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ORIGINAL PAPER

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# Evaluation of cleaved amplified polymorphic sequence markers for *Chamaecyparis obtusa* based on expressed sequence tag information from *Cryptomeria japonica*

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**Abstract** We have developed and evaluated sequence-tagged site (STS) primers based on expressed sequence-tag information derived from sugi (*Cryptomeria japonica*) for use in hinoki (*Chamaecyparis obtusa*), a species that belongs to a different family (although it appears to be fairly closely related to sugi). Of the 417 *C. japonica* STS primer pairs we screened, 120 (~30%) were transferable and provided specific PCR amplification products from 16 *C. obtusa* plus trees. We used haploid megagametophytes to investigate the homology of 80 STS fragments between *C. obtusa* and *C. japonica* and to identify orthologous loci. Nearly 90% of the fragments showed high (>70%) degrees of similarity between the species, and 35 STSs indicated homology to entries with the same putative function in a public DNA database. Of the 120 STS fragments amplified, 72 showed restriction fragment length polymorphisms; in addition, the CC2430 primers detected amplicon length polymorphism. We assessed the inheritance pattern of 27 cleaved amplified polymorphic sequence markers, using 20 individuals from the segregation population. All the markers analyzed were consistent with the marker inheritance patterns obtained from the screening panel, and no markers (except CC2716) showed significant ($P < 0.01$) deviation from the expected segregation ratio. In total, 136 polymorphic markers were developed using *C. japonica*-based STS primers without any sequence modification. In addition, the applicability of STS-based markers developed in one species to other species was found to closely reflect the evolutionary distance between the species, which is roughly concordant with the difference between their *rbc*L sequences. We plan to use these markers for genetic studies in *C. obtusa*. Most of the markers should also provide reliable anchor loci for comparative mapping studies of the *C. obtusa* and *C. japonica* genomes.

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## Introduction

Sugi (*Cryptomeria japonica*) and hinoki (*Chamaecyparis obtusa*) are the two most widely cultivated timber conifers in Japan. Both species are economically important, and nationwide forest-tree breeding programs were initiated for them in the 1950s. Taxonomically, *C. japonica* belongs to the Taxodiaceae, whereas *C. obtusa* belongs to the Cupressaceae, but this classification has been questioned in several studies (Eckenwalder 1976; Hart 1987; Price and Lowenstein 1989). Recent molecular phylogenetic studies have clarified the evolutionary relationship between the two families, using *rbc*L sequence (Brunsfeld et al. 1994), 28S rRNA sequence (Stefanovic et al. 1998), and PCR–RFLP analyses of chloroplasts (Tsumura et al. 1995). In addition, Kusumi et al. (2000) determined the phylogenetic relationship between the Taxodiaceae and Cupressaceae, using nucleotide sequences from four regions of the chloroplast DNA. The results from these studies indicate that the two families form a monophyletic group. Kusumi et al. (2000) further strongly suggested that Cupressaceae were derived from representatives of the Taxodiaceae, being most closely related to the *C. japonica*/*Taxodium*/*Glyptoctrobus* clade. Therefore, we assume that *C. japonica* and *C. obtusa* are closely related species and expect them to show high levels of synteny and colinearity.

During the last 5 years, *C. japonica* has been extensively studied under the *C. japonica* Genome project. In particular, we have obtained more than 8,000 partial sequences of expressed sequence tags (ESTs) from *C. japonica* cDNA libraries. These results were reported in part by Ujino-Ihara et al. (2000). Furthermore, many useful sequenced-tagged site (STS) markers for *C. japonica* genome mapping and population studies have been developed using previously published information (Nikaido et al. 2000; Iwata et al. 2001; Tani et al. 2003a, b).

Cleaved amplified polymorphic sequence [(CAPS) also known as PCR–RFLP)] markers based on STSs derived from ESTs have been developed, and a genetic linkage map of *C. japonica* has been constructed (Iwata et al. 2001; Tani et al. 2003a). The CAPS molecular marker system has several advantages. First, since analysis of restriction fragment length polymorphisms is based on polymerase chain reaction (PCR) amplification, it is much easier and less time-consuming, especially for species with large genomes, such as conifers [e.g., the estimated C-values are 21–23 pg in loblolly pine (Wakamiya et al. 1993), 11 pg in *C. japonica*, and 10 pg in *C. obtusa* (Hizume et al. 2001)] than analyzing alternative types of markers that require Southern hybridizations. Second, the primers for CAPS markers based on ESTs are more useful as genetic markers for comparative mapping study than those based on anonymous, non-functional sequences such as microsatellite markers, because the coding regions of functional genes are generally well conserved, not only within but also between species. Third, CAPS markers are inherited mainly in a co-dominant manner.

PCR-based markers derived from ESTs have recently been developed as anchor markers for comparative mapping studies in conifers (Brown et al. 2001). Therefore, the availability of thousands of *C. japonica* ESTs is expected to present opportunities to develop new anchor loci for other conifer species to support comparative genome analysis. Exploiting this potential would increase the effectiveness of markers that have already been developed and reduce the cost of developing new markers in *C. obtusa*. In the present study, we applied *C. japonica* STS primers directly to the conifer *C. obtusa*, which is related but belongs to a different coniferous family, and developed CAPS markers for advanced genetic research and breeding programs in *C. obtusa*.

## Materials and methods

### Plant materials

Sixteen plus tree clones, selected from artificial forests in various regions of Japan, were used to detect polymorphism in order to develop CAPS markers. The segregation population used to assess the inheritance patterns of the markers was developed from the backcross of an $F_1$ individual derived from the plus tree clones 'Fudago-2' and 'Izu-3'. The progeny were produced between an $F_1$ individual as the female parent and an 'Izu-3' individual as the male parent. A total of 20 individuals of the segregation population were used to determine the marker inheritance patterns.

### PCR amplification of STSs in *C. obtusa*, using *C. japonica* STS primers

Fresh leaves were collected from the 16 plus tree clones to evaluate the polymorphism for each CAPS marker and from the grandparents, 'Fudago-2' and 'Izu-3', the $F_1$ tree, and the 20 segregation progeny to determine the modes of marker inheritance. Genomic DNA was extracted from 50 mg of leaf material, using a Dneasy Plant Mini Kit (Qiagen), and STS primers homologous to sequences in the *C. japonica* cDNA library were used to test their transferability to *C. obtusa*. Some of the primers in this set have already been reported by Iwata et al. (2001) and Tani et al. (2003a, b). PCR amplification was performed by the method according to Iwata et al. (2001) with minor modifications, i.e., containing 10 ng DNA, 1.0 U *Taq* polymerase (Promega or TaKaRa), and carried out in a thermal cycler (GeneAmp PCR Instrument Systems 9600, Applied Biosystems) programmed for 5 min of preheating at 94°C followed, by 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min 30 s at 72°C, with a final incubation of 5 min at 72°C. For annealing, three other temperatures (55, 60, and 65°C) were tested for all primer pairs. The PCR products were separated on 2% agarose gels and stained with ethidium bromide to check whether single bands had been amplified.

### Sequence analysis and homology determination of STSs

*C. obtusa* and *C. japonica* genomic DNAs were extracted from haploid megagametophyte tissues in seeds by the CTAB method and used for PCR amplification. PCR products that showed single bands were each purified in a Multiscreen FB plate (Millipore) and used as templates for the sequencing reactions. Cycle sequencing was performed from both ends of each purified, amplified fragment with the forward and reverse STS primers and a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Each sequence was detected using an ABI377 automatic sequencer (Applied Biosystems) according to the manufacturer's instructions. We then analyzed the sequence data using Sequence Analysis Software, version 3.7 (Applied Biosystems).

### Comparison of the transferability of STS markers to closely related species and their phylogenetic relationships

To assess the transferability of STS markers to other species and their phylogenetic relationships, the chloroplast *rbc*L gene sequences of nine coniferous species were compared. To test the transferability of *C. japonica* STSs, we selected two species, *C. obtusa* and *Taxodium* (Tsumura et al. 1999), which are closely related to *C. japonica* (Kusumi et al. 2000), and for *Pinus taeda* STSs (Brown et al. 2001), we compared five species of pine and one of *Pseudotsuga*. All sequence data were obtained from the DDBJ database, and 1,258 nucleotides and 418 amino acids were compared amongst the nine species.

### Polymorphism detection from STSs

Primers that successfully supported amplification of a single fragment from *C. obtusa* were identified and used for the detection of restriction site polymorphisms in a screening panel comprising 16 *C. obtusa* plus trees, including the grandparents of the segregation progeny. Amplified fragments were first digested with each of 14 restriction enzymes (*Alu*I, *Bst*0I, *Dde*I, *Hae*II, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Nci*I, *Nde*II, *Rsa*I, *Sty*I, *Sin*I, and *Taq*I); if none of these enzymes revealed polymorphism, samples were digested with each of 12 additional restriction enzymes (*Ava*I, *Bam*HI, *Ban*II, *Bgl*I, *Bgl*II, *Dra*I, *Eco*0109I, *Eco*RI, *Hin*cII, *Hin*dIII, *Nsi*I, and *Ssp*I). Out of 36 restriction enzymes utilized in *C. japonica* (Iwata et al. 2001), the above 26 enzymes were higher in rank for polymorphism detection. We may expect effective polymorphism detection using these enzymes and supposed that time and cost could be saved in present study. The restricted PCR products were resolved by electrophoresis on 2% agarose gels containing ethidium bromide, and polymorphic STSs were detected as described for CAPS markers (Iwata et al. 2001).

To identify informative markers, we evaluated the degree of polymorphism they each detected using the polymorphic information content [(PIC) Botstein et al. 1980)] as an index for all the polymorphic markers generated by the various combinations of STSs and restriction enzymes.

### Segregation analysis

To determine the mode of inheritance of the polymorphic STSs (i.e., the CAPS markers), we randomly chose 20 individuals from the segregation population, derived (as described above) from a backcross between the $F_1$ hybrid and 'Izu-3'. Segregation data were evaluated by $\chi^2$-tests for goodness of fit to the expected Mendelian distribution.

## Results

### PCR amplification of STSs

We expected more than half of the 417 *C. japonica* STS primer pairs tested to provide single-band amplification in *C. obtusa* at one of the four annealing temperatures (50, 55, 60, or 65°C) used during the first screening. Eventually, after fine-tuning the annealing temperature, 120 primer pairs that met our criteria were obtained (Appendix). These results indicate that approximately 29% of STS primers derived from *C. japonica* EST sequences can be used directly as STS primers for *C. obtusa* species. Of the remaining 297 primer pairs, 221 yielded multiple bands, and 76 failed to support amplification. Of the 120 successful primer pairs, eight (CC1908, CC2930, CC3055, CC3106, CC3152, C3414, CC3455, and CC3861) provided single-band amplification in *C. obtusa*, but not in *C. japonica*. For 90 of the 120 STS primer pairs, agarose gel electrophoresis roughly distinguished amplified products that differed in size between *C. obtusa* and *C. japonica*.

### Sequence comparison between *C. obtusa* and *C. japonica*

We amplified STS fragments from one individual each of *C. japonica* and *C. obtusa* for sequence analysis. The partial nucleotide sequences obtained using the same primer pairs as for the amplification confirmed the similarities between these two species. Generally, orthologous loci of closely related species are theoretically expected to exhibit high nucleotide similarity. We sequenced and compared the level of nucleotide identity between these products from the two species, but excluding large indel regions (more than 100 nucleotides). Of the 80 STSs successfully compared (Table 1), 70 (87.5%) showed a nucleotide identity of 72% to 100% between *C. obtusa* and *C. japonica*, while the other ten showed low ( < 50% identity) levels of similarity between the two species. The results of a BLAST homology search using the sequence data from these 70 STSs as queries showed homology to known protein sequences for 35 of them, with BLAST values higher than 50.

### Polymorphism detection for developing CAPS

To develop CAPS markers, genomic DNA templates prepared from the 16 *C. obtusa* plus trees (including

**Table 1** Putative function of *Chamaecyparis* sequence-tagged sites (STSs) and nucleotide identity between *C. obtusa* and *Cryptomeria japonica*

| STS | Accession no. | Nucleotide identity[a] | BLAST score | Functional annotation[b] |
|---|---|---|---|---|
| CC0277 | AB127118 | 373/379 (98%) | 111 | Putative senescence-associated protein |
| CC0278 | AB127119 | 318/347 (91%) | < 50 | Low score |
| CC0281 | AB127120 | 321/331 (97%) | 79 | Unknown protein |
| CC0285 | AB127121 | 267/295 (90%) | – | No hit |
| CC0286 | AB127185 | 315/334 (94%) | 172 | Similarity to phosphate/phosphoenolpyruvate translocator |
| CC0292 | AB127122 | 591/670 (88%) | 96 | Unknown protein |
| CC0316 | AB127123 | 506/516 (98%) | 128 | Auxin-induced protein |
| CC0336 | AB127124 | 429/495 (86%) | 115 | Similar to mandelonitrile lyase |
| CC0344 | AB127125 | 241/271 (89%) | < 50 | Low score |
| CC0352 | AB127188 | Low similarity | – | Not determined |
| CC0374 | AB127142 | 631/666 (94%) | 134 | Quinone oxidoreductase-like protein |
| CC0421 | AB127187 | Low similarity | – | Not determined |
| CC0428 | AB127126 | 416/456 (91%) | 95 | Glutamate decarboxylase 1 |
| CC0438-1 | AB127127 | 450/465 (96%) | 128 | Light-independent protochlorophyllide reductase subunits |
| CC0550 | AB127184 | 349/390 (89%) | 175 | Chitinase |
| CC0674 | AB127186 | 216/254 (85%) | 77 | Unknown protein |
| CC0702 | AB127128 | 380/462 (84%) | < 50 | Low score |
| CC0708 | AB127129 | 394/448 (87%) | 50 | Unknown protein |
| CC0725 | AB127130 | 581/634 (91%) | 169 | SKIP nuclear coactivator-like protein |
| CC0731 | AB127131 | 422/499 (85%) | 409 | 60S ribosomal protein |
| CC0737 | AB127132 | 469/531 (88%) | 75 | 60S acidic ribosomal protein |
| CC0790 | AB127133 | 525/539 (97%) | 157 | Putative senescence-associated protein |
| CC0822 | AB127134 | 605/734 (84%) | 106 | Similarity to ubiquitin fusion degradation protein 1 |
| CC0831 | AB127189, AB127190 | 1,162/1,404 (83%) | 247 | Hypothetical protein |
| CC0838 | AB127135 | 412/476 (86%) | 217 | Thaumatin-like protein mRNA |
| CC0854 | AB127136 | 364/394 (91%) | 230 | 40S ribosomal protein S16 |
| CC0858 | AB127183 | 645/729 (88%) | 280 | erg-1 mRNA |
| CC0860 | AB127144 | 244/267 (91%) | 67 | HistoneH3-D |
| CC0951 | AB127191 | Low similarity | – | Not determined |
| CC0958 | AB127182 | 412/466 (88%) | – | No hit |
| CC0983 | AB127143 | 276/327 (84%) | < 50 | Low score |
| CC1015 | AB127181 | 617/675 (91%) | 53 | Similarity to storage protein |
| CC1017 | AB127145 | 475/650 (73%) | – | No hit |
| CC1104 | AB164397 | Low similarity | – | Not determined |
| CC1112 | AB127180 | 608/690 (88%) | < 50 | Low score |
| CC1135 | AB127137 | 434/501 (86%) | < 50 | Low score |
| CC1145 | AB127138 | 388/439 (88%) | 140 | Unknown protein |
| CC1147 | AB127192 | 625/693 (90%) | < 50 | Low score |
| CC1172 | AB164398 | 298/332 (89%) | 111 | 60S ribosomal protein L2 |
| CC1187 | AB127146 | 397/430 (92%) | < 50 | Low score |
| CC1287 | AB127147 | Low similarity | – | Not determined |
| CC1319 | AB127148 | 306/425 (72%) | 54 | Dehydrin mRNA |
| CC1371 | AB127149 | 443/491 (90%) | 73 | Similarity to MSP1 protein |
| CC1415 | AB127150 | 287/314 (91%) | 68 | Unknown protein |
| CC1432 | AB127193, AB127194 | 1,146/1,336 (86%) | 90 | Unknown protein |
| CC1463 | AB127151 | 594/641 (93%) | 92 | Hypothetical protein |
| CC1555 | AB127153 | 317/345 (91%) | 184 | Similarity to glycosyl transferase lgtC |
| CC1531 | AB127152 | Low similarity | – | Not determined |
| CC1606 | AB127154 | 728/837 (86%) | 361 | Myb gene for Myb transcription factor |
| CC1625 | AB127155 | 348/394 (88%) | < 50 | Low score |
| CC1697 | AB164399 | Low similarity | – | Not determined |
| CC1711 | AB127156 | 1,134/1,375 (82%) | < 50 | Low score |
| CC1787 | AB164400 | Low similarity | < 50 | Low score |
| CC1798 | AB127157 | 520/719 (72%) | 209 | Chitinase mRNA |
| CC1799 | AB127158 | 506/630 (80%) | < 50 | Low score |
| CC1974 | AB127159 | 302/305 (99%) | < 50 | Low score |
| CC2188 | AB127160 | 128/142 (90%) | 94 | Methionine adenosyltransferase |
| CC2196 | AB127161 | 499/553 (90%) | 269 | WD-repeat protein 5 |
| CC2237 | AB127162 | 596/687 (88%) | < 50 | Low score |
| CC2286 | AB127179 | 387/431 (90%) | < 50 | Low score |
| CC2288 | AB127163 | Low similarity | – | Not determined |
| CC2427 | AB127164 | 485/526 (92%) | 117 | Hypothetical protein |

**Table 1** (Contd.)

| STS | Accession no. | Nucleotide identity[a] | BLAST score | Functional annotation[b] |
|---|---|---|---|---|
| CC2435 | AB164401 | 536/650 (82%) | 60 | Similarity to zinc finger protein 216 |
| CC2448 | AB127165 | 366/407 (90%) | 83 | Flavone synthase mRNA |
| CC2478 | AB164402, AB164403 | 764/840 (91%) | 53 | Unknown protein |
| CC2483 | AB127166 | 526/613 (86%) | 68 | Hypothetical protein |
| CC2588 | AB127167 | 604/713 (84%) | – | No hit |
| CC2716 | AB127178 | 856/951 (90%) | 86 | EIN3-like protein mRNA |
| CC2777 | AB127177 | Low similarity | – | Not determined |
| CC2860 | AB127168 | 570/649 (88%) | < 50 | Low score |
| CC2865 | AB127169 | 533/552 (96%) | 140 | Putative lysophospholipase isologue mRNA |
| CC2916 | AB127170 | 626/668 (93%) | 266 | DNA-directed RNA polymerase beta'' chain |
| CC2917 | AB127171 | 440/472 (93%) | 130 | Unknown protein |
| CC2920 | AB127172 | 595/674 (88%) | 247 | mRNA for elicitor inducible chitinase Nt-SubE76 |
| CC2976 | AB127176 | 384/431 (89%) | < 50 | Low score |
| CC3133 | AB127173 | 583/625 (93%) | 254 | Unknown protein |
| CC3162 | AB127174 | 517/590 (87%) | 195 | Cell death associated protein |
| CC3807 | AB127175 | 640/640 (100%) | 125 | Putative retroelement protein |
| CC3816 | AB127195, AB127139 | 579/667 (87%) | < 50 | Low score |
| CC3872 | AB127140, AB127141 | 474/524 (90%) | 210 | Ethylene-insensitive-like mRNA |

[a]Number of nucleotides identical/number of nucleotides compared between *C. obtusa* and *C. japonica* (nucleotide identity). *Low similarity* indicates nucleotide identity less than 50%

[b]From BLAST search of GenBank with a *Chamaecyparis* STS as query. *Low score* indicates a BLAST value less than 50

the grandparents of the segregation population) were PCR-amplified using the 120 identified STS primer pairs, and the PCR products were digested with either 14 or 26 restriction enzymes. Electrophoresis in agarose gels revealed that 72 STSs (60%) showed polymorphisms after digestion with at least one restriction enzyme. In total, the combination of these 72 STSs and various restriction enzymes provided 135 different banding patterns of CAPS markers. Additionally, primer set CC2340 provided amplification products (without restriction digestion) of differing sizes amongst the 16 *C. obtusa* plus trees, and thus identified amplicon length polymorphism. The polymorphisms observed could be divided into two types of inheritance patterns, dominant and co-dominant. The PIC values calculated for the polymorphic markers ranged from 0.06 to 0.58, averaging approximately 0.30.

### Segregation analysis

We verified the marker inheritance patterns, using 20 individuals from the segregation population. Of the 72 CAPS markers identified, we expected that 27 would segregate in the progeny, because one or both of the parental genotypes was/were heterozygous (Table 2). In fact, five CAPS markers (CC0344, CC1319, CC2588, CC2920, and CC3872), which displayed dominant inheritance patterns in the screening panel, were practically co-dominant, according to the results of the segregation analysis. All the 27 markers analyzed were inherited in a Mendelian manner. Furthermore, no significant ($P < 0.01$) deviation from the expected segregation ratio was found for any markers except CC2716.

## Discussion

### Transferability of EST-based STS markers in conifers

Primers that are widely transferable across taxonomic borders are very attractive, because they offer the potential to avoid duplication of many complicated, costly, and time-consuming processes (e.g., constructing genomic/cDNA libraries, sequencing clones, designing and synthesizing primers). In this study, we directly applied more than 400 STS primer pairs designed from *C. japonica* EST sequences to PCR amplification in *C. obtusa*.

Tsumura et al. (1997) previously evaluated the rate of conservation of EST sequences from *C. japonica* among several conifer species on a smaller scale than here. In both our present study and that of Tsumura et al. (1997), about 30% of the tested STS primers could be transferred from *C. japonica* to *C. obtusa*. In comparison, Brown et al. (2001) evaluated 90 primer sets based on ESTs of loblolly pine (*P. taeda* L.). They observed that nearly 90% of primers, on average, were transferable at the subgenus level. Furthermore, 22% were useful in Douglas fir, which belongs to a different genus (*Pseudotsuga*) but the same family.

The genetic relationship between *C. obtusa* and *C. japonica* (which belong to different taxonomic

**Table 2** Segregation analysis of 27 cleaved amplified polymorphic sequence (CAPS) markers in 20 progeny of the cross between 'Izu-3' and $F_1$ individual 2a of *C. obtusa*

| CAPS marker[a] | | Genotype[c] | | Expected segregation ratio | Observed segregation ratio | $\chi^2$-test probability |
|---|---|---|---|---|---|---|
| STS | Restriction enzyme | 'Izu' | 2a | | | |
| CC0292 | *Msp*I | *AB* | *AA* | 1:1 | 12:8 | 0.37 |
| CC0344[b] | *Rsa*I | *AB* | *AB* | 1:2:1 | 5:9:6 | 0.75 |
| CC0530 | *Bst*0I | *AB* | *AB* | 1:2:1 | 4:8:8 | 0.44 |
| CC0550 | *Msp*I | *AB* | *BB* | 1:1 | 13:7 | 0.18 |
| CC0737 | *Dra*I | *Aa* | *aa* | 1:1 | 11:9 | 0.65 |
| CC0831 | *Nde*II | *Aa* | *aa* | 1:1 | 12:8 | 0.37 |
| CC0838 | *Hae*III | *aa* | *Aa* | 1:1 | 7:13 | 0.18 |
| CC0858 | *Sin*I | *AB* | *AB* | 1:2:1 | 5:11:4 | 0.75 |
| CC1287 | *Ssp*I | *AB* | *BB* | 1:1 | 6:13 | 0.11 |
| CC1319[b] | *Dde*I | *AB* | *AB* | 1:2:1 | 5:7:7 | 0.41 |
| CC1606 | *Alu*I | *AB* | *AB* | 1:2:1 | 4:8:4 | 1.00 |
| CC2196 | *Alu*I | *AA* | *AB* | 1:1 | 14:6 | 0.07 |
| CC2237 | *Taq*I | *AB* | *AB* | 1:2:1 | 6:10:3 | 0.55 |
| CC2427 | *Alu*I | *Aa* | *aa* | 1:1 | 12:8 | 0.37 |
| CC2478 | *Hae*III | *aa* | *Aa* | 1:1 | 10:10 | 1.00 |
| CC2588[b] | *Msp*I | *AB* | *AB* | 1:2:1 | 8:9:3 | 0.17 |
| CC2716 | *Msp*I | *Aa* | *Aa* | 3:1 | 7:12 | $<0.01$ |
| CC2752 | *Alu*I | *aa* | *Aa* | 1:1 | 9:11 | 0.65 |
| CC2839 | *Alu*I | *AB* | *AB* | 1:2:1 | 13:7 | 0.67 |
| CC2860 | *Dde*I | *AB* | *AB* | 1:2:1 | 7:10:2 | 0.30 |
| CC2920[b] | *Msp*I | *AB* | *AB* | 1:2:1 | 6:11:3 | 0.58 |
| CC2930 | *Hae*III | *AB* | *AB* | 1:2:1 | 5:8:5 | 0.68 |
| CC3133 | *Eco*RI | *aa* | *Aa* | 1:1 | 10:10 | 1.00 |
| CC3152 | *Hin*fI | *AB* | *AB* | 1:2:1 | 8:7:4 | 0.22 |
| CC3455 | *Rsa*I | *AB* | *AB* | 1:2:1 | 4:7:9 | 0.12 |
| CC3816 | *Rsa*I | *AB* | *BB* | 1:1 | 8:12 | 0.37 |
| CC3872[b] | *Nde*II | *AB* | *AB* | 1:2:1 | 7:9:4 | 0.58 |

[a]CAPS markers developed from the combination of the listed STSs (from *C. japonica*) and restriction enzymes
[b]The markers were re-evaluated as co-dominant after segregation analysis
[c]Parental genotypes are presented in *capital letters* for co-dominant markers and *lowercase letters* for dominant markers

families) is probably much closer than has generally been recognized. The chloroplast *rbc*L gene sequence is frequently used to evaluate phylogenetic relationships between species. Therefore, we compared nucleotide and amino acid sequences of *rbc*L among nine coniferous species (Table 3). Nucleotide differences were detected between the *rbc*L genes of *C. japonica* and *Taxodium distichum* at 18 out of 1,258 nucleotide sites evaluated. In comparison, there was a 34-nucleotide difference between *C. japonica* and *C. obtusa* and a 108-nucleotide difference between *C. japonica* and *P. taeda*. Approximately half of the STSs developed in *C. japonica* by Tsumura et al. (1999) were specifically amplified in *T. distichum*, and approximately 30% of the *C. japonica* STSs occurred in *C. obtusa*. Although the cited authors also evaluated *P. taeda*, the amplification success in this case was only 6%. As the number of differences in the *rbc*L nucleotide sequences increased between the species of interest, the transferability of markers decreased. In another example, the transferability of STSs developed in *P. taeda* was found to be over 80% in *P. elliottii*, *P. radiata*, *P. pinaster*,

**Table 3** Pairwise comparison of chloroplast *rbc*L gene sequences among species. A total of 1,258 nucleotides and 418 amino acid sites were compared. The numerals *above the diagonal* are the numbers of nucleotide differences and *below the diagonal* are the numbers of amino acid differences between the species. Sequence data for *rbc*L gene of nine species were obtained from DDBJ database

| | *C. japonica* | *Taxodium distichum* | *C. obtusa* | *Pinus taeda* | *P. elliottii* | *P. radiata* | *P. pinaster* | *P. sylvestris* | *P. menziesii* |
|---|---|---|---|---|---|---|---|---|---|
| *C. japonica*[a] | – | 18 | 34 | 108 | 107 | 109 | 109 | 113 | 104 |
| *T. distichum* | 7 | – | 32 | 100 | 100 | 102 | 105 | 103 | 101 |
| *C. obtusa* | 5 | 5 | – | 108 | 107 | 110 | 109 | 110 | 106 |
| *P. taeda* | 8 | 4 | 6 | – | 0 | 2 | 8 | 8 | 31 |
| *P. elliottii* | 9 | 4 | 6 | 0 | – | 2 | 9 | 9 | 32 |
| *P. radiata* | 10 | 5 | 7 | 1 | 1 | – | 11 | 10 | 34 |
| *P. pinaster* | 10 | 9 | 7 | 4 | 4 | 6 | – | 4 | 31 |
| *P. sylvestris* | 10 | 7 | 7 | 3 | 3 | 4 | 2 | – | 33 |
| *P. menziesii* | 7 | 7 | 5 | 5 | 5 | 6 | 6 | 6 | – |

[a]Accession numbers in DDBJ database: *C. japonica*, L25751; *T. distichum*, AF119185; *C. obtusa*, L12570; *P. taeda*, AF119177; *P. elliottii*, AB081075; *P. radiata*, AB063383; *P. pinaster*, AB019818; *P. sylvestris*, AB019809; *P. menziesii*, X52937, S53466

and *P. sylvestris* (Brown et al. 2001), in which the *rbc*L nucleotide sequence differs by only zero to eight nucleotides from that of *P. taeda*. In comparison, 22% transferability was obtained when the *P. taeda*-based STSs were applied to *P. menziesii*. These findings suggest that the level of nucleotide difference in the *rbc*L sequences is a good indicator for the transferability of markers between species. However, despite the similarity of their *rbc*L genes at the nucleotide level, the transferability of *C. japonica* markers to *C. obtusa* was slightly higher than the transferability of *P. taeda* markers to *P. menziesii* (Brown et al. 2001). This difference in transferability might reflect differences in the type of region for which the primers were designed. The primers we designed are positioned within exons (Iwata et al. 2001) whose sequences are likely to be highly conserved between related species, whereas Temesgen et al. (2001) used reverse primers that were based on sequences in the 3′ untranslated region, which is likely to be more genetically variable. Another factor that influences the transferability of markers may be genome size. The genome sizes of *C. japonica* and *C. obtusa* were estimated to be $\sim 1\times10^{10}$ bp, whereas those of pine species were 2–3 times larger (2–$3\times10^{10}$ bp). Therefore, identifying STS markers that amplify specific PCR products (i.e., amplification of a single locus) in pine would be more difficult than identifying such markers in *C. japonica* or *C. obtusa*.

## Importance of PCR-based co-dominant markers in conifers

Transferable markers not only provide economic benefits, they also accommodate comparative mapping between different species. To date, markers used for comparative mapping have mainly been heterologous RFLPs, which have been detected by conserved-sequence probes in many species (e.g., rice, maize, pearl millet, foxtail millet, sorghum, sugarcane, wheat, barley, and rye; Gale and Devos 1998). In the comparative mapping of tree species, PCR-based markers are much more convenient and useful than RFLP markers, especially for conifers, because of their large genome size. In pine species, multigene families and pseudogenes (Kinlaw and Neale 1997) also obstructed the progress of genome mapping study with RFLP markers. Recentry, Brown et al. (2001) examined anchored reference loci, using PCR-based markers in pine species for integrating pine genomics. To confirm the orthology of candidate loci, they investigated the sequence similarities of PCR amplification products between loblolly and slash pines, using a reference panel of ten anchor loci. Of the 88 STS fragments sequenced in both species, more than 90% showed high nucleotide similarity, and homologous sequences with the same putative function were found in public databases. In comparison, eight of our primer pairs (CC1908, CC2930, CC3055, CC3106, CC3152, CC3414, CC3455, and CC3861) failed to support PCR amplification of a single band in *C. japonica*. With these primers, no amplification occurred at annealing temperatures of 55°C and 60°C, and multiple bands appeared at 50°C (data not shown). Although we used primers designed to bind mainly to sites within exons, the specificity of these primer sequences with respect to the *C. japonica* genome may have been slightly lower than that for *C. obtusa* simply by chance. The level of nucleotide similarity that distinguishes paralogues from orthologues is subjective (Brown et al. 2001). Komulaine et al. (2003), and Chagne et al. (2003), for instance, loci orthology in pine species requires higher nucleotide identity (more than 95%). Therefore, we recognized that further investigation would be needed, although our results likely provide a useful indicator for comparative mapping analyses.

CAPS markers are theoretically expected to be inherited in a co-dominant manner when the marker is derived from a single-copy gene. According to Iwata et al. (2001), co-dominant inheritance encompasses the following three scenarios: (1) bi-allelic co-dominant patterns for which two homozygotic and one heterozygotic forms are found; (2) bi-allelic co-dominant patterns for which only two homozygotic (and no heterozygotic) forms occur; and (3) multi-allelic co-dominant patterns, which apparently correspond to genotypes with multiple alleles. In comparison, dominant inheritance is manifested as bi-allelic dominance patterns, which can be scored only in terms of presence or absence. In this study, 31 STSs showed co-dominant patterns, and 41 showed only dominant patterns. However, five CAPS markers that showed dominant patterns in the screening panel were re-categorized after the segregation analysis as having co-dominant inheritance, bringing the rate up to 50%. According to Iwata et al. (2001), because of the limited number of individuals assessed during the screening process, homozygotes with rare alleles at the corresponding loci may not have been sampled. The 16 plus trees we selected for this study were all from central Japan, and thus are unlikely to represent all of the genotypes, considering the broad natural distribution of *C. obtusa*, which ranges from Fukushima Prefecture (37°10′N) in the north to the Kyusyu area (30°15′N) in the south. Most of the 150 CAPS markers used to analyze natural populations of *C. japonica* showed co-dominant inheritance (Tsumura et al., unpublished data). Therefore, extended segregation analysis will likely show that the remaining 37 of our CAPS markers are actually co-dominant.

## Polymorphism of CAPS markers

The true level of polymorphisms (i.e., the development efficiency of CAPS markers) in *C. obtusa* is likely to be

higher than the level we detected. Using 26 restriction enzymes, we eventually determined that more than half of the *C. japonica*-based STSs were polymorphic in *C. obtusa*. The informativeness of these markers was measured by PIC, the average value of which was found to be 0.30. In a previous study, Iwata et al. (2001) reported that 267 of 361 STSs originally developed in *C. japonica* were polymorphic in a screening experiment based on 36 restriction enzymes and 15 individuals, and the average PIC value was 0.31 for bi-allelic co-dominant markers, corresponding to two homozygote and one heterozygote forms. Generally, the STS primers developed from one species that support DNA amplification in another may not accurately reflect the level of polymorphism in the other species. However, even though we used fewer restriction enzymes than the previous study in *C. japonica* (Iwata et al. 2001), more than 50% of our *C. japonica*-based STSs showed polymorphisms. Therefore, it seems that we could efficiently detect polymorphic regions, using the transferable primers in *C. obtusa*.

Our newly developed CAPS markers will likely be effective in diverse applications, for example, genome mapping, population analysis of *C. obtusa*, and comparative mapping studies of coniferous species (especially species of the Cuppresaseae). The genetic diversity among natural populations of *C. japonica* has been evaluated using several CAPS markers (Tsumura and Tomaru 1999). Population studies using larger numbers of CAPS markers may provide us more precise information. In this study, we successfully developed 136 polymorphic markers, using STS-based *C. japonica* primers without any modification of the primer sequences. We plan to use these markers for a genetic study of *C. obtusa*. Most of the markers developed could also provide reliable anchor loci for comparative mapping studies between the *C. obtusa* and *C. japonica* genomes.

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## Appendix

Table 4 shows size and polymorphic information content (*PIC*) of sequence-tagged site (*STS*) markers detected in hinoki (*Chamaecyparis obtusa*), using primers developed in sugi (*Cryptomeria japonica*)

**Table 4**

| STS | Forward primer | Reverse primer | Annealing temperature (°C) | Size[a] | | Restriction enzymes used, and resulting PIC[b] |
|---|---|---|---|---|---|---|
| | | | | *Chamaecyparis* | *Cryptomeria* | |
| CC0272 | GGCGTTCGACTCAGTTGATT | TTGGGCCAGCCTTTCC | 57 | 500 | 400 | *Dde*I(0.26), *Nci*I(0.16) |
| CC0277 | GCAGCCAAGCGTTCATAGC | CCGATCGAAGGTAGCCAATG | 57 | 400 | 400 | *Msp*I (0.06) |
| CC0278 | GTGCTGACATGCCAGGATTT | ATTTGACCCGCCACTCATT | 57 | 350 | 320 | – |
| CC0281 | GATAACGCAGGTGTCCTAAGA | CCTGTCTCACGACGGTCTA | 57 | 350 | 350 | *Alu*I (0.37) |
| CC0285[c] | TTGGATAAACTCTCGCAGAC | GAGGAGCCTGGCATCA | 57 | 300 | 400 | – |
| CC0286 | TGACTGTCCCTTGGCTGTT | TGTCATACTCTCGCCGTCTC | 54 | 400 | 380 | – |
| CC0292[c] | AGGCGGCGTTCAAGC | TGCCAAGCAATCTGAGAGAG | 57 | 700 | 700 | *Msp*I(0.40) |
| CC0311 | CTTCAAACCTCGCAAGACAAA | TTGGTGGATCAGTTGGAACAT | 57 | 350 | 370 | – |
| CC0316 | AGGCGTTCGACTCAGTTGAT | GCGCACTTTCCTGGTGTT | 57 | 600 | 600 | – |
| CC0336[c] | ATCAGCTGCGCTCCAGT | TCTCATCCCACTCCATCTCTC | 57 | 550 | 550 | – |
| CC0344[c] | TTATAGTAATGGCGGATTCA | TCTCCGCCATTACAAGA | 57 | 450 | 500 | *Rsa*I(0.35), *Dde*I(0.37) |
| CC0352 | GAAATTTCAAGCGGTGTT | CGAATATGGCAGATAGTCAAT | 57 | > 1,500 | 380 | *Taq*I(0.36), *Hae*III(0.37) |
| CC0374[c] | AGGTGGTGGCGTTCAAGA | TATTGCCCACGTTTCTCTCTG | 57 | 1,000 | 1,000 | – |
| CC0421 | CAGAGCTGCAAGGCCTGTATC | TCTTTCATGCAGGTGCAAGTC | 57 | 350 | 600 | – |
| CC0428[c] | CAATTCCAAAGGACCGCTGCTT | TTGCGCTTGTTCTGCCACTTA | 57 | 1,000 | 550 | – |
| CC0438-1 | ATGCAAGTCGAACGGGAAGTG | GAGTGCTTTCGCCTGTTGGTG | 57 | 600 | 700 | – |
| CC0482[c] | TGGAACGTCACTCTGCGAAAC | TTGTAATTAGCCGCCCATCCT | 57 | 800 | 900 | – |
| CC0530[c] | GGCATGCTGTAGTGAATGTC | TAGACCTCAGGTTCCCAAGTA | 57 | > 1,500 | 2,000 | *Bst*0I(0.56) |
| CC0550 | TCAAGCCAACTTCTGTGTCGT | ATGCCATCTTCCCATTCGT | 55 | 600 | 600 | *Msp*I(0.16) |

**Table 4** (Contd.)

| STS | Forward primer | Reverse primer | Annealing temperature (°C) | Size[a] | | Restriction enzymes used, and resulting PIC[b] |
|---|---|---|---|---|---|---|
| | | | | *Chamaecyparis* | *Cryptomeria* | |
| CC0592 | ATTTGAGTTAGTTTGCGATGG | AGAAGATCCTCCTGCGTAAT | 57 | >1,500 | >1,000 | *Rsa*I(0.50), *Alu*I(0.50), *Mbo*II(0.50), *Hin*fI(0.50), *Dde*I(0.50), *Nci*I(0.50), *Bst*0I(0.50) |
| CC0674 | AGCGATCGCATGTTATTCTGA | TCCATGCGCTTCCTGTAGA | 57 | >1,500 | >1,000 | – |
| CC0702 | TTTACCAACAATTACAACCCA | GTTCTGCAGAGTTTGCCTT | 62 | 450 | 500 | – |
| CC0708[c] | TCCTGGGGAGAAGATCATCA | AATGTCTGGTGTCACTGGAGC | 57 | 500 | 500 | – |
| CC0725 | CGCTTCTGGTGTTGTTGGT | GATCCAGCACTTGCCTTCATA | 57 | 700 | 650 | – |
| CC0731[c] | TGCGCGCTTTCGGAGTTT | GTCCACGCAGACGACCAGTTC | 57 | 600 | 700 | *Hin*fI(0.11) |
| CC0737[c] | TCTCTAATGTGGCAGCTCTTT | TGGAACTTATGCTACATCCCT | 57 | 600 | 600 | *Dra*I(0.28) |
| CC0738[c] | GGGCTCTCCTTCTCTCA | TAAACCCAATTACAACTTGCT | 57 | 300 | 1,400 | – |
| CC0790 | GGCCTATCGATCCTTTAGACT | GCCACTCTGCCACTTACAATA | 57 | 600 | 550 | *Taq*I(0.06) |
| CC0822 | TGTCTGCCCATTGAGAAGT | TCAGATGCCATGTTGATAAGA | 57 | 600 | 700 | *Alu*I(0.19), *Hin*fI(0.11) |
| CC0831 | GCTCGTGCATCAAGGA | TGGGTTAAAAATAACTGGACA | 54 | >1,500 | 1,200 | *Rsa*I(0.35), *Ssp*I(0.40), *Nde*II(0.33) |
| CC0832[c] | AAAGATCAAGCGTATCAATGC | CAGAAGATCCTCCTGCGTAAT | 57 | >1,500 | 1,300 | *Hae*III(0.28), *Nde*II(0.06), *Hin*fI(0.37) |
| CC0838 | TGCGGGGGATTACTCA | TCCCTATTCGACTGCATAAAG | 57 | 500 | 500 | *Rsa*I(0.11), *Hae*III(0.18), *Nci*I(0.06) |
| CC0854 | AGTAATGGCCGCAGTGGAGT | TCTCAGAACCGGGAAGATGAA | 57 | 450 | 400 | – |
| CC0858 | GGTGGAAGAAGAGCCGCTTGT | AAACTGGCGCCCATTGAGTC | 62 | 900 | 800 | *Hae*III(0.16), *Sin*I(0.38) |
| CC0860[c] | AACAGCGCGCAAGTCTACA | CTAAGCCCTTTCACCACGAAT | 57 | 1,000 | 1,300 | *Hha*I(0.06), *Hae*II(0.06) |
| CC0951 | GCGAAAGGCATTTGATGATAA | CAGACCAGCCGTCACAGTT | 60 | 1,300 | 850 | *Rsa*I((0.30) |
| CC0958[c] | CTCAATGGCCGCTATTTCACT | GAAGCCAAACGAGAAGCAATG | 57 | 500 | 500 | *Sty*I(0.48) |
| CC0973[c] | CCGGTGTGGTTTACAGG | AGTACACAGCTATGGCAGACT | 57 | 700 | 600 | *Msp*I(0.38), *Dde*I(0.19) |
| CC0983 | GTTCGATCTGGGATCAACA | AACATGACTCCTTACCACCAC | 62 | 700 | 500 | *Alu*I(0.36), *Hae*III(0.25) |
| CC0991[c] | ATGCCAGGCTGATTTCTACAA | GACACAAGCCAAACATTCACA | 57 | >1,500 | 1,600 | *Rsa*I(0.21) |
| CC1015[c] | GGAGAAATTGGAGGGCATAG | AAGTCTTAAGCAACCGAATGG | 62 | 1,200 | 1,200 | – |
| CC1017 | GAAGAGAGCCGCATCGTG | GCAAGCGCTCCATAACTGATA | 60 | 650 | 600 | *Alu*I(0.16) |
| CC1104 | TTTCTACATGCGTTCCAATGC | GCTGAGATGTCCCGACCCTA | 57 | 800 | 600 | – |
| CC1112[c] | GGGACACTAAAGAGAAGAAAC | ATTCAGAGTCCAGCTATCAA | 55 | 800 | 800 | – |
| CC1135 | TGCTGGCTGGAATTAATCAGA | GCTGCAGTGGCCTTTACACAT | 57 | 550 | 600 | – |
| CC1145 | TCAACAATTGGCTGAGAACGA | GTCTTCATGCCTTGCCTCTG | 57 | 500 | 500 | – |
| CC1147 | GCACCCATCTCACACTTGTC | CCACCCTGTTCATGTGATTCT | 57 | 700 | 650 | – |
| CC1172[c] | GCGCGCTTTCGTAGTTTGGAC | GGAGCATCACGACGCACTGTA | 60 | 600 | 600 | *Hin*fI(0.40) |
| CC1187[c] | CAGATCCTGCACCATGTGGAA | CAAACTGCCATGGCCAGACT | 62 | 450 | 450 | *Rsa*I(0.45) |
| CC1195 | ATGGTGCATTTGGGTGTA | ATGCTGGATCTATTGGCTTTA | 57 | 1,200 | 1,100 | – |
| CC1204[c] | CGAGCGCTCTGGAACATT | TCTGATTGATTGCCTTACGAG | 57 | >1,500 | 1,300 | – |
| CC1262[c] | GAGAAACGGGCATAGTGAG | ACATATTCTTTCCCAACTCCA | 62 | >1,500 | 1,300 | *Dra*I(0.43), *Bgl*II(0.29) |
| CC1287[c] | TCAACTACTACTGGGCATCTG | ACCACTTGGCTCCTTCTT | 57 | 500 | 1,500 | *Ssp*I(0.35) |
| CC1319[c] | ATAGTAATGGCGGATTCAAAT | CGCCATTACAAGAACATAAGC | 60 | 450 | 400 | *Dde*I(0.37), *Ssp*I(0.35) |
| CC1371 | TCCGCAAGCATTTGAAGTAGG | GCCACACTCCCACAACCACT | 60 | 700 | 650 | *Taq*I(0.38) |
| CC1415 | AATTAACCTTATCCGCTCGTT | ACAATAAGAGTGGCGATGC | 57 | >1,500 | 1,500 | *Alu*I(0.37), *Hin*fI(0.37), *Sty*I(0.38) |
| CC1432[c] | TGGTGATGTACACGCCTGGTT | CGTTGAAATTCTCGGGAGCAT | 62 | 1,200 | 1,500 | *Msp*I(0.32), *Rsa*I(0.32) |
| CC1463 | TGGATGATGCGTCGTTGC | TCGAGAGAAATGACCCGTTTG | 57 | 800 | 850 | *Hae*III(0.35) |
| CC1531[c] | AAACGCAGTCGAGAGGATGAG | CACCGGCAGATTCCAGTCTAC | 62 | 600 | 650 | – |
| CC1555 | CTGCCGCCTTGTGTGGA | CAGTCTGTGGCGAAGAGCAAA | 57 | 750 | 700 | – |
| CC1606[c] | TAACCAGCTTTGCCCTCAG | ATACAATTCGCGGCTACCATA | 62 | 1,000 | 900 | *Alu*I(0.12) |
| CC1625[c] | CTCCAGACAGCGGTTCTTATG | TGCCATGGACTTCCTCTTCT | 62 | 1,100 | 1,100 | *Rsa*I(0.46) |

| | | | | | | |
|---|---|---|---|---|---|---|
| CC1689[c] | CAGGAAGCAACTCGCCACTA | ATTCCCGGTACCCACCATTTA | 56 | 1,000 | 1,100 | *Hae*III(0.58), *Nde*II(0.50) |
| CC1697 | GCCAGGCTGATTTCTACAA | AGACACAAGCCAAACATTCAC | 62 | >1,500 | 1,500 | *Rsa*I(0.06), *Dra*I(0.06) |
| CC1711 | GGCAAGCATGAATACTGTT | AATGAAGACTTGGGAAGAAAC | 55 | >1,500 | 1,500 | – |
| CC1787 | ACTAAGGCGTGGGAGGAACTG | CTGCCACCGCCATTCTTTAT | 62 | 400 | 800 | – |
| CC1798[c] | GGCGGCGGAGATTACTGTA | TAGAAGACGCGCATTTGAGAA | 57 | 800 | 800 | *Alu*I(0.06), *Hae*III(0.06), *Hae*II(0.06) |
| CC1799 | AAACGCAGTCGAGAGGATGAG | CACCGGCAGATTCCAGTCTA | 57 | 600 | 600 | *Hha*I(0.16) |
| CC1908 | AGGCCTGCACTGGATGAC | ATCGGCTCCTTGATGTTCTTT | 62 | 450 | – | *Rsa*I(0.36) |
| CC1944 | GAAGGATTATGTGGCACTT | GGGCGTAATTACCTACTATTG | 62 | >1,500 | 2,000 | *Rsa*I(0.37) |
| CC1974 | TCACCCTCGCCTCTTTCA | CAACATACCCACTTCGCAAAC | 55 | 400 | 380 | – |
| CC2051 | GCGAAATTATTTGTAGCAGAA | GAACCGGAACCAGACACT | 57 | 400 | 800 | – |
| CC2188[d] | AGCTGTGCGATCAAGTTTCTG | ATGGGCGTGCCTCCTAA | 62 | 1,200 | 1,400 | – |
| CC2196 | CGGCGTATTCACCTTACAGAC | GCCAGAATAAACTTGCCATTG | 62 | 600 | 600 | *Alu*I(0.35), *Hha*I(0.35) |
| CC2237 | AGAAATGGCCAACTGCTGAG | TTGCCCTGAAGTGTAACCAAA | 57 | 700 | 700 | *Taq*I(0.37) |
| CC2286[d] | ATAATGCCACCTCCAGGAC | AGGCCAGTTTAACAAATGTCA | 57 | 1,100 | >1,500 | *Sin*I(0.11) |
| CC2288 | TGTAGCGGAGAATACCAAG | CTAGACCATGGCATAAACATT | 57 | 600 | >2,000 | *Sin*I + *Nci*I(0.19) |
| CC2340[d] | TACAGGAGGCGGAGGAC | CTCAAACTGCCAAACAACAA | 63 | 450 | 1,000 | ALP[e](0.19) |
| CC2388 | ATACAGAACATGACGACCC | AAATAGACCACCATGAAACC | 57 | 600 | 2,000 | *Hin*fI(0.30) |
| CC2427 | TGTTGCTGTCTTGGCTAATC | CATCAAAAGGCAGGAAGAG | 57 | 550 | 550 | *Alu*I(0.19) |
| CC2435[d] | GCAGGCAGTTCAGAGTTTT | TCCCGAAGAGAGTTTTATGG | 57 | 800 | 900 | *Msp*I(0.42) |
| CC2448[d] | ATCCTAAGTCCCCAGAAAGT | GAATTGGAATGGCATAAAGA | 57 | >1,500 | 2,000 | *Rsa*I(0.34), *Hae*III(0.34), *Taq*I(0.26) |
| CC2478 | CGGGCAAGCATTTCTGTTCT | AGTTTGAGGAGGCATTATTT | 57 | 900 | 800 | *Hae*III(0.35), *Ban*II(0.36) |
| CC2483 | CTAAAAATTACGGCACCGAC | GCGACTGTAACATCATCTGG | 62 | 900 | 800 | – |
| CC2588[d] | CTGCCGCTGCCGTTTATTCC | TTATCCACGACGTACACACC | 57 | 700 | 900 | *Msp*I(0.30) |
| CC2646 | GAGTACCGAGAAGGCTGTGA | TGACGCCTCTTCTGATGCTG | 57 | 650 | 600 | – |
| CC2702[d] | TTCGCCAAGCCACCATAGAC | CTGCCACCACAACACCCTCC | 57 | 500 | 500 | – |
| CC2716[d] | GTTGACATGATCCGAAAGAG | CAAACGCAAATACTGAAAGG | 57 | 1,000 | 1,000 | *Msp*I(0.30), *Dde*I(0.30), *Taq*I(0.30), *Bst*0I(0.30), *Nci*I(0.30) |
| CC2750[d] | GGCAGCACACAGACAACACA | GATACTTCTCAGGCCCAACT | 62 | >1,500 | >1,500 | *Rsa*I(0.06), *Taq*I(0.26) |
| CC2752[d] | CCGCACTGCCATCTACGACT | AACCTCTCCTCCAACTCACC | 62 | 900 | 1,000 | *Msp*I(0.36), *Rsa*I(0.06), AluI(0.30) |
| CC2777 | AGAAGCAGAGGAGGATGGAG | CGACAGCGAATGATTTGACC | 62 | 350 | 700 | – |
| CC2831[d] | GGCGATGGCAGCAAACGAAG | CACGCACCACTCCACCCTAC | 62 | 500 | 500 | – |
| CC2839 | TACGTACGATGCGGTGGTTG | GCTCCTCTGCTTTCCTTTCT | 57 | 600 | 2,000 | *Msp*I(0.32), *Alu*I(0.32) |
| CC2857 | ATCAGAGGCAGTAGCAGAGG | TGGCATCATGAGAGGACGAG | 60 | 600 | >2,000 | – |
| CC2860[d] | CTAAAGGGAAACAAATCAGG | TACTCGTCTTCTAACCGTCA | 57 | 800 | 1,100 | *Dde*I(0.30), *Taq*I(0.37) |
| CC2865 | TTACAGAGGGAAGGGGAGGA | GGCAAGTAAGAAAGGGCACA | 57 | 800 | 1,200 | – |
| CC2916 | TCCGAAGATAAACAACTCCA | CCTTCAAGCCCTTCAACCAA | 57 | 700 | 700 | – |
| CC2917 | GGCACTCCTCCTGAAAAAGC | TGGGAAGAGAGACAAAAGAC | 57 | 500 | 500 | – |
| CC2920 | CCGCCACATTCACGCCCTCT | CGCAGTTCCAGTAGTTTCTC | 62 | 900 | 800 | *Msp*I(0.36), *Bst*0I(0.35), *Dde*I(0.19) |
| CC2921[d] | TTTTGGCGGTGGGAGGAATG | CAAGAATCGGTGAAGAACAG | 62 | >1,500 | 1,400 | *Hae*III(0.29), *Taq*I(0.16) |
| CC2930 | TTCTAGAGCTGGCCAAGAGC | AGGGAACCCTGCTCTGTCTT | 50 | 500 | – | *Hae*III(0.43), *Nde*II(0.41), *Bst*0I(0.32), *Nsi*I(0.32) |
| CC2946[d] | GTATCCAGGGATGCTCGAAA | AAATTGCCATCCTTCCTCCT | 50 | 900 | 1,200 | – |
| CC2976 | TTCTCATGGGAGGACAAACC | CTACCGATGCAGAAGGCTCT | 60 | 450 | 500 | *Ban*II + *Ava*I(0.38) |
| CC3055[d] | GCCCTCAGAAAGTTGCATTC | TAGAGGTTCAATGGAAGGCG | 51 | 500 | – | *Taq*I(0.36), *Ssp*I(0.35) |
| CC3106 | AACACTGCAAGGGAGATTGG | CATGCAGAATCGTTGCATTT | 52 | >1,500 | – | *Rsa*I(0.40), *Hae*II(0.28), *Hha*I(0.32) |
| CC3133[d] | AAGGTTCATCGCCCTATGTG | AGCTCCAACCTCAAAGACCA | 60 | 800 | 900 | *Eco*RI(0.06) |
| CC3142 | CTAGGGTTGCTGTGGTTTCC | CCGATCCAAATGTTATTCCG | 50 | 700 | 600 | – |
| CC3152 | TGGCATCTTCATGTGTTTGG | CCAGATGTGCCACCCTTAGT | 50 | 1,000 | – | *Hin*fI(0.44), *Bst*0I(0.21), *Sin*I(0.45) |
| CC3162 | ATGGAGTCGCCACAAAAGAC | GTCTCGCATCAAATCTCCGT | 60 | 700 | 700 | *Hha*I(0.26) |
| CC3367[d] | AGAGATGGCGCTCACTCATT | TACTACACCACCGCTTGCAG | 65 | 1,200 | 900 | *Nsi*I(0.30) |
| CC3397[d] | ATGAGAATTCGTCGTAGCGG | AACTAGCCATGTGAGCAGGC | 55 | 300 | 300 | – |

**Table 4** (Contd.)

| STS | Forward primer | Reverse primer | Annealing temperature (°C) | Size[a] | | Restriction enzymes used, and resulting PIC[b] |
|---|---|---|---|---|---|---|
| | | | | *Chamaecyparis* | *Cryptomeria* | |
| CC3414 | TATTTTGGAGCTTTTTGCGG | TGGGTTGGTGTTGGAAGAAT | 50 | 600 | – | *Msp*I(0.26), *Taq*I(0.53) |
| CC3455 | TAACAAGGGAAAACACAGCA | CCGCATCCCATTGAACAGCA | 60 | 1,000 | – | *Rsa*I(0.36), *Hin*fI(0.28) |
| CC3583 | CCCACATCATCTTGTAGGGC | GCAGGGCTCCAAGTTTACAG | 60 | > 1,500 | > 2,000 | *Taq*I(0.42), *Dde*I(0.42) |
| CC3807 | CAATCCCGTAACCAAAAACG | GCGTTGCTGATATGCTTTGA | 50 | > 1,500 | 1,000 | – |
| CC3816[d] | AGTCAGAGCTGCCTGGAAAG | GCCACGAAGGGATTCATTTA | 60 | > 1,500 | 2,000 | *Rsa*I(0.32), *Dde*I(0.06) |
| CC3823[d] | CCCCACAGGACATCAAAACT | ACGCATTCTCCATCACTTCC | 50 | 1,000 | 900 | – |
| CC3839[d] | CTGCATTTCCTCTGGAATCG | TTGGGATAAACCTTTTTGCG | 50 | > 1,500 | 2,000 | – |
| CC3861 | CTGACACTGATGAGGCCAGA | TGCAAAGATGGAAAACACCA | 57 | 800 | – | *Msp*I(0.37), *Rsa*I(0.37), *Alu*I(0.36), *Hae*III(0.35), *Dde*I(0.36), *Bam*HI(0.36) |
| CC3872[d] | AGCGGAAGTACCCTTTGGAT | GGTTCCCAGTGATTTCCTGA | 60 | > 1,500 | 1,600 | *Dde*I(0.36), *Nde*II(0.26) |

[a]Approximate size of STS fragment amplified (bp). –: No PCR amplification
[b]The names of restriction enzymes that detected the polymorphisms. – No polymorphism detected, using any of the 26 restriction enzymes, among the panel of 16 individuals. *Numbers in parentheses* indicate PIC
[c]Iwata et al. (2001)
[d]Tani et al. (2003a, b)
[e]*ALP* Amplicon length polymorphism

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