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A linkage map for sugi (*Cryptomeria japonica*) based on RFLP, RAPD, and isozyme loci

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Abstract A linkage map for sugi was constructed on the basis of restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and isozyme loci using a three-generation pedigree prepared for genetic analysis of heartwood color. A total of 128 RFLP (123 cDNA and 5 genomic probes), 33 RAPD, 2 isozyme, and 1 morphological (dwarf) loci segregated in 73 progeny. Of the 164 segregating loci, 145 loci were distributed in 20 linkage groups. Of these loci, 91 with confirmed map positions were assigned to 13 linkage groups, covering a total of 887.3 cM. A clustering of markers with distorted segregation was observed in 6 linkage groups. In the four clusters, distortions with a reduction in the number of homozygotes from one parent only were found.

Key words RFLP · RAPD · Linkage map · Sugi (*Cryptomeria japonica*) · Three-generation pedigree

Abbreviations *MAS* marker-assisted selection, *PAGE* polyacrylamide gel electrophoresis, *QTL* quantitative traits of loci, *RAPD* random amplified polymorphic DNA, *RFLP* restriction fragment length polymorphism

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Introduction

Linkage maps based on molecular markers, such as restriction fragment length polymorphisms (RFLP; Botstein et al. 1980), have been reported for a number of crop plants, and the application of molecular markers and linkage maps to plant genetics and breeding has been suggested (for review, see Tanksley et al. 1989). In conifers, development of molecular markers and linkage maps has been somewhat slow. Long generation times, the large sizes of genomes, and the outbred mating systems of conifers are the major barriers to the construction of linkage maps. Recently, a linkage map based on random amplified polymorphic DNA (RAPD; Williams et al. 1990) markers was reported for *Picea glauca* (Moech) Voss. (Tulsieram et al. 1992).

Sugi, *Cryptomeria japonica* D. Don (Taxodiaceae) is one of the most important conifers in Japan. Forty-five percent of all the cultivated forests of Japan are comprised of this species, and it is one of the species of Japanese conifers to which mentions of genetic and breeding research have been applied most extensively. Although morphological markers have been well described (for review, see Ohba 1980), they are usually recessive and in the rare allelic frequencies. Isozyme markers have been used for the evaluation of genetic diversities in sugi populations and for fingerprinting of breeding materials, because they allow the identification of heterozygotes and are neutral with respect to natural selection (Tsumura et al. 1989; Okuizumi et al. 1990; Tomaru et al. 1992). However, the number of heterozygotic isozyme loci within a single cross is quite small. As a result, no genetic linkage map of sugi has yet been constructed. We postulated here that RFLP and RAPD techniques might provide a large number of segregating markers. Such genetic markers could be applied to genetic studies and breeding programs, such as the evaluation of genetic variations in terms of quantitative traits, and be used for comparing genomic organization among species in Taxodiaceae. Therefore, we have

begun to construct a basic linkage map for a three-generation pedigree of sugi using RFLP, RAPD and isozyme markers. Although the current linkage map is not yet saturated, the molecular markers reported here may allow genetic and breeding research that was previously impossible.

Materials and methods

The segregating population of 73 individuals was a self-pollinated array of progeny of an F_1 hybrid from a cross between 'Kumotooshi' (female) and 'Okinoyama-sugi' (male), which are local cultivars that are propagated by cutting. Establishment, begun in 1978, of this three generation pedigree (Fig. 1) included the setting up of a field experiment with cloned parents and F_1 plants, and was performed by Ohba and coworkers (Kawasaki et al. 1984; Ohba et al. 1988) as part of an effort to clarify heritable traits of heart-wood color (red and black). 'Komotooshi' was found to have a dwarf gene and a mutant gene for chlorophyll, expressed as a light-green leaf color. Both genes were recessive. 'Okinoyama-sugi' was also heterozygous for the same dwarf gene mentioned above. The F_1 plant used in this linkage analysis was also a heterozygote for this gene. The parents had different genotypes for some isozyme loci and possible quantitative trait loci (QTL) for male flower bearing (Tsurumi et al. 1989) and color of heartwood.

DNA was extracted from young needle buds of individual specimens by the method described by Murray and Thompson (1980).

A complementary DNA (cDNA) library was prepared from total RNA isolated from 3-day imbibed embryos and current needles by the method of Murray and Thompson (1980). Poly(A)$^+$RNA was fractionated through oligo-(dT) affinity chromatography. Complementary DNA was synthesized using a cDNA synthesis kit (Pharmacia, Uppsala). *Eco*RI/*Not*I adaptors were added, and cDNA was ligated into prepared vector pT7T3 18U (Pharmacia, Uppsala) and transformed into *E. coli* strain JM 109. Recombinant clones were screened using IPTG/X-gal.

A genomic DNA library was prepared from sugi total DNA extracted by the CTAB method described above. DNA was digested with *Hin*dIII, sized between 500 to 3,000bp, and ligated into *Hin*dIII-digested pUC 19. Plasmids were transformed into *E. coli* strain JM 109, and recombinant clones were screened using IPTG/X-gal.

Complementary DNA (cDNA) and genomic DNA were used as probes to detect RFLPs. Genomic DNA and cDNA clones, prepared by the rapid plasmid preparation method (Sambrook et al. 1989) were digested with appropriate restriction enzymes, and the fragments were separated by electrophoresis on agarose gels. Gel pieces containing inserts were excised and fragments of DNA recovered from the pieces were labeled with α-[^{32}P]dCTP (Amersham; 110 TBq/mmol).

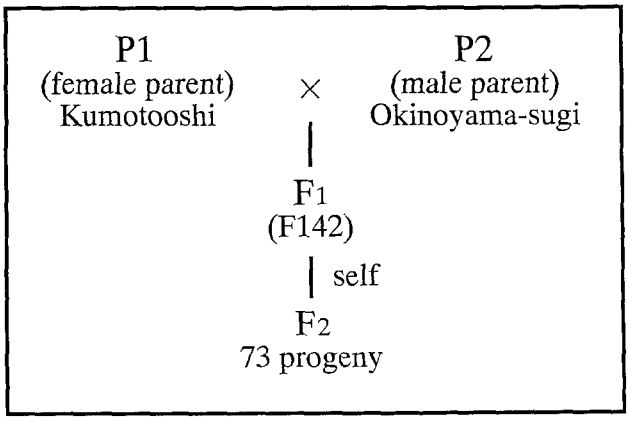
Genomic DNAs were digested with four restriction enzymes (*Bam*HI, *Eco*RV, *Eco*RI, and *Hin*dIII), with four units of enzyme per microgram of DNA. Aliquots of 5 µg of digested DNA were fractionated by a electrophoresis in a 0.7% agarose gel, and fragments were transferred to a Biodyne B membrane (PALL, N.Y.) by the alkaline transfer method (Reed and Mann 1985). Blots were prehybridized for several hours at 42 °C in 50 m*M* Tris-HCl (pH 7.5), 6 × SSC, 50% formamide, 1% SDS, 10% dextran sulfate, and 100 µg/ml denatured DNA from salmon testis. Labeled probes were denatured and directly added to the prehybridized membranes. After hybridization at 42 °C overnight, the membranes were rinsed in 2 × SSC, 0.1% SDS and washed twice for 15 min with 0.5 × SSC, 0.1% SDS at 42 °C and twice for 15 min with 0.1 × SSC, 0.1% SDS at 60 °C. Blots were then exposed to Kodak XOMAT-AR5 films with two intensifying screens at −80 °C for several days.

DNAs from the parents, a F_1 plant (designated F142), and 73 individual F_2 progeny were amplified with random 10-mer primers (Kit A to Z and AA to AC; Operon, Alameda, Calif.). The cycling parameters (40 cycles) for denaturation, annealing and extension were: 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C, respectively. Amplification was carried out with 25 ng of total DNA in 25 µl of a solution that contained 10 mM Tris-HCl (pH8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.01% gelatin, 0.1% Triton × 100 0.1% m*M* of each dNTP, 100 µmol primer, and 0.5 U *Taq* polymerase (Nippon Gene, Tokyo). Ten µl of the solution of the products of the polymerase chain reaction (PCR) was fractionated on agarose gel. Polymorphisms were scored as the presence or absence of a specific band.

Isozyme assays were performed as described by Tsumura et al. (1989). The crude extracts from the inner bark tissues were fractionated by polyacrylamide gel electrophoresis and scored for polymorphic isozyme loci for aconitase (ACO; EC 4.2.1.3) and leucine aminopeptidase (LAP; EC 3.4.11.1).

Segregating of the markers was scored only in the case of well-defined bands that obviously represented polymorphisms between the parents. Segregation data were analyzed with the MAPMAKER/EXP 3.0 computer program (Lander et al. 1987; Lincoln et al. 1992). To identify the linkage groups, pairwise comparisons and grouping of markers were performed under the following conditions: (1) the LOD score was equal to or greater than 4.0; and (2) the map distance was below 40 cM. In order to establish the most likely order of markers within each linkage group, we made comparisons between the best order and the second-best order using the exclusion threshold of an LOD score of 2.0, and the ripple command was used to confirm the orders of the markers. Two-point linkage analysis was performed with the G-MENDEL 1.0 program (Liu and Knapp 1990) to confirm the linkage relationships among markers with distorted segregation.

Fig. 1 The three-generation pedigree of sugi (*Cryptomeria japonica*) that was analyzed in this study



Results and discussion

In order to exclude the clones that originated from plastid DNA and/or repeated sequences within nuclear DNA, the genomic library was screened with total DNA by colony hybridization. Four restriction enzymes (*Bam*HI, *Eco*RV, *Eco*RI, and *Hin*dIII) and approximately 1,000 randomly chosen cDNA clones and 250 randomly chosen genomic clones were used for screening RFLPs. Approximately 30% of the cDNA clones and 12% of the genomic clones revealed RFLPs between the parents. It is generally assumed that genomic DNA clones that originate from single-copy or low-copy-number sequences reveal more polymorphisms than cDNA clones. In contrast, Landry et al. (1987) found that in lettuce cDNA clones revealed 2.5 times more polymorphisms than genomic clones. We are not certain that there is an advantage in using cDNA clones for

detecting RFLPs, because some genomic DNA clones used in the screening of polymorphisms were revealed to be moderate repeats by Southern hybridization. To obtain RAPD markers, 580 primers (29 sets of Operon 10-mer kits) were screened. Approximately 30% of the primers represented polymorphisms between the parents.

A total of 164 (128 RFLPs, 33 RAPDs, 2 isozymes, and 1 morphological) markers segregated in 73 individual F_2 progeny (Figs. 2 and 3). The majority of the RFLP markers and all of the isozyme markers segregated in a co-dominant manner (1:2:1), while all of the RAPD markers segregated in a dominant manner (3:1). A total of 39 (31 RFLPs, 7 RAPDs, and 1 isozyme) markers showed distorted segregation ($P < 0.05$). Because it has been reported that the segregation of markers in non-cultivated plant species shows some distortions, all of the segregation data were analyzed with the MAPMAKER computer program (Lander et al. 1987).

Of the 164 segregating loci, 145 (116 RFLPs, 26 RAPDs, 2 isozymes, and 1 morphological trait) loci were distributed into 20 linkage groups. Of these loci, 91 (77 RFLPs, 12 RAPDs, 1 isozyme, and 1 morphological trait) loci with confirmed map positions were assigned to 13 linkage groups, covering a total of 887.3 cM (Fig. 4). Neale and Williams (1991) estimated the size of the genome of loblolly pine in terms of map units to be approximately 2,500 cM. On the basis of an estimation of the haploid DNA contents of gymnosperms (Price et al. 1974), the size of the sugi genome, in terms of haploid DNA content, is smaller (50–66%) than those of species in Pinaceae. While loblolly pine has 12 pairs of chromosomes, sugi has 11 pairs. The size of the sugi genome in terms of map units has not yet been reported. From a comparison of the DNA content and chromosome number between sugi and loblolly pine, it seems that the sugi genome, in terms of map units, may be a little smaller than that of loblolly pine.

In order to map DNA sequences of known genes in the current linkage map, we examined which of our cDNA clones showed homology to a cDNA clone for

Fig. 2 Segregation of an RFLP marker (CD511). Total DNA extracted from 'Okinoyama-sugi' as the male parent (P_1), from 'Kumotooshi' as the female parent (P_2), from F142 as F_1 progeny (F_1) and from F_2 progeny (F_2) was digested with *Hin*dIII and probed with the [^{32}P]-labeled insert of sugi cDNA clone, pCjc511

Fig. 3 Segregation of an RAPD marker (T17b). An *arrow* shows the site of segregation of the PCR products produced with the OPT-17 primer. Symbols are as in Fig. 2. *M* Fragments of *Hin*dIII-digested bacteriophage λDNA

Fig. 2

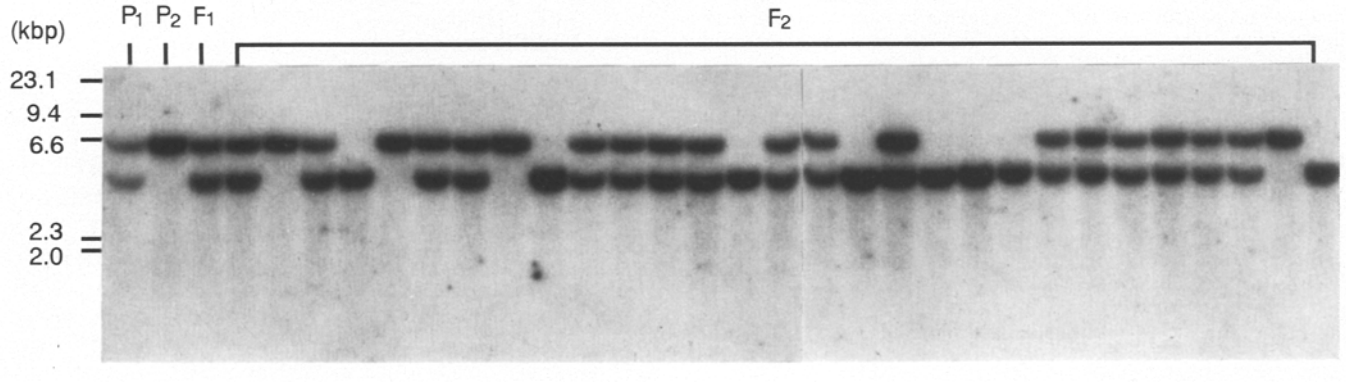
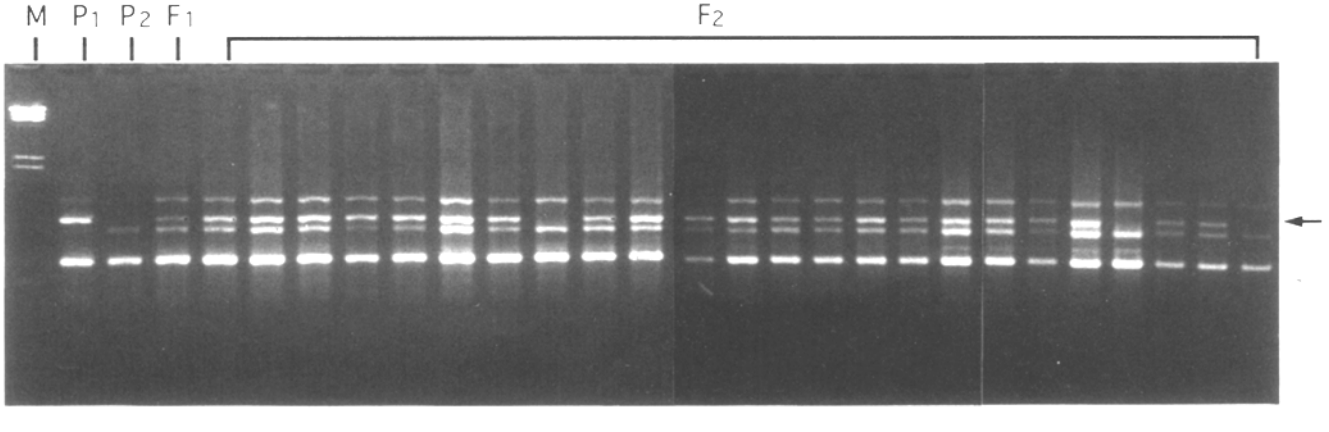


Fig. 3



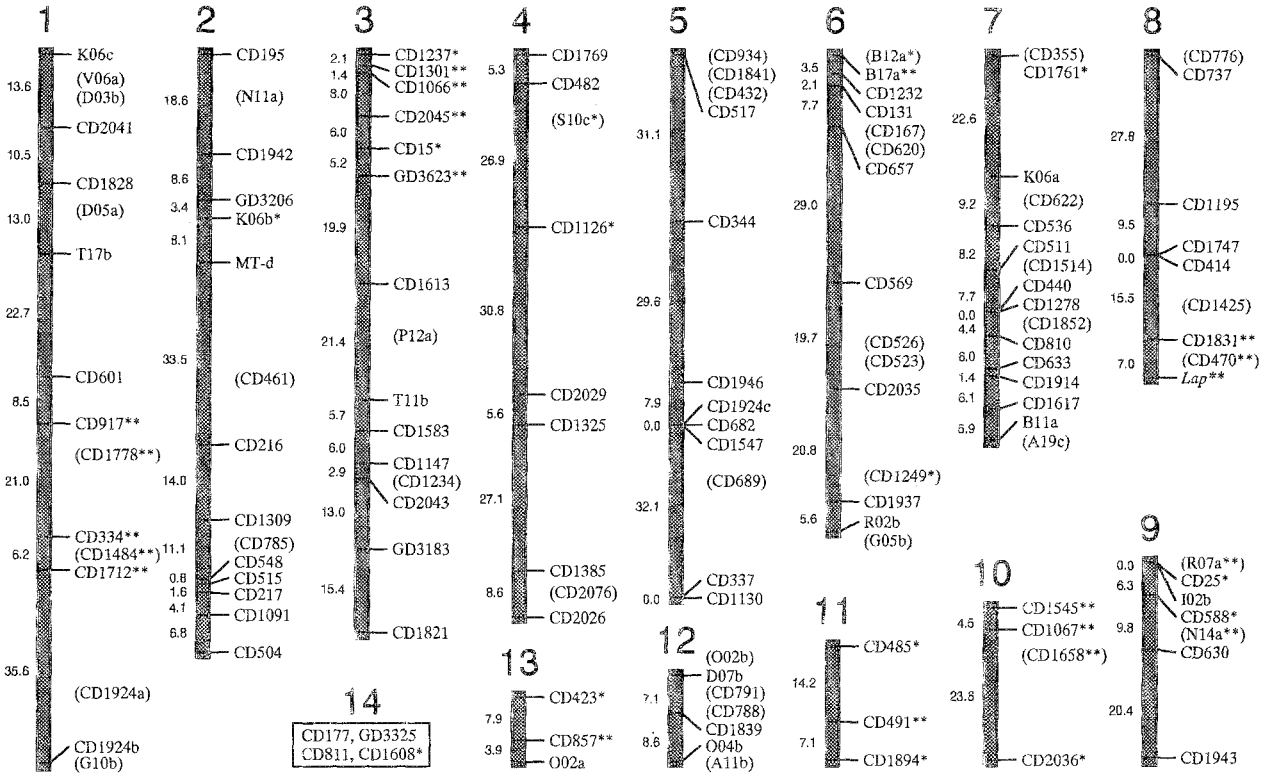


Fig. 4 A partial linkage map for the three-generation pedigree of sugi showing RFLP, RAPD, isozyme and morphological loci. A total of 145 linked loci (116 RFLPs, 26 RAPDs, 2 isozymes 1 morphological trait) were distributed into 20 linkage groups. Six linkage groups that consisted of only 2 markers were not included in the figure. The loci are listed on the *right* and map distances in cM are shown on the *left*. The prefixes *CD*, *GD*, and *single capital letters* indicate loci detected by cDNA and genomic DNA probes and by the RAPD technique, respectively. An isozyme locus is indicated by *three letters*. A morphological trait, dwarf, is shown as MT-d. An asterisk (*) and asterisks (**) designate loci with distorted segregation ($0.01 < P < 0.05$ and $P < 0.01$, respectively). The loci whose map positions were ambiguous are in *parentheses*. Linkage group 14 is enclosed in a *box* because the order of the loci in the linkage group was not confirmed. Ninety-one loci (77 RFLPs, 12 RAPDs, 1 isozyme and 1 morphological trait) with confirmed map positions (see text) were assigned to 13 linkage groups, covering a total of 887.3 cM

the light-harvesting chlorophyll *a*/*b*-binding protein (LHCP) of pine. Two cDNA clones showed homology to cDNA for pine (pPDLHC2176; Yamamoto et al. 1988) as determined by the Southern blot analysis of moderate stringency (not shown). One of the clones, CD1937, was mapped to linkage group 6. Because the dwarf phenotype segregated in the F_2 progeny, we also attempted to map the dwarf gene. Both of the parents were heterozygous for the same dwarf gene. It seems likely that the dwarf gene maps between CD216 and K06b in linkage group 2, assuming that the gene was derived from 'Okinoyama-sugi.'

Sugi has 11 pairs of metacentric or submetacentric chromosomes that show a gradual decrease in length (Toda 1979, Hizume 1988). Therefore, it was difficult to identify each chromosome, with the exception of 1 or 2 chromosome pairs with a secondary constriction. *In situ* hybridization with RFLP markers is assumed to enable the discrimination of each chromosome and the assignment of linkage groups to specific chromosome.

Thirtyfive markers with distorted segregation were mapped in 12 linkage groups. Therefore, map distances between the markers with distorted segregations may not be accurate. A distorted segregations of markers, as a result of linkage between some markers and putative "embryonic lethal gene(s)", has been reported in some conifers (Sorensen 1967; Ohba 1979; Kawasaki 1990; Ohba et al. 1992, 1993). The clustering of markers with distorted segregation has also been reported in some crop species (Heun et al. 1991; Bonierbale et al. 1988; Nodari et al. 1993; Kiss et al. 1993). We found that clustering of markers with distorted segregation in 6 linkage groups. The 5 markers in linkage group 1 (CD917, CD1778, CD334, CD1484 and CD1712) showed distortion with a reduced numbers from both parents. The 6 markers mapped to linkage group 3 (CD1237, CD1301, CD1066, CD2045, CD15 and GD3623) and the 3 markers in linkage group 8 (CD1831, CD470, and *Lap*) showed strong distortions with reduced numbers of homozygotes derived from the male parent. The 4 markers (CD25, CD588, N14a and R07a) in linkage group 9 were also associated with a reduced numbers of heterozygotes (Fig. 4, Table 1). All 4 markers in linkage group 10 (CD1545, CD1067, CD1658, and CD2036) and 3 markers in linkage group 11 (CD485, CD491, and CD1894) showed distortion with a reduced number of homozygotes from the female parent. It seems that the distorted segregation of the

Table 1 List of the clustered loci with distorted segregation

Linkage group no.	Locus	Observed segregation ratio[a]					χ^2	Probability
		aa	*a−*	*ab*	*b−*	*bb*		
1	CD917	0.127		0.704		0.169	12.10	0.002
	CD1778	0.197		0.676		0.127	9.51	0.002
	CD334	0.096		0.781		0.123	23.14	0.000
	CD1484	0.125		0.764		0.111	20.08	0.000
	CD1712	0.111		0.792		0.097	24.53	0.000
3	CD1237	0.151		0.644		0.205	6.48	0.039
	CD1301	0.123		0.685		0.192	10.67	0.005
	CD1066	0.110		0.685		0.205	11.33	0.003
	CD2045	0.104		0.672		0.224	9.81	0.007
	CD15	0.110		0.630		0.260	8.26	0.016
	GD3623	0.096		0.644		0.260	9.99	0.007
8	CD1831	0.083		0.667		0.250	12.00	0.002
	CD470	0.097			0.903		8.96	0.003
	Lap	0.090		0.642		0.269	9.69	0.008
9	R07a	0.425			0.575		11.88	0.001
	CD25	0.356		0.329		0.315	8.81	0.012
	CD588	0.380		0.338		0.282	8.83	0.012
	N14a	0.391			0.609		6.75	0.009
10	CD1545	0.389		0.556		0.056	16.89	0.000
	CD1067	0.397		0.534		0.068	16.12	0.000
	CD1658		0.931			0.069	12.52	0.000
	CD2036	0.261		0.638		0.101	8.74	0.013
11	CD485	0.268		0.620		0.113	7.48	0.024
	CD491	0.200		0.700		0.100	12.60	0.002
	CD1894	0.194		0.667		0.139	8.44	0.015

[a] Allele *a* is derived from 'Okinoyama-sugi' and allele *b* is derived from 'Kumotooshi'

markers was affected by the small size of the segreagating population and/or lethalities originating from mutant gene(s).

Although there is a discrepancy between the haploid chromosome number and the current number of linkage groups, the RFLP and RAPD markers described herein can be used immediately for the evaluation of genetic diversity in sugi populations and for the fingerprinting of breeding materials. Additional markers should be mapped to fill the gaps between linkage groups. In general, most of the practically important traits showed continuous variations, and these are assumed to be encoded by QTL. Because of the long rotation cycle of conifers, marker-assisted selection (MAS) of such quantitative traits may be an important application of linkage maps to conifer breeding programs (Neale and Williams 1991; Tulsieram et al. 1992). In a recent breeding program for sugi, features of flow- ering, such as male flower bearing and/or wood quality, have become important traits. Because the parents of the three-generation pedigree had variations in possible QTL that encoded heartwood color (Ohba et al. 1988) and features of flowering, such as male flower bearing (Tsurumi et al. 1987), it seems possible to identify such QTL.

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