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Detection of quantitative trait loci for juvenile growth, flower bearing and rooting ability based on a linkage map of sugi (*Cryptomeria japonica* D. Don)

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Abstract Quantitative traits, including juvenile growth, flower bearing and rooting ability, of a woody plant species, *Cryptomeria japonica* D. Don, were analyzed in a three-generation pedigree with 73 F_2 progenies using a linkage map with 85 genetic markers (72 RFLP, 11 RAPD, one isozyme and one morphological loci). A cluster of quantitative trait loci (QTLs) related to juvenile growth and female flower bearing was detected on linkage group 2. Some of the influence of this cluster could be attributed to pleiotropic effects of a dwarf locus located in its vicinity. QTLs related to male and female flower bearing were detected at different locations and showed different effects from each other, suggesting that the genetic systems controlling male and female flowering are different. No large QTL affecting rooting ability was detected in the material analyzed in this study.

Key words Linkage map · QTL · Juvenile growth · Flower bearing · *Cryptomeria japonica* D. Don

Abbreviations *QTL* Quantitative trait loci · *RAPD* random amplified polymorphic DNA · *RFLP* restriction fragment length polymorphism

Introduction

Sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), is one of the most important conifers in Japan, and 45% of all the cultivated forests of Japan are composed of this species. It is also one of the most extensively studied species of Japanese conifer. Sugi produces more total growth and bole growth than any other tree species in Japan, and is also easily regenerated by planting seedlings or stecklings (rooted cuttings). There are now more than 200 vegetative (clonal) cultivars (i.e. groups of trees propagated by cuttings with apparently similar phenotypes) growing in southwestern Japan (Ohba 1993).

Mukai et al. (1995) recently constructed a linkage map for sugi based on RFLP, RAPD, isozyme and morphological loci, using a three-generation pedigree derived from two clonal cultivars as parents. Ninety-one genetic markers with confirmed map positions were assigned to 13 linkage groups ($n = 11$), covering a total of 887.3 cM. Suyama et al. (1996) assigned 3 of these linkage groups to three different chromosomes using the relationship between trisomics and gene dosages based on autoradiographs of Southern blots. Furthermore, Tsumura et al. (1997) have generated 66 sequenced-tagged-site (STS) markers from the cDNA clones which Mukai et al. (1995) produced. These

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developments in sugi genetic research have made the analysis of quantitative traits of the species possible.

QTL mapping studies on several important forest tree species in recent years have strongly suggested that a few major QTLs control a large proportion of total genetic variation (Groover et al. 1995; Bradshaw and Stettler 1995; Grattapaglia et al. 1995; Grattapaglia et al. 1996; Plomion et al. 1996; Byrne et al 1997). The detection of major QTLs based on genetic linkage maps of molecular markers would offer a fine view of genetic architecture of quantitative characters and provide potential tools for effective breeding through marker-assisted selection. This report describes QTLs detected in a three-generation pedigree of sugi based on a linkage map published by Mukai et al (1995).

Materials and methods

Plant materials

The segregating population of 73 individuals was a self-pollinated array of progeny of an F_1 hybrid from a cross between 'Kumotooshi' (female parent) and 'Okinoyama-sugi' (male parent), which are local cultivars propagated by cuttings (Fig. 1). This three-generation pedigree was established by Ohba et al. (1988) as part of an effort to clarify heritable traits of heartwood color (reddish in 'Okinoyama-sugi' and blackish in 'Kumotooshi'). 'Kumotooshi' develops rather earlier than 'Okinoyama-sugi', and both maintain a good rooting ability after many repetitions of propagation by cutting. 'Kumotooshi' was found to have a recessive dwarf gene, and 'Okinoyama-sugi' was also found to be heterozygous for the same dwarf gene. The F_1 plant used in this linkage analysis was also a heterozygote for this gene, derived from 'Okinoyama-sugi' (Mukai et al. 1995). The parents had different genotypes for some isozyme loci and possible QTLs for growth, flower bearing (Tsurumi et al. 1987), rooting ability and color of heartwood.

The F_2 generation seeds were sown at an indoor nursery bed in the spring season of 1982 and were transplanted to an outdoor nursery field in a randomized block design the following spring. In the spring of 1984, 90 individuals were again transplanted to another outside field, spaced 0.75 m apart and divided into six blocks. The field was almost flat, and the six blocks were located very close to each other. Of the 90 individuals transplanted, 73 survived until 1991, when DNA was extracted from young needle buds for constructing a genetic linkage map (Mukai et al. 1995). Some quantitative characters had been measured in 1986 and/or 1987, and others were checked in 1995 and/or 1996. Details of the quantitative characters assessed will be described in the following sections.

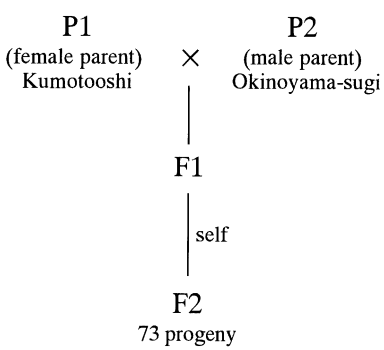

Fig. 1 The three-generation pedigree of sugi (*Cryptomeria japonica*) analyzed in this study

Genetic linkage analysis

The methods and results of the genetic linkage analysis were described in detail by Mukai et al. (1995). The segregation of 128 RFLP (123 cDNA and five genomic probes), 33 RAPD, two isozyme and one morphological (dwarf) locus in 73 F_2 progeny was analyzed with the MAPMAKER/EXP 3.0 computer program (Lander et al. 1987; Lincoln et al. 1992b). To identify the linkage groups, we assessed pairwise comparisons and groupings of markers under the condition that (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. 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. Mukai et al. (1995) concluded there were 13 linkage groups, assigning map positions to 91 genetic markers (77 RFLPs, 12 RAPDs, one isozyme and one morphological locus) covering 887.3 cM. In some cases, where several loci were located at the same map position, representative loci were chosen for the present QTL analysis. In all, 13 linkage groups with 85 genetic markers including 72 RFLPs, 11 RAPDs, one isozyme and one morphological trait were utilized. The average interval separating these markers is 12.5 cM.

Quantitative characters

In the summer of 1986, when the F_2 individuals were 4 years old, all the leaves were sprayed with 100 ppm gibberellin to promote flower-bud differentiation. Four months after the spraying, male and female flower bearing (denoted MF4 and FF4, respectively) was evaluated on a five-grade scale; grade 1 representing sparse flowering and grade 5 representing abundant flowering (Tsurumi et al. 1987).

Tree height in centimeters and diameter at stump height in millimeters were measured in the autumn of 1986 and 1987 when the F_2 individuals were 4 and 5 years old, respectively; the traits being designated H4 and H5 for height, D4 and D5 for diameter. Height and diameter at breast height were also measured at 14 years of age in 1996, being denoted as H14 and D14, respectively.

The rooting ability was evaluated by taking cuttings at 13 years of age. In the spring of 1995, 20 scions from each F_2 individual were cut and planted in an indoor nursery bed divided into two blocks (Sakamaki 1995). The nursery bed was shaded with black mesh cloth, and the plants were watered with an automatic mist spraying system during the rooting experiment. Seven months later, the rate of rooting (RR13), that is the frequency of scions which successfully rooted, and the average number of roots per rooted plant (NR13) were measured. Rooting was regarded as being successful if there was more than one root longer than 1 cm. RR13 and NR13 were averaged over two blocks. According to variance analysis, there were significant differences for both traits amongst F_2 individuals at the 1% probability level.

QTL analysis

The 13 linkage groups were scanned for the presence of a QTL effect at 2.0 cM intervals between every marker pair with the MAPMAKER/QTL 1.1 program as a free model of QTL effects (Paterson et al. 1988; Lincoln et al. 1992a). Given the scanned length (887 cM) and the average spacing between mapped markers (12.5 cM), a LOD

threshold of 2.4 was chosen following consultation of the relevant data in Fig. 4 of Lander and Botstein (1989) in order to reduce to 5% the probability of falsely assigning a QTL. The LOD score peak was used to estimate the most likely QTL position on the linkage map. For each LOD peak, we determined the LOD 1.0 support interval (that is, the region in which the LOD score remains within 1.0 unit of the peak). The percentage of phenotypic variation explained by an individual QTL, and the additive and dominance effects, were estimated at the most likely QTL position. When linked QTLs with no overlapping 1.0 LOD support intervals were detected, the locus with the highest LOD score was fixed and the chromosome scanned again for the linked effect.

The other simple method used to detect QTL effects on the linkage map was a single-factor analysis of variance for the F_2 population. Significant differences in quantitative trait means among genotypic classes of each genetic marker were determined using the F test with $P < 0.01$.

Results

Features of samples and quantitative traits

The sample size for the basic linkage analysis was 73 individuals. Although quantitative characters (H4, H5, D4, D5, MF4 and FF4) of all 90 individuals were measured at 4–5 years, only the data applying to the 73 individuals that survived until 1991 were used for QTL analysis. However, the sample size for analysis of the 13- to 14- year-old plants (H14, D14, RR13, NR13) was only 60 individuals because 13 specimens died after the basic linkage analysis. Fifteen dwarf individuals were included among the 73 individuals used to supply data for the quantitative traits analysis of the 4- to 5- year-old trees, but only 4 dwarfs survived up to 13–14 years of age. The dwarf gene did not cause significant segregation distortion in the early data. The drastic reduction of dwarfs at the later age, however, might have affected the segregation of QTLs located near the dwarf locus.

Normal distribution frequencies were observed for the population of F_2 individuals with respect to H4, H5, D4, D5, MF4, RR13 and NR13. However, the distribution of FF4 represented significantly negative kurtosis, and those of H14 and D14 showed significant positive skewing. Since the QTL analysis by MAPMAKER/QTL requires the assumption of normal distribution in the phenotypic value of quantitative traits, the parameters estimated for FF4, H14 and D14 might be affected by the skewed distribution to some extent.

QTL analysis

Significant peak values of LOD scores, the position of these peaks, the percentage of phenotypic variance explained and the estimated gene actions based on the analysis of MAPMAKER/QTL are shown in Table 1. Significant ($P < 0.01$) associations between quantitative traits and genetic markers segregating in the F_2 population detected with the one-way ANOVA are shown in Table 2. Only definitely significant QTLs detected by both of these different kinds of analysis (Tables 1 and 2) are presented on the linkage map with the 1.0 LOD support intervals shown in Fig. 2. Asterisks not in parentheses in this figure indicate loci found to have distorted segregation at the time of construction of the linkage map, asterisks in parentheses mark such loci detected at the age of 13–14 years.

Table 1 Significant QTLs evaluated with MAPMAKER/QTL

Trait	Linkage Group[a]	Interval	Length (cM)	QTL position (cM)[b]	LOD	% Var	Genetic effects[c]	
							Additive	Dominance
H4[d]	2	MT-d-CD216	33.5	2.8	8.1	48.8	29.4	55.0
H5[d]	2	K06b-MT-d	8.1	7.4	8.6	44.8	38.4	64.8
H14[d]	3	GD3183-CD1821	15.4	15.2	2.6	18.6	− 28.8	− 116.2
D4[e]	2	MT-d-CD216	33.5	8.2	3.7	40.7	2.4	13.1
	10	CD1067-CD2036	23.6	12.6	2.6	24.7	1.5	9.3
D5[e]	2	MT-d-CD216	33.5	3.6	6.3	45.4	6.2	20.2
	10	CD1067-CD2036	23.6	12.5	2.4	23.6	1.6	14.3
MF4[f]	2	CD217-CD1091	4.1	0.0	10.6	49.4	1.07	− 0.21
FF4[f]	2	MT-d-CD216	33.5	10.6	9.1	81.2	0.29	2.88
	2	CD217-CD1091	4.1	2.9	7.9	40.3	− 1.01	1.34
	5	CD1946-CD682	8.0	5.0	3.9	25.1	− 1.19	− 0.46
NR13[g]	5	CD344-CD1946	29.5	27.1	2.4	19.5	0.03	− 1.49

[a] Linkage groups were named by Mukai et al. (1995)
[b] Most likely locus position, corresponding to LOD score peak, which represents the distance from the left marker of the interval
[c] Positive values mean that the allele inherited from 'Kumotooshi' (the female parent) had a more positive effect than the allele from 'Okinoyama-sugi' (the male parent)
[d] Height in cm at 4 (H4), 5 (H5) and 14 (H14) years of age
[e] Diameter at stump height in millimeters at 4 (D4) and 5 (D5) years of age
[f] Male fertility (MF4) and female fertility (FF4); flower abundance on a five-grade scale following gibberellin treatment at 4 years of age
[g] Average number of roots per cutting which successfully rooted after cutting was taken from 13-year-old trees

Table 2 Significant associations between independent marker loci and quantitative traits evaluated by single-factor ANOVA

Trait	Linkage group	Marker locus	*P*	Genotype mean[a] ± SE		
				A/A[b]	A/B[b]	B/B[b]
H4	2	CD195	0.005	105.1 ± 9.2	150.6 ± 7.2	146.5 ± 9.4
	2	CD1942	0.009	105.7 ± 9.8	150.4 ± 7.5	145.2 ± 8.8
	2	GD3206	< 0.001	91.6 ± 7.2	161.3 ± 6.3	140.9 ± 9.0
	2	MT-d	< 0.001	85.4 ± 6.8	154.1 ± 5.1[c]	
H5	2	CD195	0.003	128.4 ± 11.7	190.6 ± 9.5	176.4 ± 11.6
	2	CD1942	0.002	124.3 ± 11.8	190.2 ± 9.9	178.3 ± 10.2
	2	GD3206	< 0.001	110.0 ± 9.2	200.8 ± 8.3	178.1 ± 10.9
	2	MT-d	< 0.001	102.7 ± 8.1	192.8 ± 6.4[c]	
H14	3	CD1821	0.003	431.3 ± 56.9	286.7 ± 17.5	373.5 ± 29.6
D4	2	MT-d	0.001	25.8 ± 1.9	35.1 ± 1.3[c]	
D5	2	MT-d	< 0.001	31.8 ± 2.1	51.0 ± 1.9[c]	
MF4	2	CD216	< 0.001	2.40 ± 0.32	3.53 ± 0.18	3.89 ± 0.17
	2	CD1309	< 0.001	2.45 ± 0.25	3.48 ± 0.17	4.17 ± 0.17
	2	CD548	< 0.001	2.45 ± 0.24	3.33 ± 0.15	4.46 ± 0.14
	2	CD515	< 0.001	2.53 ± 0.23	3.31 ± 0.15	4.60 ± 0.11
	2	CD217	< 0.001	2.48 ± 0.22	3.34 ± 0.15	4.63 ± 0.11
	2	CD1091	< 0.001	2.44 ± 0.26	3.29 ± 0.14	4.48 ± 0.14
	2	CD504	< 0.001	2.43 ± 0.24	3.51 ± 0.14	4.47 ± 0.15
	11	CD491	0.009	2.78 ± 0.26	3.55 ± 0.16	4.29 ± 0.18
FF4	2	CD1942	0.004	1.42 ± 0.29	3.08 ± 0.27	3.00 ± 0.30
	2	GD3206	< 0.001	1.20 ± 0.20	3.33 ± 0.27	2.92 ± 0.29
	2	MT-d	< 0.001	1.40 ± 0.29	3.10 ± 0.20[c]	
	2	CD548	< 0.001	3.25 ± 0.38	3.36 ± 0.24	1.46 ± 0.23
	2	CD515	< 0.001	3.37 ± 0.38	3.34 ± 0.23	1.20 ± 0.14
	2	CD217	< 0.001	3.14 ± 0.38	3.40 ± 0.22	1.11 ± 0.11
	2	CD1091	< 0.001	3.22 ± 0.41	3.47 ± 0.22	1.19 ± 0.13
	2	CD504	< 0.001	3.43 ± 0.37	3.06 ± 0.24	1.29 ± 0.21
	5	CD1946	0.001	4.00 ± 0.39	2.68 ± 0.23	1.86 ± 0.39
	5	CD682	< 0.001	4.00 ± 0.33	2.60 ± 0.24	1.87 ± 0.34
NR13	5	CD1946	0.006	5.74 ± 0.56	4.39 ± 0.21	5.82 ± 0.61

[a] Means of quantitative traits for each genotype of markers
[b] Allele A is derived from 'Okinoyama-sugi' (the male parent) and allele B from 'Kumotooshi' (the female parent)
[c] MT-d is a dominant/recessive locus and the allele from 'Okinoyama-sugi' is recessive

Since H4 and H5 values were measurements of the same trait in 2 sequential years, they would be expected to show very similar segregation patterns, as would D4 and D5 values. These expectations were confirmed by the results, and QTLs with large effects on H4, H5, D4 and D5 were detected near MT-d (a dwarf locus) on the second linkage group. The LOD scores in Table 1 are very large, and many genetic markers (CD195, CD1942, GD3206 and MT-d) showed significant association with H4 and H5; as did MT-d with H4, H5, D4 and D5 (Table 2). The alleles derived from 'Kumotooshi' increased these tree heights and diameters in the heterozygous and/or homozygous condition in comparison with the alleles from 'Okinoyama-sugi' (Table 2). A weak QTL on the tenth linkage group is suggested for D4 and D5 by the data in Table 1, but the single factor ANOVA shown in Table 2 did not support this hypothesis.

A QTL significantly affecting H14 was detected near CD1821 on the third linkage group, and the allele from 'Kumotooshi' decreased the H14 value in either the heterozygous or homogygous condition.

A QTL for MF4 was detected near CD217 on the second linkage group. Genetic markers, CD216, CD1309, CD548, CD515, CD217, CD1091 and CD504 showed significant association with this male-fertility measure. Genotypes with the allele from 'Kumotooshi' showed higher male fertility. Another association shown in Table 2, with the 11th linkage group, seems not to be so large.

QTLs for FF4 were detected on three sites, one near MT-d and another near CD217 on the second linkage group, and one near CD682 on the fifth linkage group. The allele from 'Kumotooshi' near MT-d increases female fertility. In contrast, the alleles from 'Kumotooshi' at the other two sites decrease it.

A QTL for NR13 (rooting ability) was detected near CD1946 on the fifth linkage group. At this site the allele from 'Kumotooshi' has almost same effect as the one from 'Okinoyama-sugi'.

No QTL was suggested for D14 or RR13 at all.

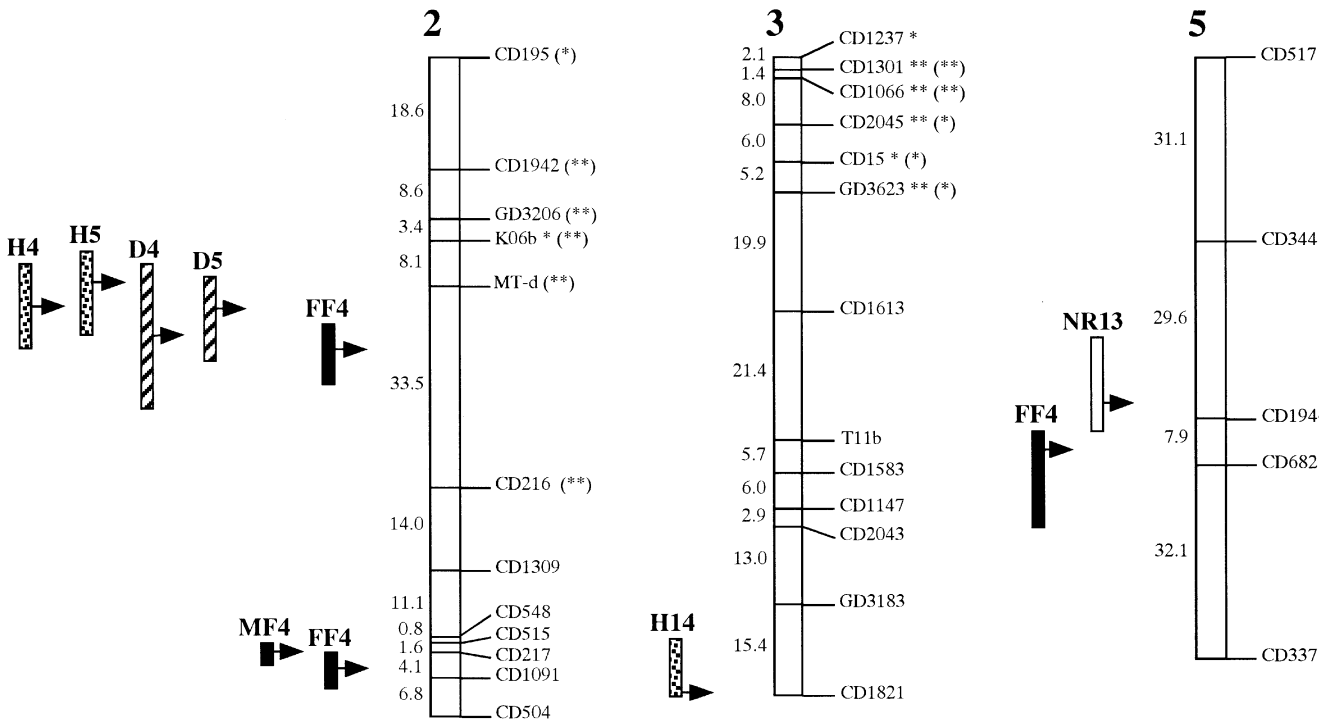

Fig. 2 Linkage map and estimated locations of significant QTLs. *Bars* to the *left* of the linkage groups correspond to the 1.0 LOD support intervals for the QTL locations. *Arrows* emitting from *bars* indicate the most likely QTL positions estimated using MAPMAKER/QTL. Only those QTLs which are supported by both the MAPMAKER/QTL analysis (Table 1) and the single factor ANOVA (Table 2) are shown in this figure. *Asterisks* indicate loci found to have distorted segregation at the construction of the linkage map, and those in *parentheses* [(*) and (**)] were found when the trees were 13–14 years old (*$0.01 < P < 0.05$, **$P < 0.01$). The prefixes *CD*, *GD*, and *single capital letters* indicate loci detected by cDNA and genomic DNA probes and by the RAPD technique, respectively. A morphological trait, dwarf, is shown as MT-d

Discussion

QTLs for many early growth characters (H4, H5, D4 and D5) and female flower bearing (FF4) were detected near MT-d on linkage group 2. The genetic effects of the alleles from 'Kumotooshi' were all positive for these traits (Tables 1 and 2). MT-d is a dwarf locus, and the recessive allele for the dwarf phenotype is derived from 'Okinoyama-sugi' (Mukai et al. 1995). It might be considered that the QTLs for growth near this locus are pleiotropic effects of the dwarf locus. The QTL near MT-d affecting female flower bearing might have some genetic relationship with those affecting growth. The most important aspect of QTL analysis with respect to breeding procedure is to detect major, previously unknown genes that have large effects on important quantitative traits. Since the dwarf locus is already known to be a major growth-controlling factor, the detection of its influence in the QTL analysis can not be regarded as a significant pragmatic advance. Other QTLs controlling growth must be detected in future studies on sugi in order to discover useful factors for marker-assisted selection in breeding.

The presence of QTLs affecting both male and female flower bearing (MF4 and FF4) was suggested near CD217 on linkage group 2, affecting the two traits in opposing ways. That is, the allele from 'Kumotooshi' increases male fertility but decreases female fertility. This phenomenon could be explained either by the presence of one locus affecting both traits, or of two different, closely linked loci (both affecting a different trait). One other QTL affecting female flower bearing was detected near CD682 on linkage group 5. Thus, male and female flower bearing are controlled by many different QTLs, which suggests that their genetic control systems are completely different. Recently, a severe allergy to sugi pollen, Japanese cedar pollinosis, has become a big social problem in Japan, and many countermeasures are required. Flower bearing is essential for plant breeding, but the QTLs with different effects on male and female flower bearing detected in this study might have potential in helping to design breeding programs to counter Japanese cedar pollinosis.

The traits measured in 13- to 14-years-old trees did not show highly significant QTLs. One of the reasons might be the relatively small number (60) of individuals that had survived to this age. Even the initial population, of 73 individuals, assessed in this study was rather small for fine linkage analysis.

The selfing of the F_1 generation used in the three-generation pedigree investigated caused segregation

distortion at many sites on linkage groups, which lowered the efficiency of detection of QTLs. The basic linkage map used in this study is partial and thought to cover about half of the total sugi genome (Mukai et al. 1995). Well-designed experimental materials with larger sample sizes (and great effort) are required to complete a genetic linkage map covering the full sugi genome. We are now preparing two-generation pedigrees with large numbers of individuals in order to carry out a fine linkage analysis using the pseudo-testcross of Grattapaglia et al. (1995, 1996). Furthermore, quantitative characters themselves vary in different environments. A complete analysis, therefore, demands that the materials be planted in blocks with different environments. Generally speaking, it takes a long time to prepare ideal materials for QTL analysis of traits that become apparent in the mature phases of long-lived woody plants. Thus, efforts should also be made to find useful materials among existing pedigrees established for other purposes before the development of modern linkage analysis. The detection of genetic markers adjacent to QTLs gives an important means for improving breeding programs through marker-assisted selection. The data presented here represents a first step towards this goal.

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