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AFLP and CAPS linkage maps of *Cryptomeria japonica*

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Abstract We have used two DNA marker systems, AFLP and CAPS, in a two-way pseudo-testcross strategy applied to an F_1 population to construct genetic linkage maps of two local sugi cultivars. The AFLP markers detected about eight polymorphisms per parent per primer combination. Using 38 primer combinations, 612 AFLPs were detected in 'Haara 4' and 'Kumotooshi', of which 305 segregated in a 1:1 ratio (P>0.05). A total of 91 markers (83 AFLP and 8 CAPS) in 'Haara 4' and 132 (123 AFLP and 9 CAPS) in 'Kumotooshi' were distributed among 19 and 23 linkage groups, respectively, each of which included 2–17 markers. Maps of 'Haara 4' and 'Kumotooshi' spanned 1266.1 cM and 1992.3 cM, and covered approximately 50% and 80% of the sugi genome, respectively. Sequences derived from cDNA, which were previously used to construct a sugi linkage map, were also placed on our linkage maps as CAPS markers. Where a 'two-way pseudo-testcross' is used, more than half of the sugi CAPS developed can be used to construct linkage maps for each parental family. The saturation of mapped markers, and the integration of several linkage maps derived from different mapping populations, is anticipated in the near future.

Key words Co-dominant marker · Dominant marker · Linkage map · Sugi

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Introduction

Sugi (*Cryptomeria japonica*) is one of the most important conifers in Japanese forestry. An active, nationwide forest-tree breeding program was started in about 1956, with sugi plus tree selection. At the same time, breeding projects for improving the species' tolerance of climatological stress, and resistance to pathogens such as sugi bark borer and sugi bark midge, were initiated (Ohba 1993). Sugi is also distinguished by the intense interest it has stimulated in many genetic researchers (Ohba 1980).

A current requirement to accelerate progress in sugi basic research and breeding programs is to construct genetic linkage maps since, for example, marker-assisted selection (MAS) is one of the most cost-effective, rapid and practical breeding strategies. Mukai et al. (1995) have developed a linkage map based on RFLP, RAPD, isozyme and morphological loci, using a three-generation pedigree derived from two clonal sugi cultivars as parents, but the map is insufficiently complete for molecular breeding.

AFLP (amplified fragment length polymorphism) analysis is fundamentally a dominant marker system based on PCR (Vos et al. 1995). AFLPs have several advantages compared with other marker techniques, including very high sensitivity to polymorphisms. In a recent study, AFLPs were also reported to give high reproducibility and low genotyping error frequencies (Hansen et al. 1999). AFLP analysis is also less time-consuming than alternative strategies because large numbers of loci can be assessed in a single assay. In addition, theory suggests that the entire genome (including both coding and non-coding regions) could be covered using AFLP markers because of the random amplification involved. With these advantages, the AFLP system should be highly appropriate not only for genetic fingerprinting, but also for the construction of linkage maps. AFLP has already been used for constructing linkage maps of various tree species such as pines (Travis et al. 1998, Remington et al. 1999), *Eucalyptus* (Marques et al. 1998) as well as other plant species.

Cleaved amplified polymorphic sequences (CAPS) can be used as PCR-based co-dominant markers (Konieczny and Ausubel 1993). Compared to RFLPs, another (frequently used) type of co-dominant marker, they are easy to handle and only small amounts of DNA are required. Where CAPS markers are developed from STS (sequenced tagged site) primers designed from cDNA, the regions amplified contain coding regions of genes. Moreover, mapping common CAPS markers is helpful for comparing and integrating different genetic linkage maps of a genome.

Here we report a first attempt to apply two marker systems, AFLP and CAPS, for constructing genetic linkage maps of sugi.

Materials and methods

Plant material

Three local cultivars of sugi, 'Haara', 'Haara 4' and 'Kumotooshi', were used for generating mapping populations. Sixty two progeny were generated from the cross 'Haara 4' (female parent)× 'Kumotooshi' (male parent), and 25 from the cross 'Kumotooshi' (female parent)×'Haara' (male parent). In total, 87 F_1 hybrids were obtained and maintained in a nursery field. All 87 F_1 hybrids were used to construct a linkage map of 'Kumotooshi' and 62, derived from the cross between 'Haara 4' and 'Kumotooshi', were used to map 'Haara 4'. DNA of the F_1 hybrids and parents was extracted from young needle buds in spring according to the modified method of Murray and Thompson (1980).

AFLP analysis

The AFLP method established by Vos et al. (1995) was slightly modified for analysing plants with large genomes. Template preparation was performed according to the protocol provided with AFLP Core Reagent Kits from Life Technologies. Total genomic DNA (500 ng) was digested with 2.5 U of *Eco*RI and 2.5 U of *Mse*I for 2 h at 37°C. Digested DNA fragments and *Eco*RI and *Mse*I adapters were ligated with T4 DNA ligase for 2 h at 20°C. The resulting DNA was used as primary template DNA in the AFLP analysis. A primer pair based on the sequences of the *Eco*RI and *Mse*I adapters with two additional selective nucleotides at the 3´ end (*Eco*RI+AC and *Mse*I+CC or *Eco*RI+AA and *Mse*I+CC combinations) was used for the first PCR step (pre-amplification). The second PCR step (selective amplification) was performed with primer pairs having three additional selective nucleotides at the *Eco*RI adapter side (E+3 primers) and four or five selective nucleotides at the *Mse*I adapter side (M+4 and M+5 primers). *Eco*RI primers for selective amplification were fluorescently labelled (Perkin-Elmer, USA). The AFLP products were amplified in a thermocycler (GeneAmp PCR Systems 9600, Perkin-Elmer). One-microliter portions of the AFLP product mixtures were denatured in formamide at 94°C for 2 min and electrophoretically separated in POP4 polymer (Perkin-Elmer) for 25 min using an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). In all, 56 primer combinations were screened for AFLP, individual hybrids being scored for the presence or absence of given fragments. The size of detected fragments was determined by the GENESCAN program using an internal standard (GS 500 ROX, Perkin-Elmer). Fragments ranging from 50 to 500 bases in size were counted and further analyzed. Polymorphic fragments that were present in one parent but absent in the other were considered to be AFLP markers. If the genotype of the markers is heterozygous in one parent and null in the other, its expected segregation ratio will be 1:1, as in a testcross.

Genotyping of CAPS markers generated by STSs of cDNA clones

A cDNA library from 3-day imbibed embryos was prepared by Mukai et al. (1995) and some of the fragments were sequenced according to Tsumura et al. (1997). Another cDNA library was prepared from inner bark. The nucleotide sequences at both ends of the cDNA inserts were determined. PCR primer pairs were designed using the OLIGO program ver. 4.0 (National Biosciences Inc.), and PCR was performed in 25-µl mixtures containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of dNTPs, 2.0 µM of the forward and reverse primers, 5 ng of DNA and 1.0 unit of *Taq* polymerase. PCR amplification was carried out in a thermal cycler (GeneAmp PCR Systems 9600, Perkin-Elmer) programmed for 5 min preheating at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 55–65°C and 1 min 30 s at 72°C for the denaturation, annealing and extension steps, respectively. There was a final incubation for 5 min at 72°C. The PCR products were analyzed on 2% agarose gels stained with ethidium bromide to check that single fragments had been amplified. Amplified fragments were digested with 32 restriction enzymes to detect CAPS and the products were resolved by electrophoresis on 2% agarose gels.

Linkage analysis

Segregation data from the AFLP and CAPS marker analyses of the F_1 progeny were evaluated by chi-square tests for goodness-of-fit to a 1:1 ratio (P>0.05). To identify linkage groups, pairwise comparisons and grouping of markers was carried out using the MAPMAKER var. 3.0 program (Lander et al. 1987) (LOD score >3.0, maximum distance=40 cM). The arrangement of markers in the linkage groups was determined using the 'order' and 'try' commands.

Results

Fifty six primer combinations were screened by selective amplification using parental template DNA sequences in AFLP analysis. Of these, 38 (30 E+3/M+4 and 8 E+3/M+5 pairings) gave reliable and reproducible polymorphisms. The number of AFLP markers obtained in 'Haara 4' and 'Kumotooshi' were similar, 309 and 303, respectively (Table 1). Three to nineteen polymorphisms were detected per E+3/M+4 combination, while E+3/M+5 combinations provided three to twelve polymorphisms. A total of 612 polymorphisms were generated using 38 primer pairings, i.e. about 16 polymorphisms per primer combination. However, not all AFLPs were used for linkage analysis. Segregation analysis of the F_1 population showed there were 155 AFLPs segregat-ing in a 1:1 (P>0.05) ratio in 'Haara 4' and 150 in 'Kumotooshi'. Although some unusable markers showed no segregation because they were homozygous in one parent and null in the other, the segregation of 50.1% of markers in 'Haara 4' and 49.5% of markers in 'Kumotooshi' was severely distorted from the expected ratio.

Sugi cDNA clones from imbibed embryos were given designations with the prefix CD (Mukai et al. 1995; Tsumura et al. 1997), and those from inner bark were given designations with the prefix CC. The PCR-amplified products were digested with 32 restriction enzymes and the enzyme that gave the clearest CAPS pattern for genotyping was chosen for segregation analysis

Table 1 Numbers of AFLP and CAPS markers obtained. Polymorphisms were detected using 30 E+3/M+4 and eight E+3/M+5 combinations. The number of linked markers was calculated using MAPMAKER software (ver. 3.0)

Cultivar	Marker (type of primer)	No. of polymorphisms (per primer)	No. of markers segregating 1:1	No. of linked markers
Haara 4	AFLP (E+3/M+4)	267 (4–19)	134	77
	(E+3/M+5)	42 (3–10)	21	6
	CAPS	10	9	8
Kumotooshi	AFLP (E+3/M+4)	252 (3–18)	125	103
	(E+3/M+5)	51 (3–12)	25	20
	CAPS	12	11	9

Table 2 CAPS markers developed by PCR using STS primers and restriction digestions. * Tsumura et al. 1997

CAPS markers	STS primer sequence (forward and reverse)	Annealing temp.	Restriction enzyme[a]	Linkage map
CD4	5' AGG AAG AAA ATG CTC CAG AC 3' 5' CAT CTT GCA CCC CTT ATT TA 3'	60	*Hae*III	Haara 4
CD470*	5' GGC CTA CGA ACA TTG AAC AT 3' 5' ACC CTT ACC GGA AAG ACC AT 3'	60	*Sal*I	Kumotooshi
CD657*	5' TCC TGA TAC TGT GGG CAA CT 3' 5' CCC CGA TAT GCT CTT CAA CT 3'	60	*Msp*I	Kumotooshi
CD1249	5' AAC TCA GTA GCC CCT GGT TA 3' 5' GCC GCA AAC TTG AAA GAG AT 3'	60	*Sty*I	Kumotooshi
CD1761*	5' AGT CAA CTC AAT GCC CTC AA 3' 5' TAA ACA GGT CGC TCC CAG AT 3'	58	*Vsp*I	Haara 4
CD1894*	5' ACC CTT TCC TCG CCT ACA TT 3' 5' GCC GAC TGA GTA AAC AAA CC 3'	62	*Rsa*I	Haara 4
CC0279	5' CCA AAG ATT AAG AGG GTT GA 3' 5' ACA CAT ACG CAG ATA CAT AGC 3'	55	*Mbo*II	Haara 4
CC0303	5' AGA AGA GAA ACC CTG AGA AA 3' 5' CCC ATC GAA GTA TAA ACC A 3'	60	*Hin*fI	Kumotooshi
CC0381	5' TGG CAT CAA AGA AAC GGT TAG 3' 5' GGG TTC GAA TGG CAT AAG AAG 3'	62	*Scr*FI	Haara 4
CC0472	5' AGT GTC ATC CGA GCT TCC T 3' 5' TCA ACC GGA TGT ATA TTT CGT 3'	55	*Msp*I	Kumotooshi
CC0498	5' GGC CGA ATC GTG ATG GA 3' 5' GGG CAA CTT AGA AGG GCA TTA 3'	55	*Hin*fI	Haara 4
CC0514	5' GAC CAG CGA TGA GGA AGA A 3' 5' GGA GAG ATC GAC CCG AAA TAC 3'	60	*Ssp*I	Haara 4
CC0576	5' AAG CCA ATT TCT TCA TTT C 3' 5' TTA CCA CCC AAG TAT GTA GAC 3'	55	*Scr*FI	Haara 4
CC0613	5' GGA ACC AAC ACC GCT GCT CTG 3' 5' GAG GGT GCC AAT GTC GGA ATC 3'	55	*Hin*fI	Haara 4
CC0616	5' CTT TGG TTG TAG TGG GCA TTC 3' 5' GAA GAC GCG CAT TTG AGA A 3'	60	*Hha*I	Kumotooshi
CC0673	5' CCG GCA GAA CCT TCC AAA 3' 5' AAG ACC ATG GCC TCC TAT CGT 3'	55	*Nsp* V	Haara 4
CC0718	5' ATA TCC ATG TCT TCC GTT CAG 3' 5' GCG GAA AGA TGG GAG CAC TTG 3'	55	*Hae*III	Kumotooshi
CC0737	5' TCT CTA ATG TGG CAG CTC TTT 3' 5' TGG AAC TTA TGC TAC ATC CCT 3'	55	*Eco*T38I	Kumotooshi
CC0958	5' CTC AAT GGC CGC TAT TTC ACT 3' 5' GAA GCC AAA CGA GAA GCA ATG 3'	60	*Alu*I	Kumotooshi
CC0990	5' AAG TAA CCG GTT CAC ATC CAT 3' 5' ATG CCA GGC TGA TTT CTA CAA 3'	60	*Rsa*I	Kumotooshi
CC1112	5' ATT CAG AGT CCA GCT ATC AA 3' 5' TCC CAA GGA TTT GAG GTT CAC 3'	55	*Bgl*II	Kumotooshi
CC1125	5' GTT TCA GAT TTC ACC CGA AGC 3' 5' AGC ACA ACA TCC CAA GCA AT 3'	55	*Dra*I	Kumotooshi

[a] Restriction enzyme which detected polymorphisms between 'Haara 4' and 'Kumotooshi'

(Table 2). Parental CAPS markers giving patterns of a backcross type, i.e. heterozygous in one parent and homozygous in the other, were selected to analyze linkage simultaneously with AFLPs. Such markers would be expected to segregate in a 1:1 ratio in F_1 populations, like the AFLPs described above. A total of 22 CAPSs were analyzed, ten of which (three CDs and seven CCs) showed heterozygous patterns in 'Haara 4' and 12 (three CDs and nine CCs) in 'Kumotooshi'. In the segregation analysis, 20 CAPSs were fitted to a 1:1 ratio (P>0.05) in the F_1 population, the other two showed distorted segregation (CD470; P=0.02, and CC0498; P=0.002).

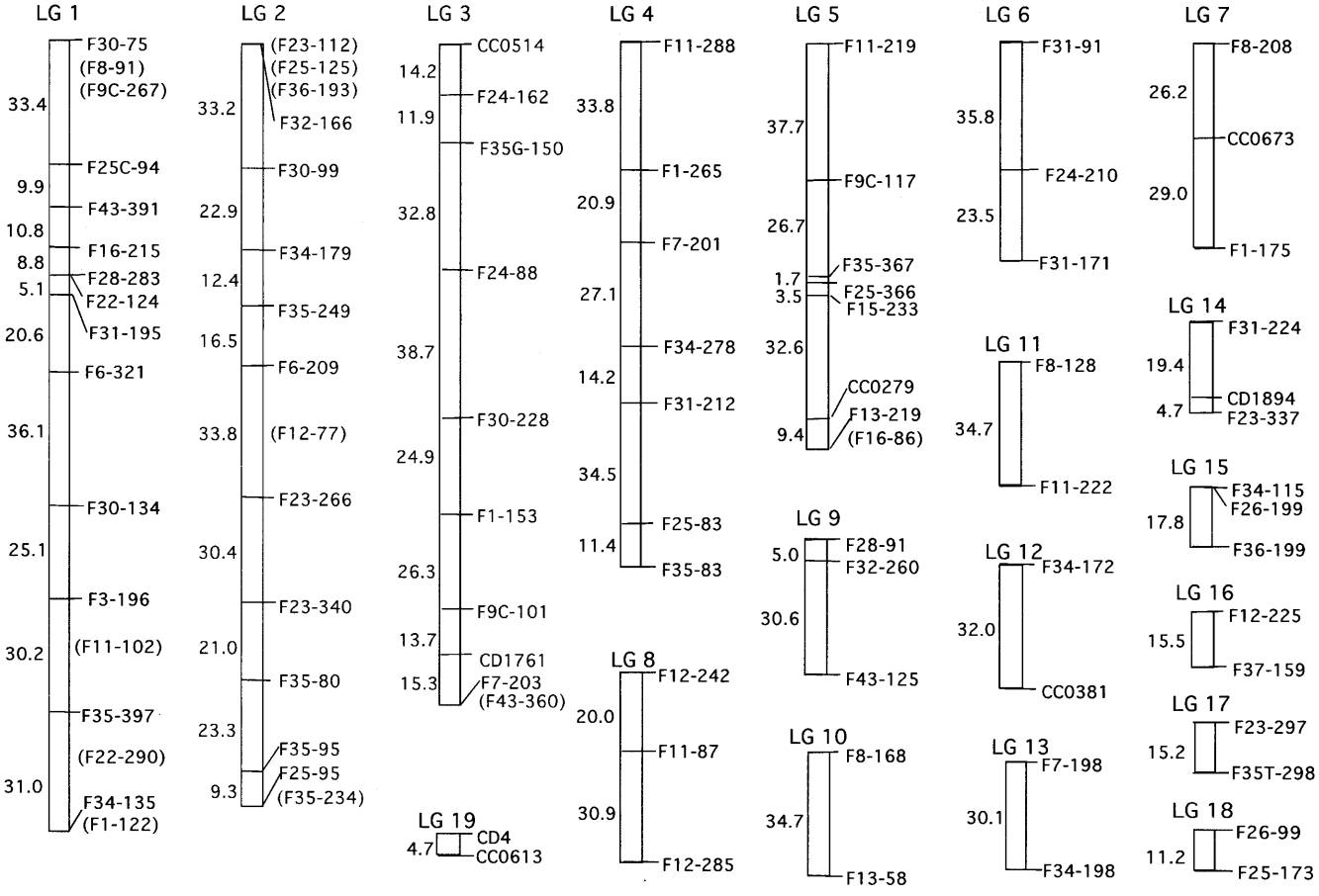

Fig. 1 Linkage map of *C. japonica,* local cultivar 'Haara 4'. AFLP and CAPS markers are indicated on the right and genetic distances in centimorgans (cM) are indicated on the left. Loci with ambiguous map positions are shown in parentheses

Construction of the genetic linkage map

Since F_1 progeny were used as a mapping population, linkage maps of 'Haara 4' and 'Kumotooshi' were independently constructed. A total of 164 markers (155 AFLP and 9 CAPS) were analyzed to construct a 'Haara 4' linkage map. Of these, 91 (83 AFLP and 8 CAPS) were assembled into 19 linkage groups, each of which included 2–17 markers. These markers covered 1266.1 cM of the 'Haara 4' nuclear genome, with an average interval of 16.0 cM between adjacent markers (Fig. 1). Also, 161 markers (150 AFLP and 11 CAPS) were analyzed in 'Kumotooshi', of which 132 (123 AFLP and 9 CAPS) were allocated to 23 linkage groups spanning 1992.3 cM. Each 'Kumotooshi' linkage group included 2–16 markers, with an average interval of 17.9 cM (Fig. 2). However, markers are not uniformly distributed in either map. There were widely spaced marker intervals in both parental maps, the maximum calculated map distance between adjacent markers being 40 cM.

Some of the linkage groups identified in this study were found to correspond to groups previously mapped by RFLP analysis, using cDNA clones from imbibed embryos, in 'Kumotooshi'×'Okinoyama' (Mukai et al. 1995). Some of the RFLP clones were converted to STSs (Tsumura et al.1997), and three cDNA markers (CD657, CD1761, and CD1894) which were mapped in 'Kumotooshi'× 'Okinoyama', were also positioned in the linkage map of 'Haara 4' or 'Kumotooshi' as CAPS markers. The segregation ratio of CD470 deviated from Mendelian expectation in both our study (P=0.02) and 'Kumotooshi'× 'Okinoyama' (P<0.01).

Discussion

AFLP markers

Sugi has a diploid genome size of 13.79 pg (Sasaki et al. 1997), which is equivalent to approximately 6000 Mbp. We confirmed the power of the modified AFLP procedure for plants with large genomes like sugi. Several precautions were taken in this study to ensure high levels of polymorphism were detected, with high reproducibility and low genotyping errors. One of the most important factors for optimizing the procedure is the selective nucleotide number added to the core primers (Vos et al. 1995). According to preliminary experiments, E+3/M+3 primer combinations gave extremely weak amplification when followed by pre-selective amplification with E+A/M+C primers (data not shown). However, both E+3/M+4 and E+3/M+5 pairings produced scorable numbers of fragments when followed by pre-amplification

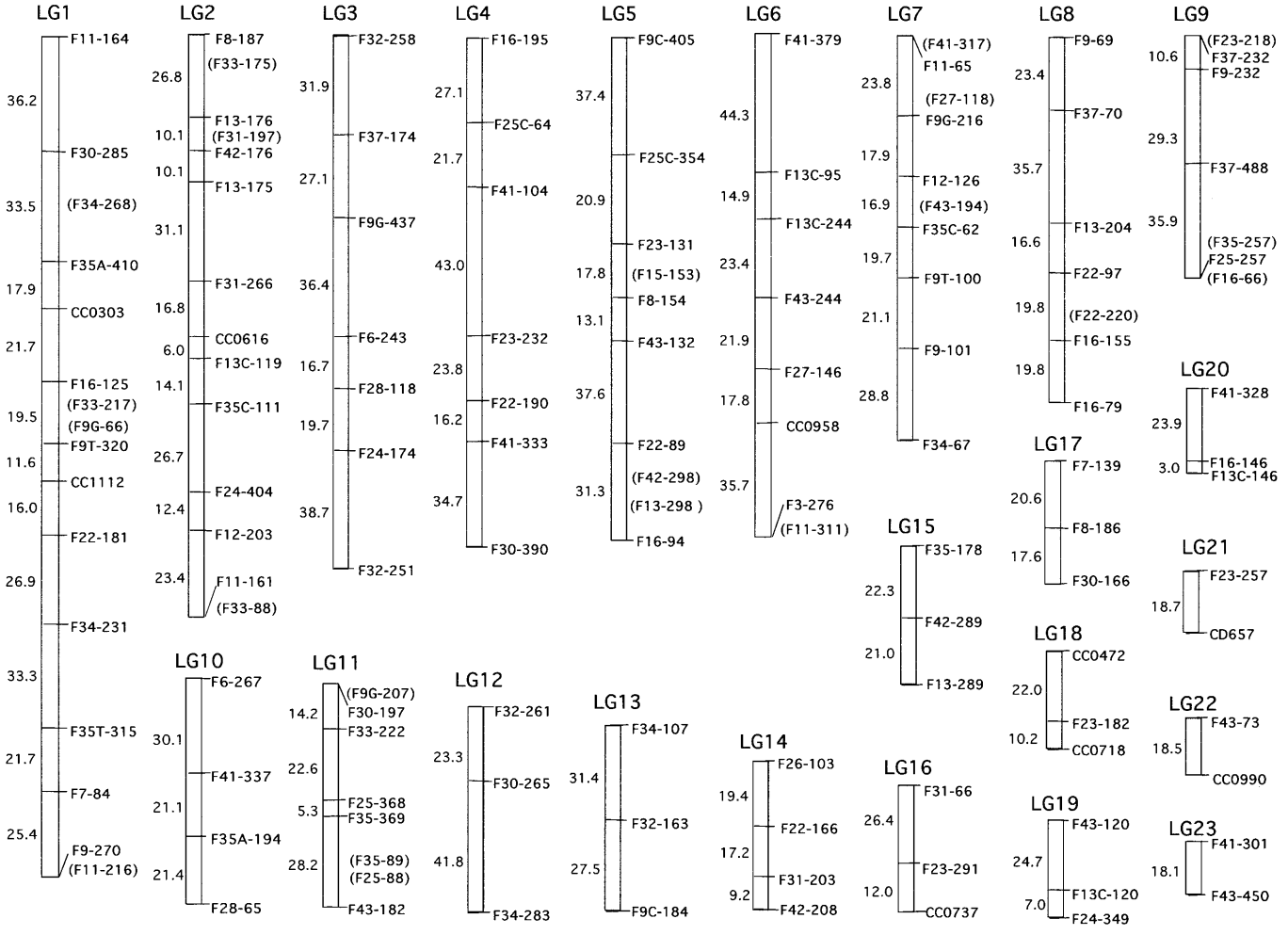

Fig. 2 Linkage map of *C. japonica*, local cultivar 'Kumotooshi'. AFLP and CAPS markers are indicated on the right and genetic distances in centimorgans (cM) are indicated on the left. Loci with ambiguous map positions are shown in parentheses

with E+AA or E+AC/M+CC combinations. This may be because the selectivity of E+3/M+3 combinations was not strong enough to reduce the number of fragments amplified in the selective amplification step to appropriate levels. In the case of *Alstromeria* (haploid genome size of 25 pg, approximately 25000 Mbp), a clear and reproducible AFLP pattern was generated using two selective nucleotides for pre-amplification and four nucleotides for selective amplification (Han et al. 1999). Although the sugi genome is only about a quarter of the size of the *Alstroemeria* genome, it can still be classed as 'large'. Finally, about eight polymorphisms were detected on average in each parent per primer combination in our study. Thus, the AFLP technique detected nearly three times more polymorphism in sugi than the RAPD method used by Kuramoto (1997). AFLP has also been shown to be highly sensitive to polymorphism in *Pinus radiata*, another coniferous species (Cato et al. 1999).

However, 50% of AFLP cannot be utilized for segregation analysis due to segregation distortion, which may occur for several reasons. Some distortion may be related to biological problems, for instance lethal genes, which are reportedly retained in various conifer species. In a study of *Pseudotsuga menziesii* var. *menziesii*, for example, it was estimated that the median tree carried about ten lethal genes per zygote (Sorensen et al. 1969). The presence of lethal genes has also been confirmed in several other studies (Ohba et al. 1979; Ohba et al. 1981), and the possible effects of lethal genes in sugi on the segregation distortion of molecular markers has been discussed by various researchers (Tsumura et al. 1989; Mukai et al. 1995; Kuramoto 1997). Kuramoto (1997) suggested that sugi possessed at least 12 lethal genes. Therefore, we should further investigate the influence of lethal genes on segregation distortion. It would be possible, for instance, to analyze regions associated with lethal genes on our linkage map for correlations with distorted markers. Another reason for segregation distortion is the presence of fragment-complexes. Nikaido et al. (1999) noted the effect of overlapping fragments consisting of identically sized fragments, which had been amplified from different loci. Approximately 30% of distorted AFLP markers were associated with fragment-complexes in sugi. Similar phenomena have been suggested to occur in other AFLP analyses by Hansen et al. (1999).

CAPS markers

CAPS molecular markers are derived from cDNA, and they include or flank coding regions for gene expression. In addition, these regions are so highly conserved that markers derived from them can provide informative anchor points on linkage maps. It is possible to map cDNA clones on genetic linkage maps using RFLP analysis. However, this requires several steps, and large amounts of DNA. To exploit the full potential of genetic linkage maps, the analysis of markers should be quick and convenient. CAPS analysis based on PCR and restriction digestion can be carried out simply and quickly, once STS primers have been developed from cDNA clones. In order to apply a DNA marker system to construct sugi linkage maps, markers with high levels of heterozygosity are required, because every sugi breeding program focuses on different, specific families, depending on where it is based in Japan. Tsumura and Tomaru (1999) demonstrated the usefulness of CAPS markers for studying diversity in *C. japonica*. According to their results, 20 out of 80 STSs showed polymorphic patterns in 11 natural populations of sugi distributed throughout Japan, and the average heterozygosity (0.281) was quite high. This implies that one-third of CAPS markers developed would show heterozygosity in one parent. Where a 'two-way pseudo-testcross' is used, as in this study, five-ninths of the CAPS markers will show heterozygosity in at least one parent. Consequently, more than half of the sugi CAPS markers developed can be used to construct linkage maps for the parental families. Moreover, previously mapped RFLP markers can be converted into CAPS markers and replaced. Therefore, this kind of PCR-based co-dominant marker has great potential, and will make construction of linkage maps easier and faster, especially when mapping populations using the 'two-way pseudo-testcross' strategy.

Map construction

AFLP and CAPS marker systems were combined for constructing genetic linkage maps. Using 'pseudo-testcross' theory (Grattapaglia et al. 1994), which applies to plants with highly retained heterozygosity, such as tree species, only the parents and F_1 progeny were required to construct sugi linkage maps. Linkage between markers was calculated using the MAPMAKER ver. 3.0 program, with the minimum LOD score set at 3.0: the default value in this program. In various previous studies in which linkage maps have been presented, higher LOD scores have been set (Grattapaglia et al. 1994, Maliepaard et al. 1998, Marques et al. 1998). However, a LOD of 3.0 corresponds to $\chi^2(1)=2.88$ ($P=0.0003$) where the markers are of backcross type (Morton 1955). Therefore, we considered LOD scores of 3.0 or more to be theoretically high enough for linkage analysis in this study.

Although similar numbers of markers were analyzed, the size of the two parental linkage maps obtained differed markedly. The 'Haara 4' map consisted of 91 markers and covered 1266.1 cM, while the 'Kumotooshi' map consisted of 132 markers and covered 1992.3 cM. The most-likely reason for this anomaly is the difference in the number of individuals used in the segregation analysis for the two families. We analyzed 62 F_1 individuals for 'Haara 4' and 87 for 'Kumotooshi'. Tests suggested that the number of linked markers in 'Kumotooshi' fell to more or less the same level as in 'Haara 4' when the number of F_1 individuals analyzed was reduced to 62 (data not shown). A linkage map based on RFLP, RAPD and isozyme markers prepared in a previous study covered 887.3 cM (Mukai et al. 1995). Both maps obtained in our study exceeded this size, especially the map of 'Kumotooshi', which covered 80% of the estimated sugi genome (2500 cM). Although the 'Haara 4' map covered only half of the genome, the average interval between adjacent markers was closer than in the 'Kumotooshi' map. Therefore, more markers will be needed to cover the 'Haara 4' nuclear genome more completely, and to construct higher-density maps of the 'Kumotooshi' genome.

In this study, the usefulness of AFLP analysis was proven and used to construct genetic linkage maps of sugi. In addition, co-dominant CAPS markers were located on these maps. Some of these markers, derived from cDNA clones, were previously placed on a map of 'Kumotooshi'×'Okinoyama' (Mukai et al. 1995), even though the marker systems employed were not the same. These markers will provide a bridge to integrate several linkage maps, although more markers must be used to improve the level of integration and quality of information. In the future, it is anticipated that the sugi linkage map presented here will be extended and become more detailed, as it is saturated with DNA markers, and the number of linkage groups will converge with the sugi chromosome number (n=11). Thus, with further work the map should become a valuable reference tool for *C. japonica* breeding programs, and for fundamental research into the genetics and physiology of the species.

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