Theor Appl Genet (1997) 94:764–772

Y. Tsumura · Y. Suyama · K. Yoshimura
N. Shirato · Y. Mukai

Sequence-tagged-sites (STSs) of cDNA clones in *Cryptomeria japonica* and their evaluation as molecular markers in conifers

Received: 8 September 1996 / Accepted: 8 November 1996

Abstract We have generated 66 sequence-tagged-site (STS) markers from cDNA clones of *Cryptomeria japonica*, and 60% of them have already been mapped into *C. japonica* linkage groups. All of the STS markers showed a single fragment following polymerase chain reaction (PCR) amplification. We investigated by polymorphism of these STS markers in a mapped F_2 population and 15 plus trees by means of a restriction endonuclease analysis. Polymorphism levels were 10.6% and 22.7% in the F_2 population and the 15 plus trees, respectively. PCR amplification levels of the 66 STS markers in 14 conifer species varied depending on their genetic relationship with *C. japonica*. *Taxodium*, which is closely related to *C. japonica*, had the most amplifications (31.82%), followed by *Sequoiadendron giganteum*, which is of the same family. The average proportion of PCR amplifications in each family gradually declined in the following order: from Taxodiaceae to Cuppresaceae, Sciadopityaceae, Pinaceae, and Taxaceae. These results are in general agreement with a molecular phylogenetic relationship based on chloroplast DNA. The 66 STS markers will be useful as on anchor point for genome mapping and population genetics, and some of them will also be useful when studying other conifers.

Key words Linkage map · RFLP · STS · Conifers · *Cryptomeria japonica* · cDNA · Polymorphism · Phylogeny · Homology

Communicated by P. M. A. Tigerstedt

Y. Tsumura (✉) · Y. Suyama[1] · K. Yoshimura · Y. Mukai[2]
Bio-resources Technology Division, Forestry and Forest Product Research Institute, Kukizaki, Ibaraki 305, Japan

N. Shirato
Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Present addresses:
[1] Sugadaira Montane Research Center, University of Tsukuba, Sanada, Nagano 386-22, Japan
[2] Faculty of Agriculture, Shizuoka University, Shizuoka 422, Japan

Introduction

Sequence-tagged-site (STS) mapping has been used to construct a physical map of the human genome (Olson et al. 1989; Hudson et al. 1995). In plant species, an STS-based physical map has also been constructing for rice (Inoue et al. 1994). Some STS primers have been used to generate polymerase chain reaction (PCR)-based markers in barley (Tragoonrung et al. 1992), lettuce (Paran and Michelmore 1993), *Populus* (Bradshaw et al. 1994), and wheat (Talbert et al. 1994).

Genetic maps of conifer species using restriction fragment length polymorphism) (RFLP) have been constructed for loblolly pine (Devey et al. 1994), sugi (Mukai et al. 1995), and radiata pine (Devey et al. 1996). RFLPs have a stable genetic background but are limited by the need for clone banks and the requirement for large amounts of DNA. Meanwhile, megagametophyte-based mapping in conifers using random amplified polymorphic DNA (RAPD) has been widely adopted because of its speed, lack of clone banks, and requirement of very little DNA (Williams et al. 1990; Tulsieram et al. 1992). However, RAPDs also pose a problem since they often do not show simple Mendelian inheritance (Echt et al. 1992; Halward et al. 1992; Reiter et al. 1992). Therefore, it is necessary to develop a reliable and convenient PCR-based marker for genomic mapping and population genetics. Complementary DNAs (cDNAs) contain the exact coding regions of genes and express some protein or some closely related morphology. Therefore, the use of STSs of cDNAs to develop molecular markers may prove most useful for providing the anchor points of genomic

mapping and other genetic studies. In the investigation described in this paper, we developed STS markers from cDNA clones of *Cryptomeria japonica* and surveyed them for polymorphisms using RFLP analysis. We also evaluated the usefulness of the STS markers in other conifers.

Materials and methods

Sequence of cDNA clones and their sequence-tagged site

We used 66 cDNA clones for primer sequencing, half of which have already been mapped on RFLP linkage groups (Fig. 1; Mukai et al. 1995). These clones were sequenced by the dideoxy termination method using universal and reverse fluorescence dye primers with an ABI DNA sequencer model 373. We determined the nucleotide sequence for at least 200 bp at both ends of the cDNA inserts. PCR primers were carefully designed using the OLIGO program (version 4.0 National Bioscience) and then synthesized (Toagousei Co.).

The PCR amplification conditions were as follows: reaction mixtures (100 µl) contained 10 m*M* TRIS-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 9.1 m*M* each dNTP, 100 pmol of each primer, 50 ng of template DNA, and 2.5 units of *Taq* polymerase. A basic PCR amplification was carried out for 5 min at 94°C, followed by 32 cycles of 40 s at 94°C, 40 s at 60°C, and 80 s at 72°C, with a final 5-min incubation at 72°C with a PC700 model of Astech Co. or a MJ RESEARCH Programmable Thermal Controller PTC100. The DNA fragments were checked on ethidium-stained 0.85% agarose gels. When there was a low reaction yield, the number of PCR cycles was increased to 36, 40, and 42 cycles or the annealing temperature was decreased to 58°C and 56°C. If multiple PCR fragments were observed, the annealing temperature was increased until only a single band remained.

Polymorphism of STS markers

PCR amplification was done using both cDNA and genomic DNA as templates for each STS, and the fragment size of the PCR products of the two templates was then compared. Nineteen STS markers which contained introns were selected from 66 STSs. The 19 STSs were investigated using the PCR-RFLP method in 15 plus trees to evaluate the polymorphism level of this kind of marker. These 15 plus trees mostly originated from locations distributed throughout their natural distribution in Japan, and they were selected from artificial stands in each region. One sample of *Cryptomeria fortunei* which originated in China was also included in the sample of 15 plus trees.

After PCR amplification, the products were purified using ethanol precipitation, and the purified DNA was then digested with seven different four base-cutter enzymes (*Alu*I, *Hae*III, *Hha*I, *Msp*I *Hin*fI, *Taq*I, and *Rsa*I) and electrophoresed on 2% agarose gels. Polymorphisms of the other 47 STSs were also investigated in the Okinoyama-sugi (♀) × Kumotooshi (♂) family (Mukai et al. 1995) and 15 plus trees using only one restriction endonuclease to make a new molecular marker in *Cryptomeria japonica*. Of the five restriction endonucleases (*Alu*I, *Hae*III, *Hha*I, *Msp*I, and *Rsa*I), we selected the one which had the most restriction sites for each STS. Because the size of each STS was not large, only a few restriction sites were

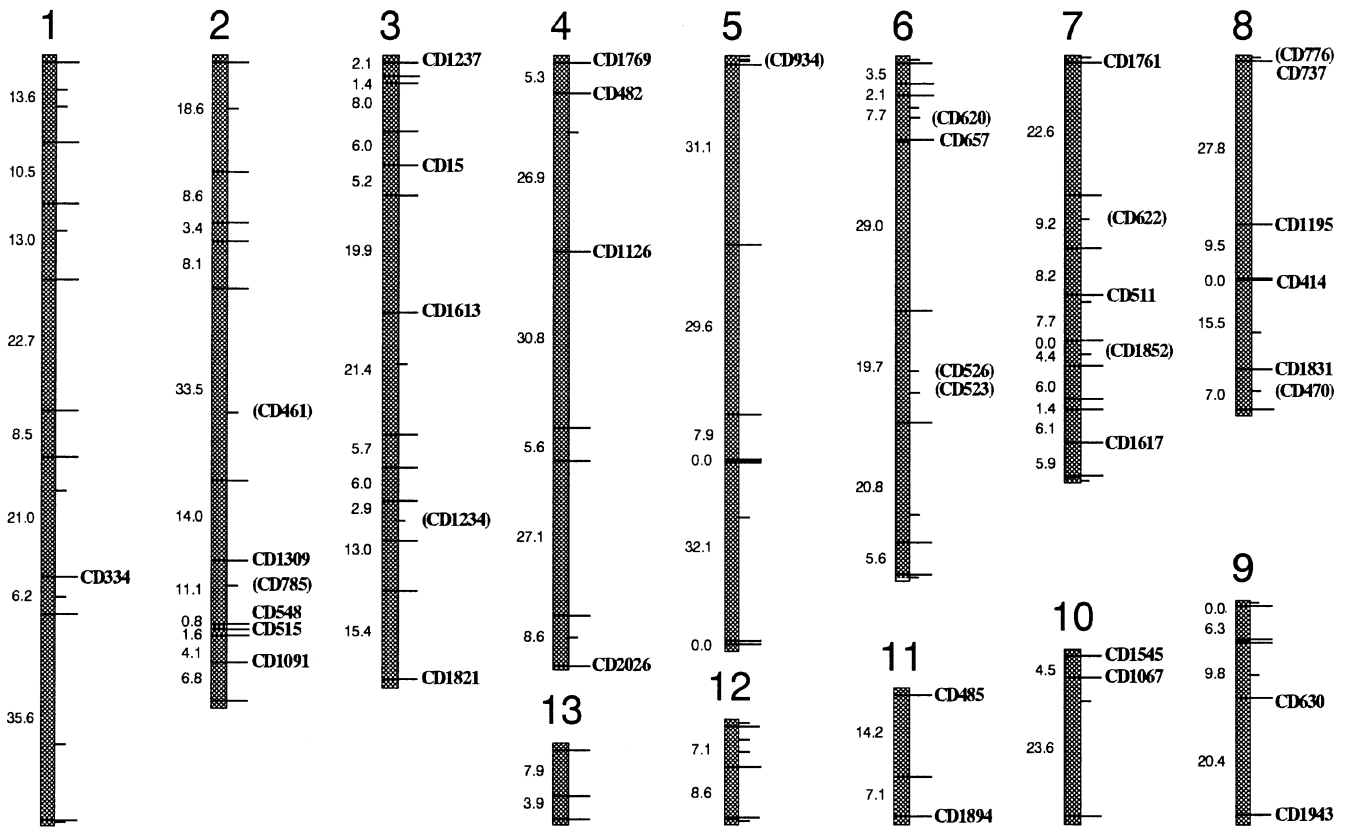

Fig. 1 The location of loci generating the STSs. *Long* and *short bars* indicate loci for which map positions were determined or ambiguous, respectively. The map distance in centiMorgans (cM) are shown on the *left* (Mukai et al. 1995)

expected even from the four-base cutters. Their sizes ranged from 196 bp to 2000 bp, with an average, size of 662.12 bp.

PCR amplifications in other conifers

PCR amplifications of the 66 STS markers were tried in 14 species of conifer under two different PCR conditions in order to determine if these markers could be useful in other conifers. The first, or high-stringency, PCR conditions were same as those found in Table 1; the second (i.e., low-stringency) PCR conditions were a modification of the basic PCR conditions as follows: annealing temperature of 52°C, 2.5 m*M* $MgCl_2$, and 40 cycles. After PCR amplification, the DNA fragments were checked on an ethidium-stained 0.85% agarose gel. The following species were investigated: 7 Taxodiaceae species (*Taxodium disticum*, *Athorotaxis cupressoide*, *Metasequoia glyptostroboides*, *Sequoiadendron giganteum*, *Sequoia sempervirens*, *Taiwania cryptomeioides*, *Cunninghamia lanceolata*), 2 Cupressaceae species (*Chamoecyparis obtusa*, *Thuja standishii*), 3 Pinaceae species (*Abies firma*, *Pseudotsuga japonica*, *Pinus taeda*), 1 Sciadopityaceae species (*Sciadopitys verticillata*), and 1 Taxaceae species (*Taxus cuspidata*).

Table 1 STS primers and their PCR amplification conditions

STS no.	Primer sequence (forward and reverse)	Annealing temp. (°C)	PCR conditions[a]		Length (bp)[c]	Linkage group[d]
			Cycles	Tm[b]		
CD334	5′ AAG GCG TGT GAG AAT CCA GT 3′	60	32	63.3	883	1
	5′ GTC ATG CAA GCC AAC AAT AA 3′			61.0		
CD1309	5′ AGC AAA ACC TTG GGA TTC TT 3′	60	32	62.8	1284	2
	5′ TAG AGC CGC ACT ATT CAG AT 3′			59.4		
CD548	5′ CAC ACC CGT TCC TTT ATT CT 3′	60	32	61.1	507	2
	5′ AGG TTG TGG ACT TGG ATT TG 3′			61.2		
CD515	5′ GCC TGG ACA ACT CAT TGC TA 3′	60	32	62.4	823	2
	5′ GAA AGT GGA AAG GGC AGT AA 3′			61.5		
CD1232	5′ TTT GTT GGA CAT TGG GTT CT 3′	58	32	61.7	927	Unlinked
	5′ GCA GAG CCT AAG TGA TTT GC 3′			61.9		
CD1237	5′ GGA ATC GGA TGG GTT ATC TG 3′	60	32	63.5	915	3
	5′ AGA ATC CGG GAC CAA ATC TA 3′			63.0		
CD1613	5′ GGT GAA CAA GAA AGG GAA AT 3′	58	32	60.2	852	3
	5′ ATG TGT TGT CTG GCT TGG TA 3′			59.7		
CD1821	5′ TGC GTA TGG ATG GTG AAA AG 3′	58	32	63.4	384	3
	5′ CCA AAA GTA GCC ACA GAA GA 3′			59.6		
CD1769	5′ CTT CCT GTT TGG CAA TGA GT 3′	60	32	61.6	791	4
	5′ GAT TAT GGA GGT GCC TGC TA 3′			61.9		
CD620	5′ AGG CCA AAC CCT CAG AAG TA 3′	56	32	63.2	333	6
	5′ GCT GGG AAG TCC TCT AAG AA 3′			60.4		
CD526	5′ TCT TGC ATG ACT TGG TTG CT 3′	60	32	62.7	1625	6
	5′ GGG GAT TTG GAG ATT TTC AG 3′			63.0		
CD523	5′ GGT TAC CCA GGG GAG GTC TT 3′	58	32	66.2	251	6
	5′ ACA AAT GCG CCT TGA AAT AC 3′			62.5		
CD622	5′ CCC TGG TAC TCC TGT GGT AA 3′	58	32	61.3	373	7
	5′ CCG GCA GTG TAA TCA CCA AC 3′			65.1		
CD1514	5′ GGT CGG TCT GAC ATT CCA TT 3′	58	32	63.2	563	7
	5′ CGA GAA GCG TCC AAA CAT TA 3′			63.2		
CD1852	5′ GCA TAG CAT TTT CCC AAT CA 3′	58	36	62.9	483	7
	5′ AAG GGA TCG AAG AGG GTC AT 3′			64.2		
CD19	5′ TCA AAC AGC GTA GTC AAC CA 3′	58	32	61.0	996	Unlinked
	5′ TGG AAG GAA AGG AGG AGG AT 3′			62.1		
CD41	5′ GAA TCC AAA ACC ACT TGC TA 3′	58	32	59.6	1067	Unlinked
	5′ ACA TTC ACG ACC CTC CGT AT 3′			63.2		
CD95	5′ GTG GGA GAG CGA GTC AAC AT 3′	60	32	63.4	805	Unlinked
	5′ GAT TGA AAT TGG CGA GAG AT 3′			61.0		
CD471	5′ AGG CTC TGC TCT CCA TCT GT 3′	60	40	62.5	443	Unlinked
	5′ GCC GCA GTG CAT AGG ATT AC 3′			65.1		
CD579	5′ CAC TGG CCT AAG AAT CAG AA 3′	60	32	59.3	639	Unlinked
	5′ GAA TCC TGC TCA CCC AAC AC 3′			63.7		
CD657	5′ TCC TGA TAC TGT GGG CAA CT 3′	60	32	61.5	703	6
	5′ CCC CGA TAT GCT CTT CAA CT 3′			63.6		
CD1761	5′ AGT CAA CTC AAT GCC CTC AA 3′	60	32	61.6	486	7
	5′ TAA ACA GGT CGC TCC CAG AT 3′			63.8		
CD630	5′ TCG AGA CGT ACT GGT GGT GT 3′	60	32	61.8	530	9
	5′ CTC CCA AGG CTG AGA AGA AA 3′			63.7		
CD1943	5′ GGG GAC AGA TCC AAC TAA CA 3′	60	32	60.9	613	9
	5′ GCC ATT TTA TTT GCC ACA GA 3′			63.1		
CD1545	5′ TTG CTG ATG AAC GGA AAC TT 3′	60	36	62.3	1995	10
	5′ TGA GCG AAA CAG AGC CAT AA 3′			63.4		

Table 1 Continued

STS no.	Primer sequence (forward and reverse)	Annealing temp. (°C)	PCR conditions[a]		Length (bp)[c]	Linkage group[d]
			Cycles	Tm[b]		
CD1067	5′ TTT AGG GTT TTG GGT TTT AG 3′	58	32	59.4	724	10
	5′ AAC ATA CCA TCT GCC CTC TT 3′			60.3		
CD1894	5′ ACC CTT TCC TCG CCT ACA TT 3′	60	36	65.0	767	11
	5′ GCC GAC TGA GTA AAC AAA CC 3′			61.5		
CD1234	5′ CCG TGG AAA GAT GAG CAC TA 3′	62	32	62.5	442	3
	5′ AGC CGT TGT TGC CAT TCT TA 3′			65.2		
CD482	5′ GGG GTT TCG TCC AAG GTT AT 3′	60	32	65.0	611	4
	5′ TCC ACA GGG TTT TTG CTT CA 3′			65.4		
CD511	5′ ATA AAC CTT GCC ACA CTC TA 3′	62	36	61.1	443	7
	5′ AAG GCA CTT GAA TCA TCC AT 3′			60.9		
CD414	5′ GCA TGA TGG AGC AAA TGG TA 3′	62	32	63.1	519	8
	5′ TAG ATG GGC AAT GCT TGG AG 3′			65.2		
CD1617	5′ TGC CAA CTC CAC CTA CTT CA 3′	62	32	62.6	1478	7
	5′ TAT GGC CCT CAA AAC AGT TC 3′			61.9		
CD737	5′ CAA CCA GGG GAG TAT TGA TG 3′	58	32	61.6	405	8
	5′ TGC ACC CAA TTA TTT TCA CA 3′			61.4		
CD1195	5′ TCC CAC TGA ATC TCC TGT GA 3′	58	40	61.5	2000	8
	5′ AAT GGC AAT GGC GTT ATT CT 3′			64.4		
CD1831	5′ GAC CAT TGT CAC CAC TGT CA 3′	60	36	59.4	360	8
	5′ ATA CCG TGC ATT GGG TTA CT 3′			61.5		
CD1126	5′ CAC AGC ATA CCC CAC TAC CC 3′	60	36	64.1	336	4
	5′ GGC TTG TAG CGG AAG AAA GA 3′			64.3		
CD461	5′ AGG CGA TGC TTA CAG AGG TG 3′	60	32	64.4	307	2
	5′ CGT AGC CAG GAT TAT GAC CA 3′			62.6		
CD470C	5′ GGC CTA CGA ACA TTG AAC AT 3′	60	32	61.7	536	8
	5′ ACC CTT ACC GGA AAG ACC AT 3′			64.6		
CD485	5′ TTG ATG CCA CAC AAA GAC AA 3′	60	40	61.4	196	11
	5′ TGA GCA ATT ATG CCA AGC AG 3′			64.0		
CD776C	5′ CTT GCT CAC CGC ATA AAC TG 3′	58	36	63.5	595	8
	5′ GTT GAT CTG CGT GGG TTT AG 3′			62.2		
CD785	5′ TGT AAT GGG TTT CGG CTT CT 3′	58	32	64.2	271	2
	5′ AGT GCG TCA TGG TTT CTT CA 3′			62.5		
CD1841	5′ ATG AGC ACC AGC AAC GAT GT 3′	60	32	64.9	543	5
	5′ GTA CGG GGT TAG AAC TTG GA 3′			61.1		
CD857	5′ ACC CTG ATG CAG CGA TTT CT 3′	60	32	66.2	421	Unlinked
	5′ ACG ACA ATT CCA AGG TCC TC 3′			62.8		
CD1712	5′ CCA GGT CAG CAG CAG ACT AA 3′	60	32	62.5	810	Unlinked
	5′ CAT TGC TGG GCT GGT GAA TA 3′			65.9		
CD133	5′ AAC AGC AAC CCC AAT GAA AG 3′	60	32	64.3	390	Unlinked
	5′ CTC TGC AGA CCC AAC TGG AA 3′			64.6		
CD618	5′ CAA GGA CAA CGG GCA AAA AT 3′	60	32	67.0	1491	Unlinked
	5′ GAA CTG GGT TCC AAG GCT AT 3′			62.7		
CD568	5′ CAA GAG CGA GAA GAG CAG AA 3′	58	36	62.2	745	Unlinked
	5′ ATT GGA ATC AGG AGG AGA AG 3′			59.7		
CD15	5′ CAG AGC AAC CGC CAC AAG AG 3′	60	32	68.0	393	3
	5′ TGA GGC GGC GAT AAC TTG TA 3′			66.1		
CD541	5′ CTG CAT GAC TGG ACT GAA CT 3′	60	36	58.7	656	Unlinked
	5′ TGA GGT GAA GAA GGA GAA CA 3′			58.7		
CD312	5′ CCC GAC GGA CCA ACA GAA CT 3′	60	32	68.6	662	Unlinked
	5′ CCC CTG TGC CGT TCC AAT AC 3′			69.2		
CD402	5′ CCT GCC CAT GGT GAA AGT AA 3′	58	36	65.0	306	Unlinked
	5′ TTG AAT CCA GAG GCT TGA AA 3′			62.3		
CD574	5′ GGA GCT TGA GAA GGA ACC TA 3′	60	32	60.4	419	Unlinked
	5′ AAA GCG CAT CTC CAA ACA CT 3′			64.2		
CD671	5′ AAA AGC ACA ACT TCC TGA CA 3′	56	36	59.5	866	Unlinked
	5′ GAG TGG AGA AGG CAA TGA GA 3′			61.0		
CD672	5′ GAG TCT GGG AAC TGG TTG TC 3′	60	32	59.9	347	Unlinked
	5′ CGA GCA CTT TCT GAG GAG AG 3′			61.3		
CD1064	5′ TGA AAA ATG TCG TTG GTG TG 3′	58	36	61.1	377	Unlinked
	5′ TGC CTC AGG GTT TAG CAG TA 3′			62.7		
CD1071	5′ GAT CGT ACC CAG CCC TTT TT 3′	58	36	65.4	583	Unlinked
	5′ CAG AGC TTC TGG GGT CTT TA 3′			61.3		
CD1117	5′ TCA CAT TCG GCA CAA GTT GA 3′	56	36	64.0	752	Unlinked
	5′ ATA TGA ACC GCC CAA AAA TC 3′			63.9		

Table 1 Continued

STS no.	Primer sequence (forward and reverse)	Annealing temp. (°C)	PCR conditions[a]		Length (bp)[c]	Linkage group[d]
			Cycles	Tm[b]		
CD1624	5′ CTC TCC GCA ACG AAG GAA AA 3′	58	36	67.3	443	Unlinked
	5′ CAC CGC GAG AAC ACG ATT AT 3′			65.2		
CD1640	5′ TCG GCA GCA TGA CAG AAA AC 3′	58	36	65.8	1204	Unlinked
	5′ GAT CTT CGG CAG CAT CTC TT 3′			63.4		
CD1675	5′ AAG ATG GGG CTC AAT AAG TT 3′	56	36	60.3	1767	Unlinked
	5′ GGC GGT CTC AGG ATT CTT AG 3′			63.2		
CD1706	5′ ATA GGC GAC GCA GGT CAA AA 3′	58	36	67.6	387	Unlinked
	5′ TCT GCG GCT GTA GTT CCA GT 3′			64.7		
CD1216	5′ GCC AAG ACC CTG AGC AAA TC 3′	58	36	66.2	739	Unlinked
	5′ CCT GTG CGA AAG CCA ATC AA 3′			68.6		
CD1179	5′ TGG GTT TGG GCA TAA GTC TG 3′	56	36	65.0	1101	Unlinked
	5′ TTG CCC CTG TTG TTT TAT CC 3′			64.5		
CD1444	5′ GGC AGA CAA GGC TTA CAA GA 3′	58	36	62.0	583	Unlinked
	5′ CCT GCG ACA TAG ACA AAT CA 3′			60.6		
CD2026	5′ TGT GAA TAT GGC GAG TGC TA 3′	56	36	61.4	784	4
	5′ GCT TTG AGA CAA GGT GGT AT 3′			58.2		
CD1091	5′ GGT TTT CTT CAG AGG CAA GG 3′	58	36	63.4	683	2
	5′ GAA ACC CTG GAC TGG CAT AG 3′			63.6		

[a] Basic PCR amplification was carried out for 5 min at 94°C, followed by 32 cycles of 40 s at 94°C, 40 s at 60°C, and 80 s at 72°C, with a final 5-min incubation at 72°C. Only modified conditions are shown
[b] Melting temperature
[c] PCR-amplified fragment size from genomic DNA
[d] Mukai et al. (1995)

Results

PCR amplification of STSs

A single fragment was observed for all 66 STS primer sets after PCR amplification using Okinoyama-sugi (♀), Kumotooshi (♂), and 142 (F_1) genomic DNAs (Mukai et al. 1995) (Fig. 2). PCR products of the three samples gave approximately the same yield and did not show any size variation. PCR conditions for each STS was decided independently, but we were able to use a basic PCR protocol, for about 40% of them. In the case of low yield and/or multiple bands, we modified the PCR conditions (Table 1).

Polymorphism detection in the Okinoyama-sugi (♀) × Kumotooshi (♂) family and 15 plus trees

PCR amplification of 66 STSs was carried out in each PCR condition in the Okinoyama-sugi (♀) × Kumotooshi (♂) family and 15 plus trees. As 2 of the plus trees did not yield any PCR products, one of the STSs, CD579, may be a deletion mutant. To evaluate levels of polymorphism, we digested the 19 STSs which contained a intron with the seven different four-base cutter enzymes. The digested products were electrophoresed on 2% agarose gels (Fig. 2). Restriction fragment length

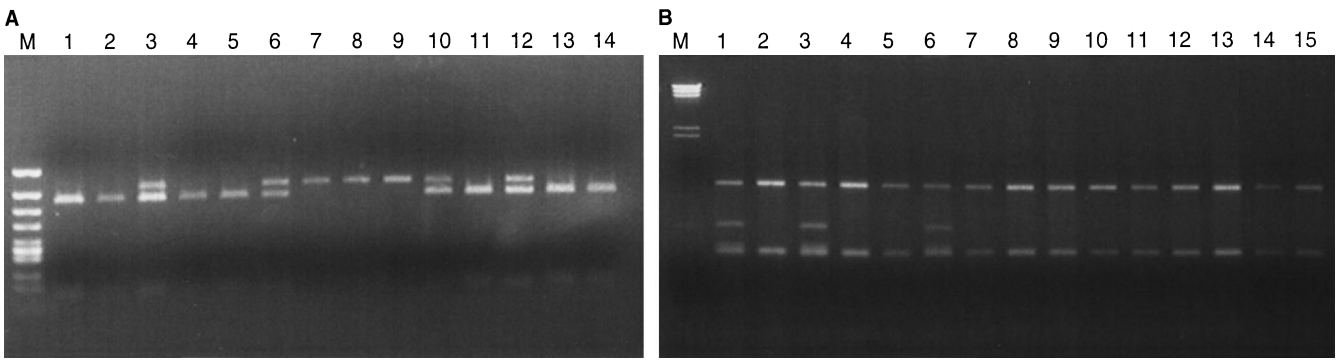

Fig. 2A, B RFLP patterns of STS markers in 15 plus trees. **A** CD657 STS digested by *Hha*I, **B** CD1309 STS digested by *Alu*I. *M* Molecular marker, *lanes* 1–15 plus trees

Table 2 Sixteen STS markers showing polymorphisms using a restriction endonuclease analysis

STS	PCR conditions[a]			Intron (bp)[b]	Length (bp)	Linkage group[c]	RFLP in plus-tree[d]	RFLP in F_2[e]	Polymorphic enzymes
	Annealing temp (°C)	Cycles	Extension time (s)						
CD41	58	32	80	147	1067	Unlinked	1		*Hha*I
CD471	60	40	80	177	443	Unlinked	1		*Hin*fI
CD526	60	32	80	0	1625	6	1	1	*Rsa*I, *Hae*III
CD657	60	32	80	112	703	6	1	1	*Msp*I, *Hae*III, *Rsa*I, *Hin*fI, *Hha*I
CD671	56	36	80	0	866	Unlinked	1		*Rsa*I
CD776	58	36	80	0	595	8	1		*Alu*I
CD1067	58	32	80	159	724	10	1	1	*Alu*I, *Hae*III, *Rsa*I
CD1091	58	36	80	0	683	2	1		*Alu*I
CD1195	58	40	120	841	2841	8	1	1	*Hha*I, *Hae*III
CD1216	58	36	80	0	739	Unlinked	1		*Hha*I
CD1237	60	32	80	265	915	3	1		*Hha*I, *Msp*I, *Rsa*I, *Alu*I
CD1309	60	32	80	442	1284	2	1	1	*Alu*I, *Rsa*I, *Hin*fI, *Msp*I
CD1545	60	36	80	1000	1995	10	1		*Hae*III, *Taq*I, *Alu*I, *Hin*fI
CD1675	56	36	80	644	1767	Unlinked		1	*Alu*I, *Hae*III, *Rsa*I, *Nde*II
CD1706	58	36	80	0	387	Unlinked		1	*Hha*I
CD1894	60	36	80	0	767	11	1		*Rsa*I

[a] PCR reaction was conducted under basic conditions (see methods) but only modified conditions are shown
[b] Length of each intron
[c] Mukai et al. (1995)
[d,e] Number 1 showed a polymorphism

polymorphisms were detected in 9 (47.4%) of the STSs (Table 2). Of these 19 STSs, 3 showed variation with only a single enzyme, while the others showed variation with at least two different enzymes. Polymorphism in the other 47 STSs was investigated by selecting an enzyme for each STS which was expected to yield the most restriction sites. In this latter survey, we found 8 polymorphic STSs (17.0%). In all, we found 16 (24.2%) polymorphic STSs using RFLP analysis. We were also able to compare STSs with and without introns (Table 3). The only difference between the two kinds of STSs was size, with the average length of an STS with an intron being larger than that without an intron. Polymorphism levels were greater in STSs with introns (26.26%) than in STSs without an intron (18.09%).

Table 3 Polymorphisms of 16 STSs in 15 plus trees using RFLP analysis

STS	Intron (bp)	Length (bp)	Polymorphism (%)[a]
With intron			
CD1675	644	1767	13.00
CD41	147	1067	20.00
CD1067	159	724	23.33
CD1195	841	2841	13.33
CD1237	265	915	13.33
CD1309	442	1284	13.33
CD657	112	703	56.66
CD471	177	443	40.00
CD1545	1000	1995	43.33
Mean	420.78	1304.33	26.26
No intron			
CD776	–	595	13.33
CD1706	–	387	20.00
CD1091	–	683	40.00
CD526	–	1625	6.66
CD1894	–	767	33.33
CD671	–	866	6.66
CD1216	–	739	6.66
Mean	–	808.86	18.09

[a] This polymorphism was estimated by the following formula: $P = (n - x)/n \times 100$, where n is the total number of individuals investigated, and x is the number of major variants

PCR amplification of STSs generated from *Cryptomeria japonica* in conifers

PCR amplifications of the 66 STSs were carried out in 14 conifers under two different PCR conditions (Fig. 3). The amplification results are shown in Table 4. Under high-stringency conditions, which were the same as those used for *Cryptomeria japonica*, the sizes of the PCR products were not different from those from *C. japonica*. In *Taxodium*, 31 STS markers could be amplified, but in the other Taxodiaceae species the number of amplified STSs was only 4–14. The average number of amplified STSs found in Taxodiaceae species was 12.00 (18.18%) out of 66 STSs; for the 2 Cupressaceae species this was 10 (15.15%) and 6 (9.09%), respectively. The 3 Pinaceae species used for STS amplification showed fewer amplifications than the Taxodiaceae and Cupressaceae species. For *Sciadopitys verticillata*, which has been a problem taxonomically, speaking only 4 STSs

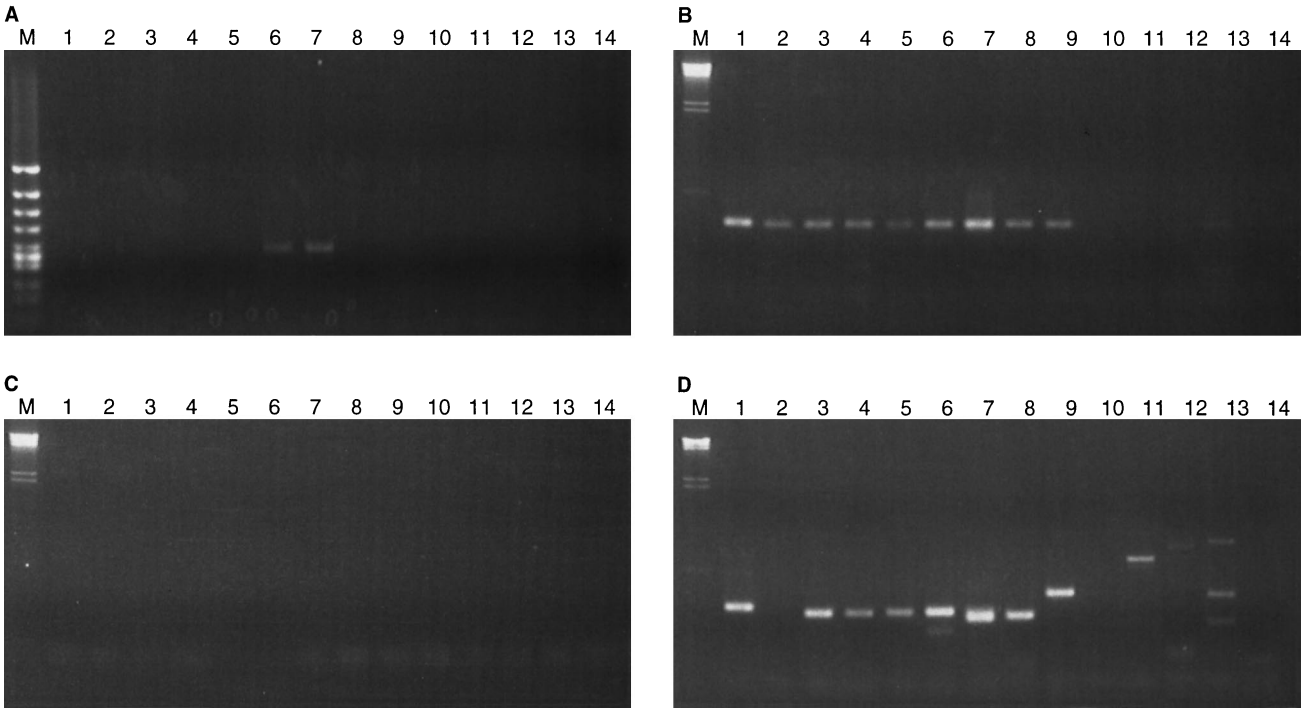

Fig. 3 PCR amplification of STS marker in 14 conifers. **A** CD622 STS under high-stringency conditions, **B** CD622 STS under low-stringency conditions, **C** CD1831 under high-stringency conditions, **D** CD1831 under low-stringency conditions. *M* Molecular marker, *lanes* 1–14 conifer species (see Table 4)

were amplified, as in Pinaceae. In Taxaceae, no STSs were amplified.

Under low-stringency conditions, more PCR fragments were observed than under high-stringency conditions. While the number of PCR amplifications increased in most species, in 4 species, *Taxodium*, *Sequoiadendron*, *Taiwania* and *Cunninghamia*, the number decreased from the point of view of a single fragment. This was especially true for *Taxodium* in which the number decreased from 31 to 18. This decreased number of single fragment STSs is offset by many with multiple fragments. In Cupressaceae, Sciadopitaceae, Pinaceae, and Taxaceae species, the number of single-fragment PCR products increased and the number of multiple PCR fragments increased even more. Overall, the proportion of amplified STSs under low-stringency conditions including a single and multiple fragments, was 60.71%, 54.17%, 53.33%, 51.11%, and 18.33% in Taxodiaceae, Cupressaceae, Sciadopitaceae, Pinaceae, and Taxaceae, respectively.

Discussion

STS of cDNA clones and their polymorphism

We generated 66 STSs from cDNA clones of *C. japonica*, half of which had already been mapped (Mukai et al. 1994). We surveyed the genetic diversity of these STSs using a restriction endonuclease analysis. Sixteen of the STSs were polymorphic with more than one restriction endonuclease. Most of the segregation patterns were just like those for co-dominant traits; however, a few segregation patterns were not. The reason these patterns were considered is that these STSs may not be single copy. While we selected for single- or low-copy cDNA clones when we screened the RFLP probes during mapping, some of them must have been multiple-copy clones. These co-dominant markers will be most useful as landmark STSs for future mapping of *Cryptomeria*. Of the 16 polymorphic STSs 9 have introns with lengths varying from 112 bp to 841 bp (Table 2). We expected STSs having introns to be more polymorphic; however, differences between STSs with and without introns were not significant (Table 3). This kind of polymorphism would be caused mainly by point mutations; therefore, these events are closely related to the mutation rate of the nuclear genome. Generally, the sequences of cDNAs are more highly conserved than other regions, and the expressed sequence regions would be more highly conserved than the introns. Accordingly, STSs with introns might be expected to have much higher polymorphism levels than those without introns, as shown in Table 3.

Application of *Cryptomeria* STS markers to other conifers

The average number of amplified STSs in observed Taxodiaceae was 12.00, which is about one-fifth of the

Table 4 PCR amplification of 66 STS markers generated in *Cryptomeria japonica* in 14 conifers

	Number of amplified STSs				Proportion of amplified STSs (%)		
	High conditions[a]		Low conditions[b]		High conditions[a]	Low conditions[b]	
	Single[c]	Multi[d]	Single	Multi		Single	Single + Multi
Taxodiaceae							
1 *Taxodium distichum*	31	–	18	27	46.97	27.27	75.00
2 *Athrotaxis cupressoides*	9	–	12	21	13.64	18.18	55.00
3 *Metasequoia glyptostroboides*	4	–	11	28	6.06	16.67	65.00
4 *Sequoiadendron giganteum*	9	–	8	21	13.64	12.12	48.33
5 *Sequoia sempervirens*	14	–	17	17	21.21	25.76	56.67
6 *Taiwania cryptomerioides*	7	–	6	24	10.61	9.09	50.00
7 *Cunninghamia lanceolata*	10	–	12	33	15.15	18.18	75.00
Average	12.00	–	12	24.43	18.18	18.18	60.71
Cupressaceae							
8 *Chamaecyparis obtusa*	10	–	21	16	15.15	31.82	61.67
9 *Thuja standishii*	6	–	15	13	9.09	22.73	46.67
Average	8.00	–	18.00	14.50	12.12	27.27	54.17
Sciadopityaceae							
10 *Sciadopitys verticillata*	4	–	8	24	6.06	12.12	53.33
Pinaceae							
11 *Abies firma*	2	–	5	26	3.03	7.58	51.67
12 *Pseudotsuga japonica*	2	–	5	21	3.03	7.58	43.33
13 *Pinus taeda*	4	–	4	31	6.06	6.06	58.33
Average	2.67	–	4.67	26.00	4.04	7.07	51.11
Texaceae							
14 *Taxus cuspidata*	0	–	3	8	0.00	4.55	18.33

[a] Basic PCR conditions (see Methods)
[b] PCR conditions were the same as basic PCR conditions except for an annealing temperature of 52°C, 2.5 m*M* Mg_2Cl_2, and 40 cycles
[c] A single fragment was amplified
[d] Several fragments were amplified

total number of STSs found under high-stringency conditions. The number of amplified STSs decreased in the following order: Cupressaceae, Sciadopyaceae, Pinaceae and Taxaceae. The number of amplified STSs shared with *Cryptomeria* is not considered to be related to the phylogeny of these families. The molecular phylogenies of the Taxodiaceae based on a PCR-RFLP analysis of chloroplast genes (Tsumura et al. 1995) and the sequence of *rbc*L (Brunsfeld et al. 1994) are also very similar to our results. On the basis of those studies, *Taxodium* is the species most closely related to *Cryptomeria*, and in our study approximately half of their STSs could be amplified, the highest of any of the species studied. The values for the 2 Cupressaceae species were not very different from those of the Taxodiaceae, indicating that these two families may be closely related. Molecular phylogenies have shown very similar results (Tsumura et al. 1995; Brunsfeld et al. 1994). In the 3 Pinaceae species, fewer STSs were amplified than in Taxodiaceae and Cupressaceae. Physical maps of cpDNA between *C. japonica* (Tsumura et al. 1993) and Pinaceae species (Strauss et al. 1988) were quite different from each other. Our results show that the nuclear genomes may not be closely related in these families either. *Sciadopitys verticallata*, a taxonomic problem, amplified as few STSs as Pinaceae species. This result may indicate that the evolutionary position of this species is not within the Taxodiaceae; molecular phylogenies based on chloroplast (cp) indicate the same DNA. However, since the amplified fragment sizes were exactly, the same as those of *Cryptomeria*, the fragments may be the same genes as found in *Cryptomeria japonica*.

Under low-stringency conditions, the number of amplified STSs with only a single fragment mainly increased, especially in Cupressaceae, Sciatoptyaceae, Pinaceae and Taxaceae, in which the number doubled. The amplified fragment sizes were mostly the same as those of *Cryptomeria*, but some did change. Under the low PCR conditions, many multiple bands were observed, although most had only a few bands. In the Taxodiaceae species, *Taxodium*, *Sequoiadendron*, and *Taiwania*, the number of amplified STS with a single fragment decreased because some amplified STSs have become multiple-banded. This is not a problem because under a modified PCR conditions these amplified fragments may be a single band. Therefore, if the STSs generated in *Cryptomeria* are to be used in other species, the PCR conditions must be decided separately for each STS and species.

The STSs of CD1613, CD1769, CD1821, CD471, CD620, CD1514, CD618, CD776, CD785, CD312,

CD1147, and CD1064 were amplified in more than 5 species. It is likely that these cDNA sequences are relatively conserved in conifers. Four of them also showed a homology at a high significance with specific genes using BLAST (Altschul et al. 1990). These are: CD471, homologous to *Arbidopsis thaliana*-transcribed sequence clone YAP078T; CD620, homologous to *Arabidopsis thaliana* cDNA clone 118A8T7; CD1514, homologous *Pinus sylvestris* CHS gene for chalcone synthase; and CD312, homologous to *Linum usitatissimum* allene oxide synthase mRNA. About 60% of STS cDNA sequences showed a hit with a BLAST homology test (data is not shown). The sequence of cDNA usually is thought to be conserved in related species, genera, or families. Therefore, if the primer sequence was changed somewhat, the probability of PCR amplification in conifer species might be increased.

We have tried to detect polymorphisms in these STSs using RFLP analysis in other conifers. The CD1237 STS digested by *Msp*I showed polymorphism in *Taxodium* (data not shown). If the RFLP analysis had been conducted in each species, variation within species might have been detect. Other methods such as SSCP or CFLP might be more efficient in detecting more variation within species. The STS markers could also be used as DNA probes for conifers, in which case, we might expect a lot of variation within species (Ahuja et al. 1994). Use of the same DNA markers as those for genome mapping will be valuable for understanding speciation and evolution within a family or genus of related species.

The STS markers which we are developing in *Cryptomeria japonica* might also be useful as anchor points of genome mapping for quantitative trait loci (QTL), and MAS (marker-assisted selection) and in the study of genetic diversity and structure of natural forests in the future.

Acknowledgements The authors thank Ms. M. Koshiba for technical assistance with the experiments and Drs. T. Kondo, H. Yoshimaru and T. Kawahara for helpful comments. We also thank Ms. Deborah R. Overath of The University of Georgia for a critical reading of the manuscript. This study was supported in part by a grant from Global Research on Biotechnological Plant Breeding of the Ministry of Agriculture, Forestry and Fisheries.

References

Ahuja MR, Devey ME, Groover AT, Jermstad KD, Neale DB (1994) Mapped DNA probe from loblolly pine can be used for restriction fragment length polymorphism mapping in other conifers. Theor Appl Genet 88:279–282

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bradshaw HD Jr, Villar M, Watson BD, Otto KG, Stewart S, Stettler RF (1994) Molecular genetics of growth and development in Populus. III. A genetic linkage map of a hybrid popular composed of RFLP, STS, and RAPD markers. Theor Appl Genet 89:167–178

Brunsfeld SJ, Soltis PS, Soltis DE, Gadek PA, Quinn CJ, Strenge DD, Ranker TA (1994) Phylogenetic relationships among the genera of Taxodiaceae and Cupressaceae: evidence from *rbcL* sequences. Syst Bot 19:253–262

Devey ME, Fiddler TA, Liu B-H, Knapp SJ, Neale DB (1994) An RFLP linkage map for loblloy pine based on a three-generation outbred pedigree. Theor Appl Genet 88:273–278

Devey ME, Bell JC, Smith DN, Neale DB, Moran GF (1996) A linkage map for *Pinus radiata* based on RFLP, RAPD, and microsatellite markers. Theor Appl Genet 92:673–679

Echt CS, Erdahl LA, McCoy J (1992) Genetic segregation of random amplified polymorphic DNA in diploid cultivated alfalfa. Genome 35:84–87

Grattapaglia D, Sederoff RR (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross mapping strategy and RAPD markers. Genetics 137:1121–1137

Halward T, Stalker T, LaRue E, Kocher G (1992) Use of single-primer DNA amplifications in genetic studies of peanut (*Arachis hypogaea* L.). Plant Mol Biol 18:315–325

Hudson TJ, Stein LD, Gerety SS, Ma J, Castle AB, Silva J, Slonim DK, Baptista R, Kruglyak L, Xu S-H, Hu X, Colbert AME, Rosenberg C, Reeve-Daly MP, Rozen S, Hui L, Wu X, Vestergaard C, Wilson KM, Bae JS, Maitra S, Ganiatsas S, Evans CA, DeAngelis MM, Ingalls KA, Nahf RW, Horton LT Jr, Anderson MO, Collymore AJ, Ye W, Kouyoumjian V, Zemsteva IS, Tam J, Devine R, Courthey DF, Renaud MT, Nguyen H, O'Connor TJ, Fizames C, Faure S, Gyapay G, Dib C, Morissette J, Orlin JB, Birren BW, Goodman N, Weissenbach J, Hawkins TL, Foote S, Page DC, Lander ES (1995) An STS-based map of the human genome. Science 270:1945–1954

Inoue T, Zhong HS, Miyao A, Ashikawa I, Monna, L, Fukuoka S, Miyadera N, Nagamura Y, Kurata N, Sasaki T, Minobe Y (1995) Sequenced-tagged sites (STSs) as standard landmarkers in the rice genome. Theor Appl Genet 89:728–734

Mukai Y, Suyama Y, Tsumura Y, Kawahara T, Yoshimaru H, Kondo T, Tomaru N, Kuramoto N, Murai M (1995) A linkage map for sugi (*Cryptomeria japonica*) based on RFLP, RAPD, and isozyme loci. Theor Appl Genet 90:835–840

Olson M, Hood L, Cantor C, Dotstein D (1989) A common language for physical mapping of human genome. Science 254:1434–1435

Paran I, Michelmore RW (1993) Development of reliable PCR-based makers linked to downy midew resistance genes in lettuce. Theor Appl Genet 85:985–993

Reiter RS, Williams JGK, Feldmann KA, Rafalski JA, Tingey SV, Scolnik PA (1992) Global and local genome mapping in *Arabidopsis thaliana* by using inbred lines and random amplified polymorphic DNAs. Proc Natl Acad Sci USA 89:1477–1481

Strauss SH, Palmer JD, Howe GT, Doerksen AH (1988) Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. Proc Natl Acad Sci USA 85:3898–3902

Talbert LE, Blake NK, Chee PW, Blake TK, Magyer GM (1994) Evaluation of "sequence-tagged-site" PCR products as molecular marker in wheat. Theor Appl Genet 87:789–794

Tragoonrung S, Kanazin V, Hayes PM, Blake TK (1992) Sequenced-site-facilitated PCR for barley genome mapping. Theor Appl Genet 84:1002–1008

Tsumura Y, Ogihara Y, Sasakuma T, Ohba K (1993) Physical map of chloroplast DNA in sugi, *Cryptomeria japonica*. Theor Appl Genet 86:166–172

Tsumura Y, Yoshimura K, Tomaru N, Ohba K (1995) Molecular phylogeny of conifers using PCR-RFLP analysis of chloroplast genes. Thor Appl Genet 91:1222–1236

Tulsieram L, Glaubitz JC, Kiss G, Carlson JE (1992) Single-tree genetic linkage mapping in conifers using haploid DNA from megagametophytes. Bio/Technol 10:686–690

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535