growth of an organism, and analytical techniques, which can extract genetic information relatively easily. The paternal inheritance of chloroplast DNA in conifers, including *Pseudotsuga*, *Pinus*, and *Chamaecyparis*, has been clarified (Neale *et al.*, 1986, 1989a, b; Ponoy *et al.*, 1994; Kondo *et al.*, 1998). Most chloroplast DNA analyses have used the RFLP method (Kondo *et al.*, 1986; Wagner *et al.*, 1987; Szmidt *et al.*, 1988; Wagner, 1992; Tsumura *et al.*, 1994; Shiraishi *et al.*, 1995; Ziegenhagen *et al.*, 1995). However, PCR-SSCP with our new primers is a simple, swift method of analysis if an automatic DNA sequencer is available (Orita *et al.*, 1989; Hayashi, 1991). DNA molecular markers are suitable for genetic and breeding research in which a great number of samples must be analyzed at a time, as in this study, to obtain basic information on seed orchard management, such as monitoring gene flow via pollen movement.

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