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Genetic diversity of *Cryptomeria japonica* using co-dominant DNA markers based on sequenced-tagged sites

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Abstract We have investigated the genetic diversity of 11 natural populations of *C. japonica* using 13 polymorphic STS markers. The average unbiased heterozygosities (H_e), the average number of alleles per locus (N_a) and the proportion of polymorphic loci (Pl) were 0.281, 1.93 and 76.92%, respectively. Coefficients of linkage disequilibrium were calculated, and no significant deviation was found except in four combinations – which might have occurred by chance alone. The fixation index (F_{IS}) for 3 loci showed statistically significant values at the 1% level. The genetic differentiation between populations was only 0.047, and there were no clear geographical tendencies in the allele frequencies or the heterozygosities among populations. Consequently, the results from STS-based co-dominant DNA marker analysis were very similar to those from a previous allozyme study. However, the resolution of the technique is greater than allozyme analysis because many loci with high heterozygosities can be evaluated, and it is very simple. Therefore, the STS-based marker approach is very useful and convenient for population genetics and genome mapping of *C. japonica*.

Key words Sugi · Genetic diversity
Sequence-tagged-site (STS) · PCR-RFLP · CAPS

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Introduction

Sequence-tagged-site (STS) primers have been used to generate polymerase chain reaction (PCR)-based markers in many plant species including *Arabidopsis* (Konieczny and Ausubel 1993), barley (Tragoonrung et al. 1992), lettuce (Paran and Michelmore 1993), wheat (Talbert et al. 1994), rice (Inoue et al. 1995), *Populus* (Bradshaw et al. 1994), *Cuphea* (Slabaugh et al. 1997) and *Cryptomeria japonica* (Tsumura et al. 1997). The STS-based co-dominant markers, also called cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993; Drenkard et al. 1997), are more reliable and convenient for genomic mapping and population genetics than such other DNA markers as random-amplified PCR markers (RAPDs). Complementary DNAs (cDNAs) contain the precise coding regions of genes and express specific peptides. Therefore, using STSs of cDNAs to develop molecular markers may be extremely useful for establishing anchor points in genomic mapping and other genetic studies.

Since ancient times *C. japonica* has been a very valuable tree species in Japan because of its excellent attributes (straight bole, rapid growth, ease of processing and pleasant color and scent) for use in house beams, rafters and boards (Ohba 1993). The modern natural forests are distributed in temperate moist regions from Aomori Prefecture (40°42′N) to Yaku Island (30°15′N) in the Japanese Archipelago (Hayashi 1960). However, the distribution is discontinuous and scattered among small, restricted areas as a result of the extensive exploitation of this species during the past thousand years (Ohba 1993).

Geographical variations among natural forests of *C. japonica* have been investigated using both morphological traits (needle length, needle curvature and other features; Murai 1947) and diterpene components (Yasue et al. 1987). These studies suggested that there are two main lines, namely, Ura sugi (found near the

Sea of Japan) and Omote sugi (located near the Pacific Ocean). Tsumura and Ohba (1992, 1993) and Tomaru et al. (1994) studied natural populations using allozymes and subsequently suggested that there was little of the genetic differentiation between populations that is generally found for conifers with widespread distribution. In this paper, we describe the application of STS-based co-dominant markers from cDNA clones of *Cryptomeria japonica* for population studies. We also discuss the genetic diversity of *C. japonica* on the basis of STS-based co-dominant marker analysis and compare our results from STSs with those of previous allozyme studies.

Materials and methods

Plant materials

Mature needle tissues were collected from 246 trees representing 11 natural populations during the winter season. All trees sampled were in national forests that were candidates for *in situ* gene-conservation programs (Fig. 1). The locations of the sampled populations covered most of the natural distribution of *C. japonica*. We collected needle tissues from individual trees with considerable space between sampled specimens to avoid sampling half-sib individuals, but we paid no attention to the age or size of the trees. All individuals were rescued by transferring cuttings to the cutting bed, and in the following spring the resulting seedlings were transplanted to the nursery. After shooting, we collected the shoot from each individual. Collected needle tissues were stored at -30°C prior to DNA extraction.

Laboratory analysis

Total DNA was extracted from these samples using the slightly modified CTAB method of Murray and Thompson (1980). Co-dominant DNA markers based on STSs of cDNA clones from *C. japonica* (Tsumura et al. 1997) were used to evaluate the genetic diversity and differentiation of the species. We screened 80 STS markers to detect polymorphic STS loci. PCR amplification took place in 100-µl reaction mixtures containing 10 m*M* TRIS-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1 m*M* of each dNTP, 100 pmol of each primer, 50 ng of template DNA and 2.5 units of *Taq* polymerase. The amplification was carried out for 5 min at 94°C, followed by 36 cycles of 40 s at 94°C, 40 s at 56–60°C and 80 s at 72°C, with a final 5-min incubation at 72°C in a PC700 instrument from the Astech Company or a PTC100 Programmable Thermal Controller from MJ Research. In our survey for polymorphic STS markers in *C. japonica*, we used 15 individuals for screening. The PCR products were digested with eight four base-cutter enzymes (*Alu*I, *Hae*III, *Hha*I, *Msp*I, *Nde*II *Hin*fI, *Taq*I and *Rsa*I) and electrophoretically separated on 2% agarose gels or 7.5% polyacrylamide gels with ethidium bromide.

Statistical analysis

To estimate within-population variation, we used five measures calculated from the allele frequencies of all loci analyzed: the proportion of polymorphic loci (*Pl*) at the 95% level, the average number of alleles per locus (N_a), and the averages expected, and an unbiased heterozygosity (H_e, unbiased H_e) (Nei 1978). The fixation index, $F_{IS}(= 1 - H_e/H_o)$, F_{IT} and F_{ST}, for polymorphic loci and its average over all loci, were determined for comparison of the observed genotype frequencies with the expectations from the Hardy-Weinberg equilibrium (Wright 1922; Nei 1977; Nei and Chesser 1983; Workman and Niswander 1970). The deviations from such expectations were analyzed using the 2-test (Li and Horvitz 1953). Coefficients of linkage disequilibrium (D) were calculated for 78 pairs of loci by the method of Weir (1979, 1990) using genotype data pooled from all of the test populations, since there were insufficient samples to calculate the coefficients for single populations. The variance of the estimates was also calculated, and differences from equilibrium were verified by the chi-square test (Weir 1990). These analyses were done using the GENESTRUT program (Constantine et al. 1994). In addition, to estimate the amount of gene flow among populations, we indirectly calculated the number of migrants exchanged per generation, N_m, from values of F_{ST} at each locus. Average values for all loci were then derived by applying Wright's infinite island formula (Wright 1931): $N_m = (1 - F_{ST})/4F_{ST}$, where N is the effective population size and m is the proportion of migrants exchanged per generation. Finally, Nei's unbiased genetic distances were calculated for all population pairs (Nei 1972, 1978). Dendrograms were also constructed by the UPGMA and Neighbor-Joining methods and bootstrap analysis was done using DISPAN (Ota 1993).

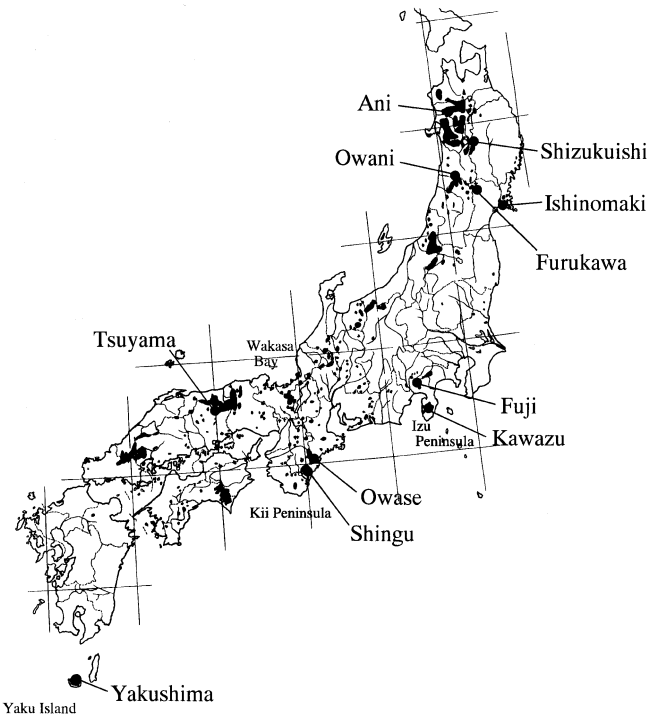

Fig. 1 Natural distribution (Hayashi 1960) and 11 surveyed natural populations of *Cryptomeria japonica* in Japan

Results

We screened the polymorphism of 80 STSs from which we obtained single fragments by PCR using eight different restriction endonucleases. Out of the 80 STSs 20 showed polymorphic patterns, and 13 of these showed co-dominant patterns (Fig. 2). Therefore, we used these 13 polymorphic STS markers to evaluate the genetic

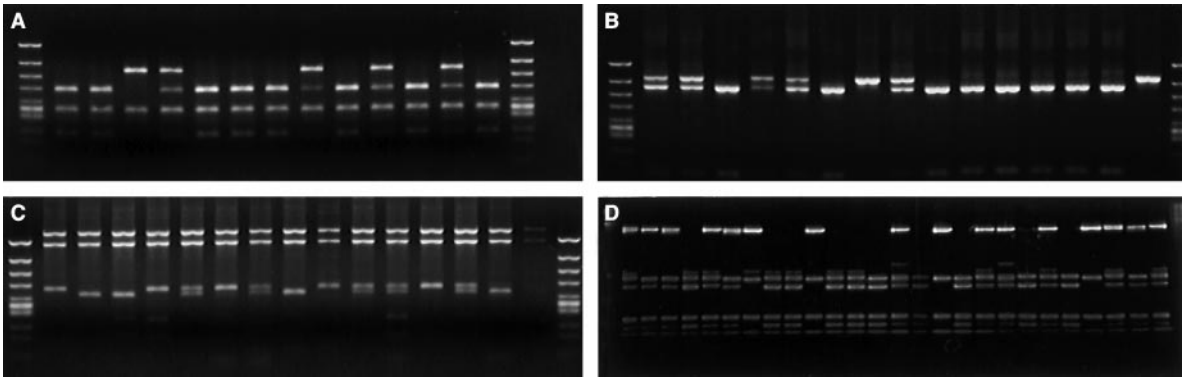

Fig. 2A–D RFLP patterns based on STS markers of *C. japonica*. **A** CD526/*Hae*III, **B** CD657/*Hha*I, **C** CD1091/*Alu*I, **D** CD1675/*Hae*III. Both *marginal lanes* in each panel indicate DNA size marker of ϕX174/*Hinc*II, and the other lanes indicate the investigated individuals. **Panels A–C** show 2% agarose gels and **D** shows 7.5% polyacrylamide gel

diversity of natural populations of *C. japonica* (Tables 1 and 2).

The average unbiased heterozygosities in each population were relatively high, ranging from 0.210 to 0.303 with an average of 0.281, because we used only polymorphic loci (Table 3). The average number of alleles per locus ranged from 1.85 to 2.08. All of the loci detected had two types of alleles except for 2 loci, CD1237 and CD1232; for these latter 2 loci 3 alleles were detected in only 2 populations, respectively. The proportion of polymorphic loci ranged from 53.85% in Yakushima to 100% in Ishinomaki, with an average of 76.92%. The average heterozygosities of 5 of the loci (CD657, CD1706, CD1067, CD1091 and CD1675) were considerably higher than those of the other loci.

There were only four significant D values among the loci. These appear to represent chance sampling events. If the coefficients were independent, then 3.9 (SE = 0.0578) significant values would be expected due to chance alone at a probability level of 0.05. The genotype data for two pairs of linked loci (CD657 and CD526, CD1309 and CD1091, Mukai et al. 1995) did not show deviation from the equilibrium.

The fixation index (F_{IS}) for 3 loci (CD657, CD1067 and CD1195) showed statistically significant values at the 1% level (Table 4). The F_{ST} values also showed statistical significance at 6 loci by the chi-square test, and the mean values of F_{ST} also showed significance.

The genetic variations were partitioned into within – (and between-population components, with proportions of 95.3% and 4.7%, respectively (Table 4). The number of migrants exchanged per generation among populations, N_m, was 5.1. The unbiased genetic distances between populations ranged from 0.000 to 0.079, with an average of 0.007. There were no clear geographical tendencies in the allele frequencies or the heterozygosities among populations.

Discussion

STS-based co-dominant markers

The polymorphic DNA markers derived from *C. japonica* cDNA STS primers could also be used in studies of other coniferous species (Tsumura et al. 1997). When screening for suitable markers, we excluded those which showed no co-dominance and those which were clearly multi-copy genes. The selected markers were considered to represent expressed genes because they were derived from cDNA sequences. These regions might be usable in other conifers because cDNA sequences tend to be conserved between closely related taxa like Taxodiaceae and Cuppresaceae (Brunsfeld et al. 1994; Tsumura et al. 1995). The inheritance of these markers has been confirmed in some families of *C. japonica*.

STS-based DNA markers can detect polymorphisms much more sensitively than allozyme analysis because they detect nucleotide differences directly. Another advantage is that they do not require the fresh materials needed for isozyme analysis. However, there are high labor and cost requirements in developing such PCR-based markers because identifying polymorphisms involves cloning, sequencing, PCR primer synthesis, PCR and screening by many restriction endonucleases. However, once suitable polymorphisms have been identified, this approach provides a rapid and convenient procedure for surveying Mendelian polymorphism in large numbers of individuals. This technique has several advantages over the standard Southern blotting approach to restriction fragment length polymorphism (RFLP) analysis (Karl et al. 1992). Firstly, only a small amount of DNA is required for the PCR reaction. Secondly, since the fragment size of each PCR product is conserved, restriction site variants can be distinguished from indel variants. Thirdly, there is no interference from the methylation of genomic DNA because we use PCR products to survey the polymorphisms. Fourthly, PCR-amplified DNA can be digested directly and visualized in EtBr-stained agarose gels or polyacrylamide gels in just a few hours.

Table 1 PCR-based co-dominant markers developed from cDNA clones of *C. japonica*

Locus	Primer	PCR conditions[a]	Fragment size (bp)	Enzyme[b]	Linkage group[c]	Homology[d]
CD657	5′ TCC TGA TAC TGT GGG CAA CT 3′ 5′ CCC CGA TAT GCT CTT CAA CT 3′	60°C, 36 cycles	800	*Hha*I	6	
CD1237	5′ GGA ATC GGA TGG GTT ATC TG 3′ 5′ AGA ATC CGG GAC CAA ATC TA 3′	60°C, 36 cycles	1300	*Hha*I	3	*Arabidopsis thaliana* cDNA clone 120C7T7
CD526	5′ TCT TGC ATG ACT TGG TTG CT 3′ 5′ GGG GAT TTG GAG ATT TTC AG 3′	60°C, 36 cycles	1000	*Hae*III	6	
CD1309	5′ AGC AAA ACC TTG GGA TTC TT 3′ 5′ TAG AGC CGC ACT ATT CAG AT 3′	60°C, 36 cycles	1400	*Alu*I	2	
CD1894	5′ ACC CTT TCC TCG CCT ACA TT 3′ 5′ GCC GAC TGA GTA AAC AAA CC 3′	60°C, 36 cycles	800	*Rsa*I	11	Oat TUB1 mRNA for beta-tubulin (partial)
CD1216	5′ GCC AAG ACC CTG AGC AAA TC 3′ 5′ CCT GTG CGA AAG CCA ATC AA 3′	58°C, 36 cycles	800	*Hha*I	Unlinked	*P. abies* (L.) Karst. Lhcb1*1 mRNA for light-harvesting
CD1706	5′ ATA GGC GAC GCA GGT CAA AA 3′ 5′ TCT GCG GCT GTA GTT CCA GT 3′	58°C, 36 cycles	400	*Hha*I	Unlinked	*A. thaliana* ferrodoxin mRNA, complete cds.
CD1067	5′ TTT AGG GTT TTG GGT TTT AG 3′ 5′ AAC ATA CCA TCT GCC CTC TT 3′	58°C, 36 cycles	1000	*Hae*III	10	*Arabidopsis thaliana* cDNA clone 35A3T7
CD1091	5′ GCC CTC CCT GGT TTT CTT CA 3′ 5′ GAA ACC CTG GAC TGG CAT AG 3′	58°C, 36 cycles	700	*Alu*I	2	
CD41	5′ GAA TCC AAA ACC ACT TGC TA 3′ 5′ ACA TTC ACG ACC CTC CGT AT 3′	58°C, 36 cycles	1900	*Hha*I	Unlinked	*Arabidopsis thaliana* cDNA clone 113J6T7
CD1232	5′ TTT GTT GGA CAT TGG GTT CT 3′ 5′ GCA GAG CCT AAG TGA TTT GC 3′	58°C, 36 cycles	1500	*Hha*I	Unlinked	
CD1675	5′ AAG ATG GGG CTC AAT AAG TT 3′ 5′ GGC GGT CTC AGG ATT CTT AG 3′	58°C, 36 cycles	2300	*Hae*III	Unlinked	*Arabidopsis thaliana* cDNA clone 43B3T7
CD1195	5′ TCC CAC TGA ATC TCC TGT GA 3′ 5′ AAT GGC AAT GGC GTT ATT CT 3′	56°C 36 cycles	2800	*Hha*I	8	

[a] Annealing temperature and PCR cycles
[b] Polymorphic enzyme
[c] Mukai et al. (1995)
[d] Significant by BLAST homology test

Table 2 Allele frequencies of 13 loci in 11 natural populations of *C. japonica*

	Allele	Population											Mean
		Owani	Ani	Shizukuishi	Ishinomaki	Furukawa	Kawazu	Fuji	Shingu	Owase	Tsuyama	Yakushima	
CD657	*a*	0.278	0.375	0.268	0.288	0.260	0.318	0.400	0.174	0.316	0.071	0.375	0.275
	b	0.722	0.625	0.732	0.712	0.740	0.682	0.600	0.826	0.684	0.929	0.625	0.725
CD1237	*a*	0.963	0.939	0.911	0.935	0.940	0.864	1.000	0.978	0.974	0.929	1.000	0.953
	b	0.037	0.062	0.089	0.065	0.060	0.114	0.000	0.000	0.026	0.071	0.000	0.042
	c	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.022	0.000	0.000	0.000	0.006
CD526	*a*	0.093	0.021	0.000	0.078	0.180	0.043	0.125	0.022	0.000	0.167	0.000	0.077
	b	0.907	0.979	1.000	0.922	0.820	0.957	0.875	0.978	1.000	0.833	1.000	0.923
CD1309	*a*	0.889	0.958	0.804	0.839	0.800	0.857	1.000	0.826	0.944	0.738	0.917	0.865
	b	0.111	0.042	0.196	0.161	0.200	0.143	0.000	0.174	0.056	0.262	0.083	0.135
CD1894	*a*	0.759	0.875	0.893	0.938	0.900	0.957	0.958	0.978	0.947	0.881	1.000	0.945
	b	0.241	0.125	0.107	0.062	0.100	0.043	0.042	0.022	0.053	0.119	0.000	0.055
CD1216	*a*	0.037	0.125	0.036	0.141	0.060	0.043	0.000	0.043	0.211	0.071	0.042	0.076
	b	0.963	0.875	0.964	0.859	0.940	0.957	1.000	0.957	0.789	0.929	0.958	0.924
CD1706	*a*	0.593	0.667	0.518	0.547	0.360	0.591	0.625	0.652	0.868	0.524	0.625	0.599
	b	0.407	0.333	0.482	0.453	0.640	0.409	0.375	0.348	0.132	0.476	0.375	0.401
CD1067	*a*	0.500	0.479	0.429	0.391	0.520	0.304	0.292	0.326	0.368	0.405	0.292	0.362
	b	0.500	0.521	0.571	0.609	0.480	0.696	0.708	0.674	0.632	0.595	0.708	0.638
CD1091	*a*	0.463	0.313	0.357	0.313	0.440	0.370	0.375	0.391	0.342	0.571	0.208	0.376
	b	0.537	0.687	0.643	0.687	0.560	0.630	0.625	0.609	0.658	0.429	0.792	0.624
CD41	*a*	1.000	1.000	0.982	0.919	0.960	0.886	0.583	0.957	0.789	0.952	0.958	0.876
	b	0.000	0.000	0.018	0.081	0.040	0.114	0.417	0.043	0.211	0.048	0.042	0.125
CD1232	*a*	0.926	0.938	0.786	0.911	0.760	0.913	0.958	0.957	0.974	0.714	1.000	0.898
	b	0.074	0.062	0.214	0.089	0.220	0.087	0.042	0.000	0.026	0.262	0.000	0.091
	c	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.043	0.000	0.024	0.000	0.011
CD1675	*a*	0.574	0.979	0.440	0.469	0.500	0.273	0.375	0.457	0.395	0.262	0.500	0.404
	b	0.426	0.021	0.560	0.531	0.500	0.727	0.625	0.543	0.605	0.738	0.500	0.596
CD1195	*a*	0.426	0.000	0.161	0.145	0.000	0.286	0.125	0.091	0.000	0.075	0.125	0.106
	b	0.574	1.000	0.839	0.855	1.000	0.714	0.875	0.909	1.000	0.925	0.875	0.894

Table 3 The average heterozygosity (H_e), average number of alleles per locus (N_a), proportion of polymorphic loci (Pl) and unbiased heterozygosity

Population	n	Pl 95% level	N_a(SE)	H_e(SE)	Unbiased H_e(SE)
Owani	27	76.92	1.92 (0.08)	0.305 (0.101)	0.303 (0.055)
Ani	24	61.54	1.85 (0.14)	0.237 (0.061)	0.210 (0.054)
Shizukuishi	28	76.92	1.92 (0.08)	0.279 (0.085)	0.291 (0.051)
Ishinomaki	32	100.00	2.00 (0.00)	0.304 (0.100)	0.294 (0.043)
Furukawa	25	84.62	2.00 (0.17)	0.260 (0.073)	0.299 (0.050)
Kawazu	23	76.92	2.08 (0.08)	0.283 (0.087)	0.292 (0.044)
Fuji	12	61.54	1.77 (0.19)	0.272 (0.080)	0.275 (0.062)
Shingu	23	53.85	2.00 (0.00)	0.231 (0.058)	0.235 (0.052)
Owase	19	69.23	1.85 (0.14)	0.247 (0.066)	0.239 (0.054)
Tsuyama	21	92.31	2.08 (0.08)	0.275 (0.082)	0.298 (0.045)
Yakushima	12	53.85	1.69 (0.23)	0.199 (0.43)	0.218 (0.058)
Mean	22.36	76.92	1.93 (0.10)	0.277 (0.700)	0.281 (0.049)

Table 4 *F*-statistics

Locus	H_O	H_S	H_T	F_{IS}	Chi-square test			F_{ST}	Chi-square test		F_{IT}
					χ^2	df	P		χ^2	P	
CD657	0.236	0.404	0.408	0.416	42.60	10	< 0.01	0.009	4.40	ns	0.421
CD1237	0.090	0.098	0.099	0.081	1.60	20	ns	0.005	5.30	< 0.05	0.086
CD526	0.101	0.119	0.124	0.147	5.30	10	ns	0.042	20.40	< 0.05	0.183
CD1309	0.205	0.220	0.226	0.071	1.20	10	ns	0.027	13.30	ns	0.096
CD1894	0.146	0.148	0.153	0.012	0.00	10	ns	0.031	15.10	ns	0.042
CD1216	0.124	0.133	0.137	0.066	1.10	10	ns	0.026	12.90	ns	0.090
CD1706	0.467	0.465	0.482	− 0.004	0.00	10	ns	0.036	18.00	ns	0.032
CD1067	0.659	0.471	0.477	− 0.401	39.50	10	< 0.01	0.013	6.60	ns	− 0.382
CD1091	0.495	0.464	0.471	− 0.066	1.10	10	ns	0.013	6.60	ns	− 0.052
CD41	0.169	0.142	0.167	− 0.185	8.40	10	ns	0.150	73·70	< 0.01	− 0.007
CD1232	0.160	0.180	0.191	0.111	3.00	20	ns	0.061	60.40	< 0.01	0.166
CD1675	0.468	0.442	0.500	− 0.059	0.90	10	ns	0.116	57.00	< 0.01	0.064
CD1195	0.098	0.204	0.227	0.516	65.60	10	< 0.01	0.104	51.30	< 0.01	0.567
Mean	0.263	0.268	0.282	0.020	170.40	150	ns	0.047	345.10	< 0.01	0.067

Genotype distribution within each population

F_{IS} values of 3 loci showed deviation from Hardy-Weinberg equilibrium; 2 of these (CD657 and CD1195) had an excess of homozygotes and 1 (CD1067) an excess of heterozygotes. As a general explanation, excesses of homozygotes or heterozyotes may be the result of inbreeding or selection. The allele frequencies of CD657, CD1067 and CD1195 loci showed significant deviations from Hardy-Weinberg equilibrium by the G-test in 8, 10 and 6 populations, respectively. The mean F_{IS} values across populations for each locus showed similar tendencies. Generally, inbreeding can be expected to affect all loci equally, but it is likely that there are additional causes. These may include subdivided population structure and mixed sexual and asexual reproduction (by seed and natural layering, respectively) – a phenomenon typical of regions with heavy snow (Honda 1919; Taira et al. 1995, 1997). Some allozyme loci also showed some deviation, but there were no clear tendencies across populations (Tomaru et al. 1994).

Coefficients of linkage disequilibrium were calculated from genotype data pooled from all populations for 78 pairs of loci. No significant deviations were found, except for 4 combinations, which might have occurred by chance alone. In fact, 2 pairs of loci (CD657-CD526 and CD1309-CD1091) are linked, but their map distances are not small (Mukai et al. 1995) and no significant distortion was detected. Linkage relationships of 5 (CD1216, CD1706, CD41, CD1232 and CD1675) of the loci have not yet been confirmed by linkage analysis (Table 1).

Genetic diversity within and between populations

Tomaru et al. (1994) found the amount of genetic variation within the species and within populations of *C. japonica* to be somewhat higher than those reported for

other long-lived species of woody plants on the basis of allozyme analyses. In general, long-lived trees have a high degree of genetic variation within species and within populations compared to other plant taxa (Hamrick and Godt 1989; Hamrick et al., 1992). Hamrick et al. (1992) showed that the genetic variation parameters of long-lived trees (>191 species) had high average values. However, these values are mostly lower than those for *C. japonica* based on the results of the allozyme study (H_T, 0.196; *Pl*, 48.5%; N_a, 2.31; H_e, 0.189). In our STS-based study, we used only polymorphic loci to evaluate the genetic diversity of *C. japonica*, so we are not able to directly compare these data to those from the allozyme study. The average *Pl*, N_a and unbiased H_e values were 76.92%, 1.93 and 0.281, respectively, and only the N_a values was smaller than the corresponding value found in the allozyme analysis. The main reason for this lack of correspondence is the difference in the numbers of populations and individuals in the allozyme and STS-based studies investigated; in the allozyme study, 859 individuals from 17 populations were investigated (Tomaru et al. 1994), while we examined 246 individuals from 11 populations in this study, because the N_a value depends on the number of individuals investigated (Nei 1987). The average fragment size resulting from the restriction endonuclease digestions was 1286 bp. Using longer DNA fragments to survey nucleotide site differences between individuals by several kinds of enzymes, we may expect to detect more alleles per locus.

Natural forests of *C. japonica* have retained little differentiation among populations, as indicated by average F_{ST} values of 0.067, which are generally similar to that found in allozyme studies (Tsumura and Ohba 1992, 1993; Tomaru et al. 1994). Furthermore, our average estimate of unbiased genetic distance, 0.007, and the corresponding value from the isozyme study of 0.010 (Tomaru et al. 1994) also suggest that little genetic differentiation exists among populations. The Yaku Island population is considered to have been isolated at a great distance from the natural distribution on the mainland since before the last glacial period (Tsukada 1980, 1982, 1986). The genetic differentiation between populations on the mainland and Yaku Island was, however, found to be very small. In addition, two main lines, Ura sugi and Omote sugi, that were distinguished by both morphological variation and variation in diterpene components (Murai 1947; Yasue et al. 1987), did not appear to be so clearly distinct in the STS-based DNA marker analysis as they did in the allozyme study. The reasons for little genetic differentiation between populations in *C. japonica* are the following. Firstly, *C. japonica* is a wind-pollinated, outcrossing species with wind dispersal of seeds. Long-lived woody species, especially species with a large geographical range, tend to have low levels of genetic variation among populations (Hamrick et al. 1992). In coniferous species particularly, most of which are widely distributed now (Dancik and Yeh 1983; Lagercrantz and Ryman 1990) or have a history of having been widely distributed (Hiebert and Hamrick 1983; Niebling and Conkle 1990), the population structures tend to exhibit little genetic differentiation. This phenomenon is considered to be a result of a high rate of gene flow among populations. We indirectly estimated the mean number of migrants exchanged per generation, N_m, obtaining a mean value of 5.1 in this study and 7.1 in the allozyme study. Although the estimates should be treated with caution (Slatkin and Barton 1989), our average estimate of N_m is similar to those found in many other studies of conifers (Govindaraju 1989; Schuster et al. 1989). Secondly, the gene flows between natural-/artificial forests may be high. Afforestation with the species has been documented since the Muromachi era, about 500 years ago. Tsukada (1982, 1986) also postulated from the results of pollen analysis that for approximately 2500 years humans have planted *C. japonica* in moist, temperate climatic regions. The present plantations occupy 45% (4 436 000 ha) of the total plantation area in Japan, mainly in mountainous regions and areas surrounding the limited natural forests of the species. Consequently, the borders between natural and artificial forests have become blurred; the gene flow may have occurred frequently, probably even between natural and artificial forests of *C. japonica* (Tomaru et al. 1992). The genetic diversity between natural populations and plantations established by seedlings was also found to be very similar (Tomaru et al. 1992, 1994).

In the allozyme study, geographical differences were evident between the northern populations on the Japan Sea side, which had *6Pg-1*^{a} and *6Pg-1*^{e} as higher-frequency alleles, and the other populations, which had these alleles at lower frequencies or did not have them at all. However, in the STS-based DNA marker analysis, we observed no such geographical pattern.

We have demonstrated that there is little genetic differentiation among natural populations of *C. japonica* on the basis of STS marker analysis, essentially the same result as found in the allozyme study. Therefore, we may conclude that STS-based co-dominant markers are very useful not only for population genetics and genome mapping studies, but also for breeding programs like management of seed orchards and plus-tree clones. Use of these markers does not require special instruments like auto sequencers, just a PCR machine, an incubator and simple electrophoresis equipment. Of course, microsatellite DNA markers are also very useful if an auto sequencer is available, or if gels can be sequenced manually. However, we believe that PCR-RFLP based on STS markers is much more convenient and straightforward for small labs and breeding stations, where it may become a widely-used and popular method.

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