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Assignment of RFLP linkage groups to their respective chromosomes in aneuploids of sugi (*Cryptomeria japonica*)

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Abstract Aneuploids of sugi (*Cryptomeria japonica*) were found in the open-pollinated progenies of triploid-plus tree clones. Seven trisomics and one hypotriploid were used to assign the chromosomes to the RFLP linkage groups constructed previously. The Southern blots containing their genomic DNA were hybridized with the labeled DNA clones corresponding to the loci in the linkage map. The additional dosage in autoradiographs showed that the cloned DNA fragment was located on the extra chromosome in the trisomics. On the other hand, the extra chromosome in two trisomics and the chromosome lacking the triplet in the hypotriploid were cytologically identified as chromosome 10 by consistent presence of a secondary constriction in the proximal region of its short arm. As a result, three linkage groups were assigned to their respective chromosomes, namely chromosome 10 and two other chromosomes.

Key words RFLP linkage map · Chromosome · Trisomics · *Cryptomeria japonica*

Introduction

Sugi, *Cryptomeria japonica* D. Don, is the most widely-planted forest tree in Japan. The area planted with sugi occupies over 4.5 million ha, or 44% of all the Japanese cultivated forest. Because of the importance of sugi in forestry, the genetic and breeding research of this species has been applied extensively. Recently, a linkage map for sugi based on RFLP, RAPD, and isozyme loci has been reported (Mukai et al. 1995). While sugi has 11 pairs of chromosomes (Sax and Sax 1933), the linkage map comprises 20 linkage groups; thus, so far there is no correspondence with chromosomes.

Genetic linkage maps are constructed based on the calculation of segregation data of genetic markers. In a fully saturated map, the number of linkage groups should agree with the number of chromosome pairs. To corroborate a genetic linkage map, it is essential to assign the linkage groups to their respective chromosomes.

In organisms with some chromosome-specific markers, it is possible to assign linkage groups to chromosomes by linkage analysis. And in the major crop plants with aneuploid stocks, such as trisomic or monosomic series, it is possible to assign cloned DNA fragments to their specific chromosomes by looking for changes in gene dosage. This technique has been applied successfully in some crop species including maize (Helentjaris et al. 1986), tomato (Young et al. 1987), rice (McCouch et al. 1988), and pea (Ellis and Cleary 1988). Neither chromosome-specific markers nor aneuploids have been reported in forest trees except for some trisomics ($2n = 23$) of sugi found in the progenies of triploids (Matsuda 1980; Sasaki and Kuroki 1987). However, their genetic analysis has not yet been carried out.

In the present study, we found trisomics and a hypotriploid in the open-pollinated progenies of triploid-plus tree clones, and specific chromosomes from these were identified cytologically. Using these aneuploids and cloned DNA fragments corresponding to the loci of the linkage map, the linkage groups were assigned to chromosomes. We report here the first assignment of a linkage group to a specific chromosome of sugi.

Materials and methods

Identification of aneuploids

Open-pollinated seeds were collected from triploid-plus tree clones, Kuji-30 and Higashikamo-1 (Sasaki 1982; Kondo 1988), and ger-

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minated. The seedlings were planted in plastic pots and grown for 7 years. Root tips were collected from the 79 progenies of Kuji-30 and the 31 progenies of Higashikamo-1. They were immersed overnight in 2 mM 8-hydroxyquinoline, fixed with ethanol-acetic acid (3:1), and stored in 70% ethanol. Then, they were macerated in 45% acetic acid at 60 °C for 4 min and squashed on glass slides in acetocarmine. The chromosomes were observed at metaphase through a microscope.

DNA preparation and Southern analysis

Using the method of Murray and Thompson (1980), DNAs were extracted from needle tissues of individual specimens, including the mother trees as well as the aneuploids found in the progenies, using the diploid cultivers Okinoyama-sugi and Kumotooshi as standards. The amount of DNA used in the Southern-blot analysis was calculated to equalize the amount of DNA per chromosome without regard to differences between chromosomes. That is, the amounts of DNA analyzed were 5.0 μg, 5.22 μg, 7.28 μg and 7.5 μg for diploid, trisomics, hypotriploid and triploid, respectively. The methods for restriction-endonuclease digestion, agarose-gel electrophoresis, Southern transfer, hybridization and autoradiography have been described previously (Mukai et al. 1995).

The autoradiographs were scanned with a densitometer (Toyo, DMU-33C) to support the tentative visual assignments and to provide a quantitative estimate of the additional dosage.

Results and discussion

Identification of aneuploids

Polyploids, particularly triploids, are the most dependable sources of primary trisomics (Khushu 1973). Therefore triploids were used as the source of trisomics. While trisomics are rare in gymnosperms (Owens 1967; Ching and Doerksen 1971), Matsuda (1980) found them among the progenies of triploid cultivars of *C. japonica*. In the present study, the germination rates of the seeds from Kuji-30 and Higashikamo-1 were 0.5% and 0.3%, respectively. Most of the progenies from these were diploids ($2n = 22$), whereas there were nine trisomics (12%) among 74 progenies of Kuji-30 and two trisomics (7%) among 29 progenies of Higashikamo-1. The only other aneuploid was a hypotriploid ($2n = 32$). Because three of those trisomics were too small to extract DNA, one trisomic (H-1-25) from Higashikamo-1, six trisomics (K-30-2, K-30-10, K-30-22, K-30-25, K-30-62 and K-30-70) and one hypotriploid (K-30-17) from Kuji-30 were used in this study.

C. japonica shows variation in both the number and position of secondary constrictions (Toda 1979 a, b, c) which appear on chromosomes 6 and 10. Chromosome 10 always possesses a secondary constriction in the proximal region of the short arm. Chromosome 6 appears either without a secondary constriction or with a secondary constriction in the interstitial region of the short arm (Kondo et al. 1985). With the exception of the chromosomes 6 and 10, it is difficult to identify individual chromosomes in *C. japonica* because most of them are similar in shape (i.e. metacentric or submetacentric) and length (Sax and Sax 1933; Mehra and Khoshoo 1956). In the present study, chromosome 10 was identified by the invariable presence of a secondary constriction, whereas chromosome 6 could be identified only when a secondary constriction was present.

The number of identified chromosomes in seven trisomics and one hypotriploid is shown in Table 1. A triplet of chromosome 10 was observed in trisomics K-30-2 and K-30-25 (shown in Fig. 1), while the other trisomics (excepting K-30-62) had two representatives of chromosome 10. This indicates that the extra chromosome of the two trisomics is chromosome 10. By contrast, the extra chromosome of the four trisomics K-30-10, K-30-22, K-30-70 and H-1-25 is not chromosome 10. However, due to the limited availability of root-tip material, observations of secondary constrictions were not possible for trisomic K-30-62. Additionally, in the hypotriploid K-30-17 only two representatives of chromosome 10 were observed.

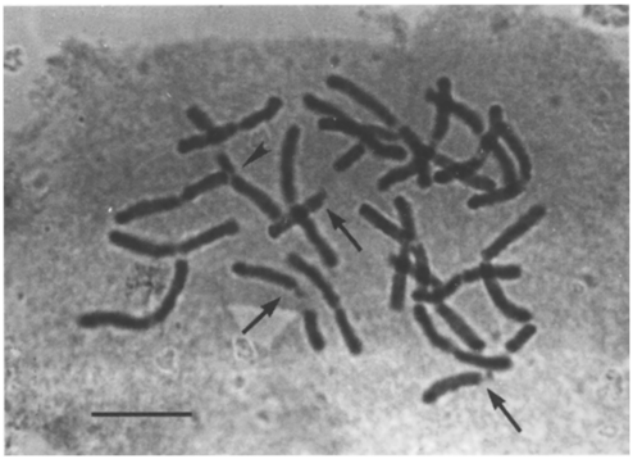
Assignment of linkage groups to chromosomes

In trisomics, the hybridization signal with DNA clones originating from the extra chromosome results in the additional dosage detected on autoradiographs of

Table 1 The number of identified chromosomes for seven trisomics and one hypotriploid of sugi (*C. japonica*)

Specimens	Chromosome 6 with secondary constriction	Chromosome 10
Trisomics		
K-30-2	0	3
K-30-10	1	2
K-30-22	1	2
K-30-25	1	3
K-30-62	–	–
K-30-70	0	2
H-1-25	2	2
Hypotriploid		
K-30-17	0	2

Fig. 1 Photomicrographs of chromosomes at mitotic metaphase in root-tip cells of trisomic K-30-25 *Cryptomeria japonica*. *Arrows* and *arrowhead* indicate chromosome 10 and chromosome 6 with secondary constrictions, respectively. The *bar* indicates 10 μm



Southern blots either in the form of the additional intensity or the number of bands. For Southern analysis we selected the 20 cloned DNAs that show single- or low-copy RFLP probes and that correspond to 20 loci distributed in all 14 linkage groups consisting of more than three loci in the linkage map (Mukai et al. 1995). The combinations of the probe and restriction enzyme used for hybridization are listed in Table 2.

The clones residing in linkage groups 1, 2, 3, 4, 6, 7, 8, 11, 12, 13 and 14 (namely, pCjc2041, 548, 1066, 482, 569, 511, 1425, 1894, 788, 857 and 177, respectively) did not demonstrate any additional dosage in the hybridization pattern observed with the trisomics's DNA (Table 2). Therefore, these 11 linkage groups do not correspond to the extra chromosome in any of the trisomics. On the other hand, additional dosage was observed in the hybridization pattern with the clones residing in linkage groups 5, 9 and 10. Typical results are shown in Figs. 2 a–c.

The autoradiograph with pCjc1841 (linkage group 5) is shown in Fig. 2 a. Four of the seven trisomics indicated a heterozygotic pattern (lanes 3, 5, 6 and 7), whereas the other three showed a homozygotic pattern (lanes 4, 8 and 9). The homozygotic band of the three trisomics showed the same intensity as that of the diploid (lane 2). In other words, they did not exhibit any additional dosage. One of the four trisomics portraying a heterozygotic band pattern had two bands of the same intensity (lane 5). The other three gave two bands with different band intensity in a 1:2 ratio (lanes 3, 6 and 7). That is, the band intensity of increased hybridization was equal to that of the homozygotic band from the diploid (lane 2) and the band pattern was the same as the heterozygotic pattern from a triploid (lane 10). The densitometric analysis indicated a dosage shift (Fig. 3). The clear additional dosage in the three trisomics suggests the triplication of the chromosome corresponding to the DNA fragment. When the other clones (pCjc337, 517, 682 and 934) residing in linkage group 5 were used, the three trisomics also gave evidence of additional dosage (Table 2). Consequently, it was concluded that linkage group 5 corresponds to the extra chromosome in these three trisomics (K-30-2, K-30-25 and K-30-62). Additionally, the hypotriploid (2n = 32) did not indicate a triplet pattern when the clones residing in linkage group 5 were used (lane 12 in Fig. 2a and K-30-17 in Table 2). Therefore, the chromosome lacking the triplet in the hypotriploid also corresponds to linkage group 5.

When pCjc1545 (linkage group 10) was used (Fig. 2 b), the other three trisomics (K-30-10, lane 4; K-30-70, lane 8; H-1-25, lane 9) showed additional dosage. The other clone (pCjc2036) residing in the linkage group 10 also gave evidence of additional dosage in these trisomics (Table 2). Therefore, it is considered that linkage group 10 corresponded to the extra chromosome in these three trisomics.

When pCjc1943 (linkage group 9, Fig. 2c) was used, the other trisomic (K-30-22, lane 5) revealed additional dosage. The other clone (pCjc25) residing in linkage group 9 also gave additional dosage in this trisomic (Table 2). The results show that linkage group 9 corresponds to the extra chromosome in the trisomic K-30-22.

Table 2 Allelic status of 20 RFLP loci for two triploids of sugi (*C. japonica*) and their aneuploid progenies. Alleles were designated in alphabetical order corresponding to their fragment size

Linkage group	Locus	Probe-enzyme[a]	Triploid (mother tree) 2n = 33		Trisomic 2n = 23							Hypotriploid 2n = 32
			Kuji-30	Higashikamo-1	K-30-2	K-30-10	K-30-22	K-30-25	K-30-62	K-30-70	H-1-25	K-30-17
1	*CD2041*	pCjc2041-B	*bbb*	*abb*	*bb*	*ab*	*bb*	*bb*	*bb*	*ab*	*ab*	*bbb*
2	*CD548*	pCjc548-H	*aab*	*aaa**	*aa*	*aa*	*bb*	*bb*	*bb*	*aa*	*ab*	*abb**
3	*CD1066*	pCjc1066-V	*abb*	*abb*	*ab*	*ab*	*ab*	*ab*	*ab*	*ab*	*aa*	*abb*
4	*CD482*	pCjc482-B	*bbc*	*aac*	*ab**	*bc*	*ac*	*bc*	*ac*	*ab*	*aa*	*bcc*
5	*CD337*	pCjc337-B	*abc*	*bbc*	*bcc*	*ac*	*ab*	*abc*	*aac*	*aa*	*cc*	*cc*
	CD517	pCjc517-I	*acc*	*acc*	*acc**	*cc*	*ac*	*abc*	*aac*	*ac*	*cc*	*ab*
	CD682	pCjc682-V	*aab*	*abb*	*aab*	*ab*	*ab*	*aab*	*aab*	*ab*	*ab*	*aa*
	CD934	pCjc934-H	*bbb*	*abb*	*bbb**	*bb*	*ab*	*bbb*	*abb*	*bb*	*aa*	*bb*
	CD1841	pCjc1841-V	*aac*	*aaa*	*aac*	*aa*	*ac*	*aac*	*aac*	*aa*	*aa*	*bc*
6	*CD569*	pCjc569-V	*acd*	*bdd*	*cd*	*aa*	*bc*	*ad*	*ab*	*ad*	*ab*	*abc*
7	*CD511*	pCjc511-H	*bbb*	*aab*	*ab*	*bb*	*bb*	*ab*	*ab*	*bb*	*ab*	*abb*
8	*CD1425*	pCjc1425-V	*aaa**	*aaa**	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aaa**
9	*CD25*	pCjc25-V	*abc*	*bbd*	*bc*	*bc*	*abc*	*cc*	*bd*	*ab*	*bc*	*abc*
	CD1943	pCjc1943-V	*bcc*	*bbb*	*ac*	*bc*	*bcc*	*bb*	*ac*	*cc*	*bc*	*bbc*
10	*CD1545*	pCjc1545-V	*abc*	*bcc*	*bb*	*aac*	*ac*	*ac*	*bd*	*abb*	*bbc*	*acc*
	CD2036	pCjc2036-I	*cdd*	*bbc*	*bd*	*ccd*	*cd*	*cd*	*cd*	*bcd*	*bbc*	*cdd*
11	*CD1894*	pCjc1894-V	*acd*	*cdd*	*cd*	*dd*	*cd*	*cd*	*ad*	*ad*	*cd*	*add*
12	*CD788*	pCjc788-V	*abb*	*aaa*	*aa*	*ab*	*ab*	*ab*	*aa*	*ab*	*aa*	*aab*
13	*CD857*	pCjc857-H	*abc*	*aab*	*ac*	*ab*	*bb*	*bc*	*ab*	*ab*	*ab*	*abc*
14	*CD177*	pCjc177-H	*abb*	*aaa*	*ab*	*aa*	*ab*	*aa*	*ab*	*ab*	*ab*	*aab*

[a] Probe-enzyme refers to the DNA clone used as a probe and the restriction endonuclease used for genomic DNA digestion in Southern hybridization. B, H, I and V indicate *Bam*HI, *Hin*dIII, *Eco*RI and *Eco*RV, respectively

* Unconfirmed findings

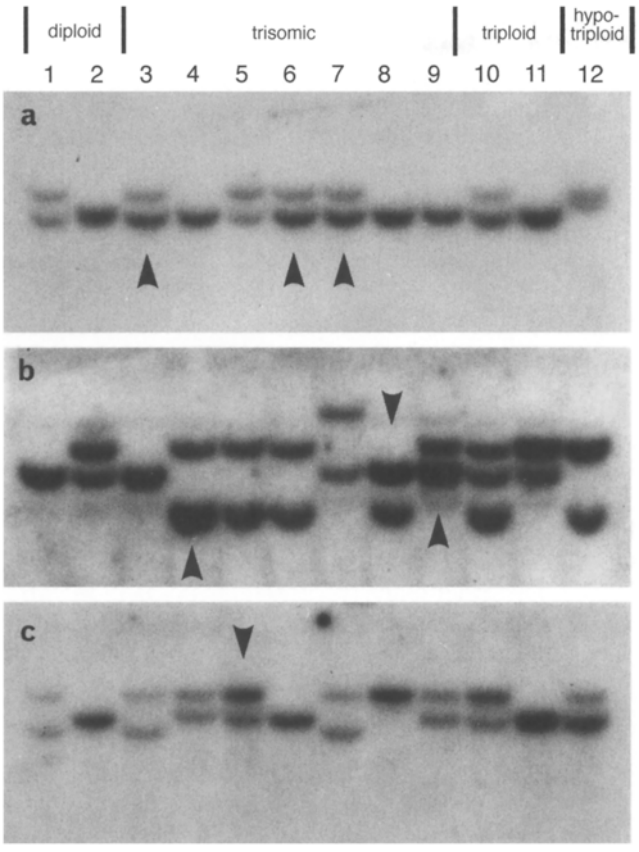


Fig. 2a–c Southern-blot analysis for the total DNAs extracted from diploid (lane 1, Okinoyama-sugi; lane 2, Kumotooshi), trisomic (lanes 3–9; K-30-2, K-30-10, K-30-22, K-30-25, K-30-62, K-30-70 and H-1-25, respectively), triploid (lane 10, Kuji-30; lane 11, Higashikamo-1) and hypotriploid (lane 12, K-30-17) sugi. **a** probed with pCjc1841 (linkage group 5); **b** probed with pCjc1545 (linkage group 10); **c** probed with pCjc1943 (linkage group 9)

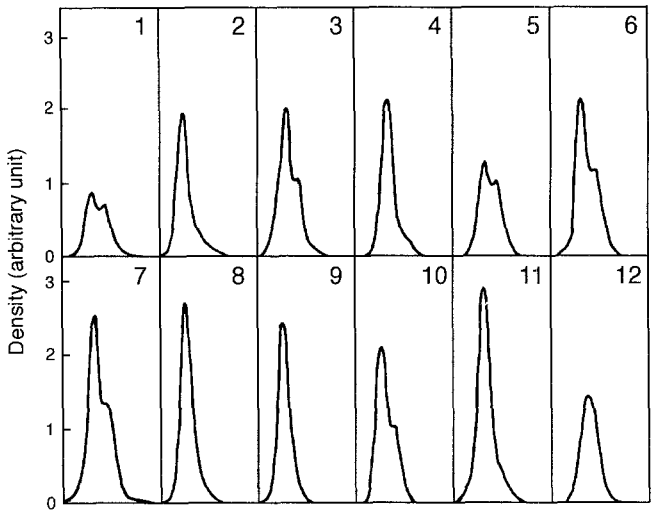


Fig. 3 Densitometric scans of autoradiography detected by the Southern blot, probed with pCjc1841 (linkage group 5). The autoradiography used in this analysis (shown in Fig. 2a) was scanned with a densitometer. *Numbers* in the upper right of the panels correspond to the lane numbers in Fig. 2a

Thus, 3 of the 14 linkage groups were assigned to the extra chromosome of trisomics. That is, linkage groups 5, 10 and 9 corresponded to the extra chromosome of trisomics group 1 (K-30-2, K-30-25 and K-30-62), group 2 (K-30-10, K-30-70 and H-1-25) and group 3 (K-30-22), respectively. The results also suggest that the other unassigned linkage groups correspond to the other chromosomes. This is important information for the unsaturated linkage map at this stage. Moreover, the extra chromosome of group-1 trisomics (excepting K-30-62) and the chromosome lacking the triplet in the hypotriploid were confirmed both genetically and cytologically (Tables 1 and 2). Therefore, it is suggested that linkage group 5 be assigned to chromosome 10.

This is the first assignment of a linkage group to a specific chromosome in sugi. Because of the limitation of cytological technique for chromosomal assignment, only one specific chromosome could be assigned to a linkage group. The remaining two linkage groups would be assignable to chromosomes only by the cytological identification of the extra chromosomes in the trisomics. To assign all the linkage groups to chromosomes by this method, it is necessary to create a complete trisomic series and identify all the chromosomes. On the other hand, in situ hybridization techniques would enable us to identify specific chromosomes and directly assign linkage groups to them. However, few reports have detected any single-copy clones in plant species using in situ hybridization (e.g. Mouras et al. 1987; Huang et al. 1988). We hope to develop this technique using the high-resolution laser microscope and employing a high sensitivity fluorochrome for DNA labeling.

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