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Cleaved amplified polymorphic sequence markers in sugi, *Cryptomeria japonica* D. Don, and their locations on a linkage map

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Abstract Sugi, *Cryptomeria japonica* D. Don, is one of the most important forestry species in Japan. We here report the development of 217 CAPS markers derived from sugi cDNA libraries. More than half of a set of STS markers produced could be converted into CAPS markers using restriction endonuclease analysis. Of the 217 markers, 71 showed different patterns of polymorphism when they were digested with a range of endonucleases and, in total, 347 polymorphisms were found in the various combinations of STSs and endonucleases. When the polymorphisms gave co-dominant patterns in a screening program, the polymorphic information content (PIC) used to evaluate the value of the polymorphisms was relatively high (0.33, on average) compared to the information yielded by commonly used markers, like isozymes. The results of a segregation analysis suggest that approximately 80% of the CAPS markers developed here will show co-dominant inheritance. From logistic regression analysis, the polymorphisms were found to be associated more strongly with intron than with exon regions. Sixty two markers were subsequently mapped on the previously reported linkage map, 15 of which showed abnormal segregation, presumably caused by linkage with lethal factors.

Keywords Cleaved amplified polymorphic sequence (CAPS) marker · *Cryptomeria japonica* · Sequenced-tagged-site (STS) · Expressed-sequence-tag (EST) · Linkage map

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Introduction

Informative DNA markers showing co-dominant inheritance are valuable for constructing linkage maps and studying population genetics, especially for allogamous plant species. Plant geneticists have sought to map the genome, and study its organization, in diverse species by various methods including: evaluation of specific breeding lines such as recombinant inbred lines (RIL; Lister and Dean 1993) and doubled-haploid lines (DH; Powell et al. 1992), development of new theories (Grattapaglia and Sederoff 1994) and analysis of molecular markers. However, for allogamous plant species subject to strong inbreeding depression like conifers it is difficult, or even virtually impossible, to make a RIL or DH line. Thus, developing informative molecular markers is a much more effective way to resolve genetic questions.

In the last decade, with the advances of molecular biology, various types of molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, have been developed and applied to the genetic studies of plant species. Amongst these, RAPD and AFLP markers have the virtues of speed and efficiency, due to their use of polymerase chain reaction (PCR) systems. However, they are mostly inherited in a dominant manner and so are less-informative than co-dominant marker systems. On the other hand, RFLP markers are generally co-dominant, but they require several time-consuming, tedious steps and a large amount of DNA per assay. Moreover, for species with large genomes, like conifers, the signal of a single-copy gene in RFLP analysis is generally weak, and the DNA is not usually well-digested because of the inhibitory effects of methylation on the endonucleases used.

Cleaved amplified polymorphic sequence (CAPS), also known as PCR-RFLP, markers utilize amplified DNA fragments digested with a restriction endonuclease to display restriction site polymorphisms (Konieczny and Ausubel 1993; Drenkard et al. 1997) and they have been

developed in several plant species, such as *Arabidopsis thaliana* (Konieczny and Ausubel 1993), rice (Williams et al. 1991; Ghareyazie et al. 1995), barley (Tragoonrung et al. 1992; Mano et al. 1999), wheat (Talbert et al. 1994) and loblloly pine (Harry et al. 1998). In comparison with other molecular-marker systems, CAPS markers have several advantages. They are quick and easy to assay, they require only a few nanograms of DNA owing to the PCR amplification, and they are inherited mainly in a co-dominant manner. CAPS markers are robust because an amplified product is always obtained, whereas RAPD and AFLP markers have inherently null alleles. Furthermore, where CAPS markers are developed from sequenced-tagged-site (STS) primers with designs based on cDNA (i.e. expressed-sequence-tags; ESTs), they represent real functional genes and are more useful as genetic markers than those based on anonymous non-functional sequences, such as simple sequence repeat (SSR) markers. To detect polymorphisms of STS fragments, we can also use other marker systems or techniques such as SSCP or DGGE. Although SSCP and DGGE can detect DNA sequence-alterations as small as a single nucleotide change in the entire segment, they are more sensitive to the experimental conditions and need more difficulty in their manipulation than CAPS markers.

Sugi, *Cryptomeria japonica* D. Don, is one of the most important forestry tree species on account of its excellent characters, such as rapid growth, a straight bole, ease of regeneration, and softwood with a pleasant color and scent. In sugi, several mapping projects have been undertaken using different marker systems and types of segregating populations, such as a full-sib F_1 population (Nikaido et al. 2000) and a three-generation pedigree derived from the full-sib cross or self-pollination of F_1 individuals (Mukai et al. 1995). Therefore, the information contained in the different maps should now be integrated to correlate the loci identified on them. Such integration should enable the construction of a complete map of sugi and a comparison of the quantitative trait loci (QTLs) detected on different maps. The EST markers, such as CAPS and RFLP markers derived from cDNA, should provide good anchor points (i.e. putatively identical points of reference between different maps) in integrating the information contained in multiple maps derived from different mapping populations.

The coding regions of functional genes are generally well preserved not only within species but also between species. Tsumura et al. (1997) and Tsumura et al. (1998) showed that STS primer pairs derived from sugi cDNA were successfully amplified in related species within *Taxodium* and *Chamaecyparis*. This suggests that the sugi cDNA-derived CAPS markers could be efficiently applied to related species, such as hinoki [*Chamaecyparis obtusa* (Sieb. Et Zucc.) Endl.], which is the second most-widely cultivated tree species in Japan. This would increase the effectiveness of developed markers and reduce the cost of developing them in related species. Moreover, sugi cDNA-derived CAPS markers would provide good anchor points in comparative mapping among related species and a means of locating homologous regions of maps (i.e. synteny) among the related species.

Following these considerations, Tsumura et al. (1997) generated 66 STS markers from sugi cDNA and found that 16 of them showed restriction-site polymorphisms (i.e. CAPS). However, the number of CAPS markers was insufficient for use as anchor points in integrating or comparing the linkage maps derived from other families or other related species. Nikaido et al. (2000) also developed CAPS markers, which have been mapped in an F_1 population, but these markers were not evaluated. In this study, we designed STS primer pairs based on sugi cDNA sequences and screened their potential as CAPS markers. Some of the developed markers were further used in the construction of a linkage map, together with the segregation data previously reported by Mukai et al. (1995). We also characterized the polymorphisms of the CAPS markers, together with 16 previously published CAPS markers (Nikaido et al. 2000), in relation to the exon, intron and total lengths of the STS fragments and the putative functions of the cDNAs from which the primer pairs were designed, in order to make the future development of CAPS markers more efficient.

Materials and methods

Designing primers and testing for the amplification of STSs

We designed 55 and 716 pairs of primers, based upon sugi cDNA sequence libraries derived from 3-day imbibed embryos (Mukai et al. 1995) and inner-bark tissues (Ujino-Ihara et al. 2000), respectively, using OLIGO ver. 4.0 or 5.1 (National Biosciences). The pairs derived from these libraries were given designations with the prefixes “CD” and “CC”, respectively. These 771 pairs were tested for PCR amplification of STSs. The amplification was performed in 20-µl reaction volumes containing 0.2 µM of each primer, 0.2mM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 or 2.0 mM $MgCl_2$, 0.4 U of *Taq* polymerase and 4 ng of template DNA using thermal cyclers (a GeneAmp PCR Systems 9600, Applied Biosystems PE, or a PTC100 Programmable Thermal Controller, MJ Research) programmed for 5 min at 94°C, 35–45 cycles of 1 min at 94°C, 1 min at 50–65°C and 1 min 30 s at 72°C; finishing with 5 min at 72°C. The concentration of $MgCl_2$, the annealing temperature and the number of cycles were optimized for the amplification of STS fragments.

Screening of polymorphisms

Primer pairs that amplified STSs were then screened for their ability to detect restriction-site polymorphisms, with a screening panel comprising 15 individuals (11 plus trees, i.e. elite trees selected according to their excellent phenotype from various parts of Japan, and four parental trees of segregating populations). PCR products amplified from the 15 individuals were subsequently digested with either 24 endonucleases (*Alu*I, *Bam*HI, *Bgl*I, *Bgl*II, *Dra*I, *Eco*T38I, *Hae*III, *Hha*I, *Hin*cII, *Hin*dIII, *Hin*fI, *Mlu*I, *Msp*I, *Nci*I, *Nsi*I, *Rsa*I, *Scr*FI, *Spe*I, *Sph*I, *Ssp*I, *Sst*II, *Sty*I, *Vsp*I and *Xho*I) or 36 of them (the 24 just listed, plus *Bcl*I, *Bst*XI, *Eco*O109I, *Eco*RI, *Eco*RV, *Mbo*II, *Nhe*I, *Nsp* V, *Pst*I, *Pvu*II, *Sal*I and *Sau*96I). The products were then checked for restriction patterns through electrophoresis in 2% agarose gels stained with ethidium bromide. The 24- and 36-endonuclease sets are referred to hereafter as sets A and B, respectively.

Characterization of polymorphisms

To identify informative markers, we evaluated the degree of polymorphism they detected, using the polymorphic information content (PIC) (Botstein et al. 1980) as an index, for all the different polymorphisms observed by the various combinations of STSs and endonucleases.

For the STSs derived from inner-bark libraries, we characterized the observed polymorphisms in the following two respects. Firstly, we evaluated the influence of the lengths of the exon and intron contained in each STS on the level of polymorphism. Exon lengths were obtained from the size of the amplified fragments of the corresponding cDNA clones, and intron lengths were derived by subtracting the length of the exon from the total length of the STS. STSs longer than 2,000 bp were excluded from the analysis because their lengths could not be precisely measured by reference to the molecular size markers used in the electrophoreses. To clarify the contribution of exon and intron lengths to the polymorphism, we applied the following logistic regression model to their relationship:

$$\log\left(\frac{p(x_e,x_i)}{1-p(x_e,x_i)}\right)=\beta_0+\beta_e x_e+\beta_i x_i\,,$$

where x_e and x_i are the exon and intron lengths, respectively, and $p(x_e,x_i)$ is the conditional probability that the STS shows the polymorphism through digestion by at least one endonuclease contained in set A or B, given the lengths of the exon and intron found. The goodness of fit of the model was tested based on deviance and Pearson χ^2 statistics, and the significance of each explanatory variable was tested by calculating Wald χ^2 values. We also applied logistic regression to the relationship between the total length of each STS and the polymorphism, in order to evaluate the contribution of the fragment lengths themselves. StatView ver.5.1 (SAS Institute) was used in the analyses.

We also evaluated the relationship between the polymorphisms and the putative functions of the cDNAs from which the STS primer pairs were disigned. We classified the STSs into 13 functional classes (related to metabolism, energy, cell growth, transcription, protein synthesis, protein destination, transport facilitation, intracellular transport, cellular organization, cell-wall formation, signal transduction, stress response and organelle-specific proteins) according to their putative functions, based on homology with proteins identified in a BLAST search at a threshold score of 50 or more (Ujino-Ihara et al. 2000). We then determined the proportions of the polymorphic STSs in each functional class. Differences in the proportions among the classes were evaluated by a χ^2 test based on the resulting contingency table. Because the degree of polymorphism depends on the number of endonucleases used in the screening, these analyses were separately performed with two data sets; one derived from STSs screened with endonuclease set A, and the other from STSs screened with set B (hereafter, designated as data sets I and II, respectively).

Segregation analyses and construction of a linkage map

Some of the polymorphic STSs were investigated further to determine their mode of inheritance and location on a linkage map. In this analysis, we used the segregating population that had been previously assessed in the construction of the linkage map generated by Mukai et al. (1995). This population consists of 73 individuals derived as a self-pollinated array of progeny (F_2) from an F_1 hybrid of a cross between two local cultivars, 'Kumotooshi' (female) and 'Okinoyama-sugi' (male). Markers considered to be heterozygous in the F_1 hybrid were employed for segregation tests and segregation patterns were scored for the markers. The segregation data were analyzed using the MAPMAKER/EXP 3.0 program (Lander et al. 1987), including information concerning 164 markers (128 RFLPs, 33 RAPDs, two isozymes, and one morphological) used in Mukai et al. (1995). Linkage thresholds for grouping markers at loci were set at LOD scores of 4.0 or more, and a maximum map distance of 40 cM. The "Order" command was used to decide the orders of the loci and the Haldane (1919) map function was used in the analysis.

Results

Test for the amplification of STSs

Of 771 primer pairs designed, 361 successfully amplified STSs of which 31 were based on sequences in the cDNA library from 3-day imbided embryos, and 330 on sequences in the inner-bark tissue library. In most cases, suitable PCR conditions were obtained solely by varying annealing temperatures. The size of the STSs ranged from 300 bp to over 2,000 bp and most (approximately 70%) varied from 500 bp to 1,500 bp. In most cases of failure in amplifying STSs, plural sizes of fragments were amplified even in a stringent condition.

PCR amplification of these STSs was carried out in optimized conditions with 15 individuals included in the screening panel. Of these STSs, ten did not yield any PCR products in several (1–7) individuals and ten showed a clear length polymorphism of amplified fragments, i.e. amplicon length polymorphism (ALP) (Ghareyazie et al. 1995) (see Appendix). The former type of polymorphism seemed to be caused by variations within the priming site and the latter mainly by insertion/deletion (indel) mutations within the amplified region.

Screening for restriction-site polymorphism

Of the 361 STSs, 267 (74%) were revealed to be polymorphic in the screening, following digestion with restriction endonucleases. In cases in which we applied endonuclease sets A and B, 70% and 77% showed polymorphisms, respectively. Of these, 217 were found to have clear polymorphisms and 71 showed different polymorphic band patterns when they were digested with different endonucleases. In total, we found 347 clear polymorphisms using the different combinations of STSs and endonucleases (see Appendix).

The polymorphisms observed in the screening program were classified into the following five types based on their observed band patterns: (1) bi-allelic co-dominant patterns, which seemed to correspond to two homozygotes and one heterozygote, (2) as in the first case, except that no heterozygote was found, (3) bi-allelic dominant patterns, which could be scored only in terms of presence or absence, (4) multi-allelic co-dominant patterns, apparently corresponding to genotypes with multiple alleles, and (5) patterns for which the allelic relationship could not be inferred. These five types accounted for 119, 6, 183, 16 and 23 of the polymorphic combinations, respectively (see Appendix).

Characterization of the observed polymorphisms

We calculated PIC values for all the observed polymorphisms except for those in the above-mentioned class 5. In class 1, PIC was generally high, its average value be-

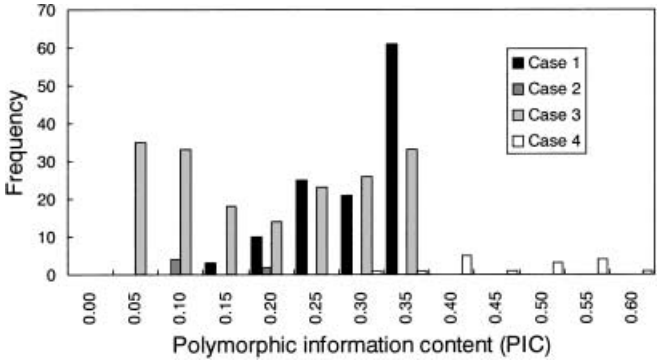

Fig. 1 Polymorphic information content (*PIC*) of the polymorphisms observed with the different combinations of STSs and endonucleases. *Cases 1 to 4* indicate four types of band patterns observed in the screening panels. Case 1: bi-allelic co-dominant patterns, which seemed to correspond to two homozygotes and one heterozygote. Case 2: patterns like case 1, except that no heterozygote was found. Case 3: bi-allelic dominant patterns, which can be scored only in terms of presence or absence. Case 4: multi-allelic co-dominant patterns, which seemed to correspond to genotypes with multiple alleles

ing 0.31 (see Fig. 1, and the Appendix). In class 2, PIC was generally low (0.15, on average), while PIC values in class 3 were diverse (0.21, on average). In class 4, where multiple alleles were observed, PIC was generally higher than that observed in the other three classes listed (0.46, on average).

For the primer pairs derived from the inner-bark libraries, we evaluated the relationship between the STS lengths and the restriction-site polymorphisms. For data set I, most of the STSs showed polymorphism when the intron length exceeded 200 bp (Fig. 2b). However, the length of the exon had a less-marked influence on the number of polymorphic STSs (Fig. 2a). We applied logistic regression to these data and obtained the following regression equation:

$$\log\left[\frac{p(x_e,x_i)}{1-p(x_e,x_i)}\right] = -0.354 + 0.144(\pm 0.065)\,x_e \times 10^{-2} + 0.348(\pm 0.117)\,x_i \times 10^{-2}.$$

Neither the deviance nor Pearson χ^2 values of this model were significant (χ^2_{79}=49.72, p=0.996 and χ^2_{79}=62.83, p=0.909, respectively), indicating that there were no grounds for deciding that this model did not fitt well to the data. The coefficient of determination (R^2) for this model was 0.122. The Wald χ^2 test indicated that the contributions of the exon and intron length were significant at the 5% and 1% probability levels (χ^2_1=4.89, p=0.027 and χ^2_1=8.91, p=0.003, respectively). The intron length gave higher values for the coefficient and the Wald χ^2 than the exon length, suggesting that the intron region in each STS contributes to restriction-site polymorphism more strongly than the exon region. We also applied logistic regression to the relation between the total length of the STSs (x_t) and the polymorphism, obtaining the following equation:

$$\log\left[\frac{p(x_t)}{1-p(x_t)}\right] = -0.667 + 0.214(\pm 0.053)\,x_t \times 10^{-3}.$$

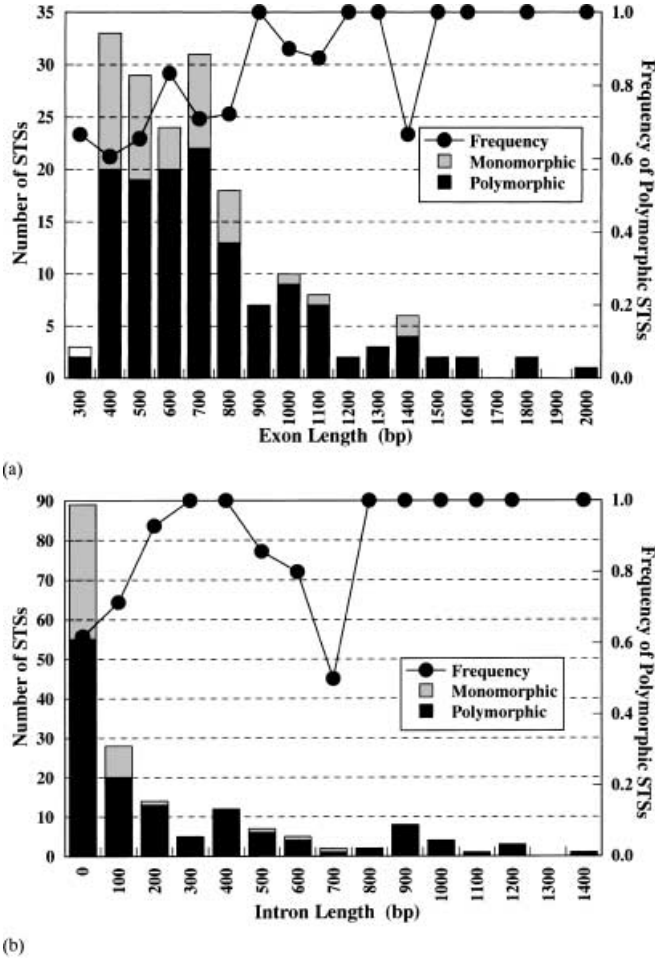

Fig. 2 Numbers of "polymorphic" STSs, i.e. those that showed restriction-site polymorphism with at least one endonuclease in the screening with endonuclease set B (see text), and "monomorphic" forms, i.e. those that did not show polymorphism throughout the screening, within the length of (**a**) the exon and (**b**) the intron. Line graphs indicate the proportion of polymorphic STSs within each class

Neither the deviance nor the Pearson χ^2 values were significant (χ^2_{22}=11.68, p=0.964 and χ^2_{22}=9.56, p=0.990, respectively) and the Wald χ^2 was significant at the 0.1% probability level (χ^2_1=16.40, p<0.001). The R^2 value for this model was 0.110. For data set II, the results were very similar to those for data set I, except that the effect of exon length was not significant at the 5% level.

We also evaluated the relationship between the putative function of the amplified sequences and the polymorphisms. We found that differences in the proportion of polymorphic STSs among the functional classes were not significant at the 5% probability level in either data set (χ^2_{12}=20.60, p=0.057 for data set I; χ^2_{12}=10.50, p=0.311 for set II). This indicates that there is no clear relationship between the putative functions and the polymorphisms.

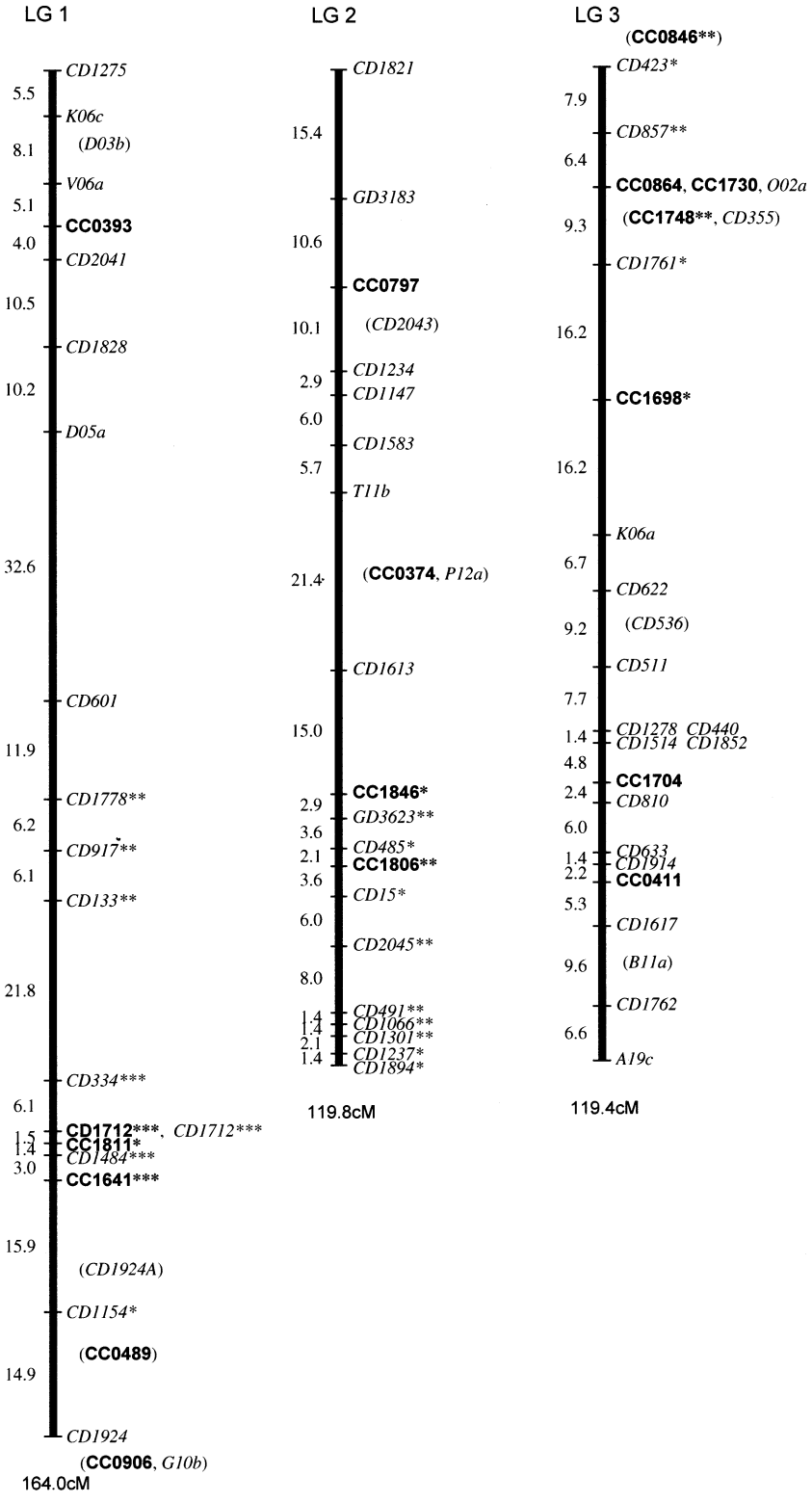

Fig. 3 Sugi linkage map based on the F_2 segregating population between "Kumotooshi" (female) and "Okinoyama" (male) showing CAPS, RFLP, RAPD, isozyme and morphological loci. Four linkage groups, each consisting of just two markers, were not included in the figure. The loci are listed on the right, and map distances in cM are shown on the left. CAPS loci are indicated in *bold letters* and the *prefixes* CD and CC indicate markers derived from the cDNA libraries of 3-day imbibed embryos and inner-bark tissues, respectively. The other loci are indicated in *italic letters.* RFLP loci are indicated by the *prefixes* CD and GD, for loci detected by cDNA and genomic DNA analysis, respectively. Loci designated by *single capital letter prefixes*, by *three letters* and by the *prefix MT* indicate RAPD, isozyme and morphological trait loci, respectively. *Single, double and triple asterisks* indicate loci with segregation that is distorted significantly at the 5%, 1% and 0.1% probability levels, respectively. Loci with ambiguous locations are shown in *parentheses*; 167 loci (identified by 46 CAPS, 101 RFLP, 17 RAPD and two isozyme markers, and one morphological marker) with confirmed map positions were assigned to 15 linkage groups, with a total size of 1,109.1 cM

Segregation test and construction of the linkage map

Sixty markers, which showed heterozygous patterns in the F_1 hybrid, were subsequently used in segregation analysis. Of these markers, all 30 that showed co-dominant band patterns in the screening programm (classes 1 and 4) were found to segregate in a co-dominant manner among F_2 recombinants. Of the 27 markers that showed dominant type band patterns in the screening (class 3), 16 segregated in a co-dominant manner and the others in a dominant way. The other three markers showed band patterns with allelic relationships that could not be

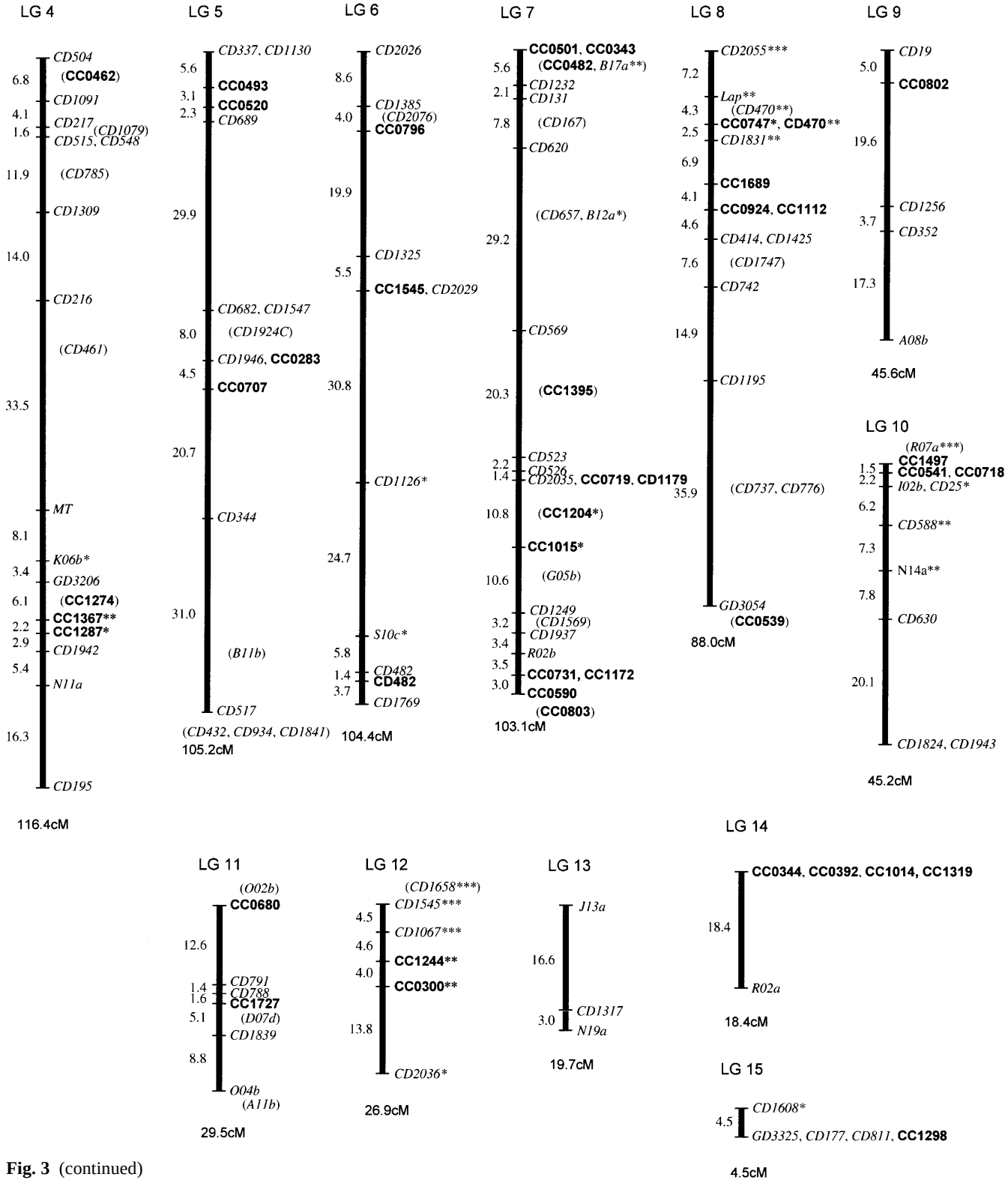

Fig. 3 (continued)

inferred from the screening results (class 5); two segregated in a co-dominant manner and the other in a dominant fashion. In total, 48 markers out of 60 segregated in a co-dominant manner. The segregation data concerning the 60 markers were evaluated by χ^2 tests for goodness-of-fit to the expected ratios, i.e. 1:2:1 in co-dominant cases and 3:1 in dominant cases. Fifteen out of sixty of the markers showed a significant deviation from the expected segregation ratios at the 5% level. Of these, two markers (CD1712, CC1641) showed a severe distortion, which was significant at the 0.1% probability level.

Whether they fitted to the expected ratio or not, all of the segregation data were used in the linkage analysis, because the abnormal segregation of markers has been already reported in a study using the same population (Mukai et al. 1995). In the analysis of the 226 segregating markers, all those except for one CAPS and three RAPD markers were distributed into 18 linkage groups, of which 15 contained more than three markers (Fig. 3). The total map distance covered by these 15 linkage groups was 1,109.1 cM, and the average distance of intervals between marker loci was 8.7 cM. The CAPS markers were distributed amongst all of these 15 linkage groups except for linkage group 14, and the positions of 46 CAPS markers could be confirmed.

All of the 15 CAPS markers with distorted segregation were located in the neighborhood of markers with distorted segregation that have already been reported in Mukai et al. (1995) (Fig. 3). The two markers with severe segregation distortions (significant at the 0.1% probability level) were mapped, together in a cluster of markers with distorted segregation, on the first linkage group (LG1).

Discussion

In this study, we developed 217 CAPS markers in *C. japonica*. The total number of CAPS markers available for sugi is now 233, including those previously developed by Tsumura et al. (1997). This represent the second largest number currently available for any plant species after *Arabidopsis thaliana*, and the largest number for any tree species.

The CAPS markers developed in this study are expected to be inherited mainly in a co-dominant manner. Although 53% (183/347) of the polymorphism showed dominant band patterns in the screening, i.e. they could only be scored in terms of their presence or absence, a number of these seemed to be inherited in a co-dominant manner, because they showed low PIC values. Low PIC values suggest that homozygotes with rare alleles at the corresponding loci may not have been observed, purely by chance, owing to the limited number of individuals assessed in the screening process. Furthermore, in the segregation test, of 26 markers that showed dominant band patterns in the screening, 16 were found to segregate in a co-dominant manner. In total, 80% (48/60) of the markers segregated in a co-dominant way in the segregation test. Although we did not check the segregation of all the developed markers, this proportion should be a good reflection of the total ratio.

The screening panel used in this study contained 11 plus trees selected from various locations in Japan. Hence, the genetic heterogeneity in the screening panel would be expected to reflect the heterogeneity in the Japanese sugi populations as a whole, at least to some degree. Thus, the degree of polymorphism observed in the panel should reflect the value of the markers in further genetic studies. The markers that showed bi- or multi-allelic co-dominant band patterns in the screening (classes 1 and 4), which comprised 39% (135/347) of the total observed polymorphisms, had comparatively high PIC values, indicating that these markers should be generally informative in practice. Moreover, 33% (71/217) of the developed markers showed different polymorphic band patterns when they were digested with different endonucleases. These markers could be treated as multi-allelic markers, which should make them more-informative in further investigations.

In this study, 15 out of 60 CAPS markers showed a departure from expected segregation ratios in the mapping population. This, however, is unlikely to have been due to the nature of the developed markers. The main cause of the segregation distortions is probably linkage with deleterious or lethal alleles, because all of these markers were clustered on the linkage map with markers that have already been observed to segregate abnormally (Mukai et al. 1995). If observed distortions are caused only by linkage with viability loci, the locus positions and effects can be estimated statistically from the distortions (Cheng et al. 1996). In practice, viability loci causing inbreeding depression have been detected from mapping data in conifers (Kuang et al. 1998, 1999; Remington and O'Malley 2000). In other words, the distorted segregation of CAPS markers will provide good information for detecting deleterious or lethal alleles.

Although the CAPS markers developed in this study are expected to have valuable practical features, their development is not very efficient (using current techniques) because of the costs, time and labor involved. Therefore, improving the efficiency of their development would be an important step. From the logistic regression analyses, we found that the intron length of STSs contributed more to the polymorphism than the exon length. This indicates that a strategy whereby amplification primers were placed so that STS fragments always included some intron regions should increase their efficiency in detecting STS polymorphism. This strategy could be exploited when similar gene sequences in other plants are found from DNA databases, and possible intron positions can be identified. We also derived a logistic regression equation concerning the relationship between the total length of STS markers and polymorphism. Based on this equation, the probability that we get a polymorphic STS will exceed 0.8 when its total length is 1,000 bp (Fig. 4). However, although the efficiency of obtaining polymorphic STSs will increase as their size increases, the PCR amplification will become less efficient and the pattern of the cleaved fragments will become more difficult to score since multiple bands will be produced. Hence, polymorphic STSs are expected to be obtained most efficiently when primer pairs are designed that amplify fragments with sizes ranging from 1,000 bp to 1,500 bp. No clear relationship between the polymorphism and putative functions of the cDNA used for designing the primer pairs was found. This suggests that if we consider only the degree of polymorphisms of the STS markers, there is no need to consider the function of the cDNA when developing CAPS markers.

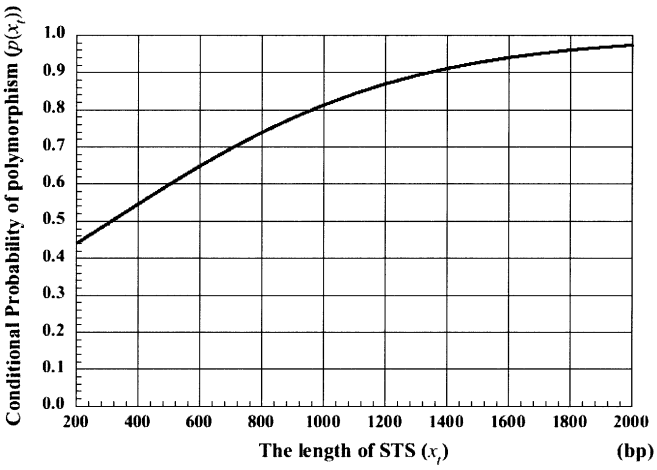

Fig. 4 Conditional probability [$p(x_t)$] that a STS with given total length (x_t) is revealed to be polymorphic by at least one endonuclease when the STS is screened with endonuclease set B (see text). The probability calculation was based on the estimated logistic regression equation of polymorphism on the total length of the STSs

In this study, we also found length polymorphisms of amplified fragments, i.e. ALPs. ALP-type STS markers are appealing because developed markers need not to further manipulation of PCR products when assaying polymorphisms, a feature which also helps reduce the cost of screening polymorphisms. In this study, however, the frequency of primers that detected ALPs (10 primer pairs out of 438) was musch lower than expected, in comparison with studies in black spruce (Perry and Bousquet 1998) and rice (Ghareyazie et al. 1995). Several possible reasons could be postulated for the low frequency of ALPs we detected: a major one being that we designed the primers so as to bind mainly to sites within the exons of the STSs, in contrast to the strategy used by Perry and Bousquet (1998), who designed their primers to bind to sites within 3´ untranslated regions (UTR). Although the strategy of developing STS markers based on ALPs is appealing, in several respects they have some disadvantages in comparison to CAPS markers. First, the detectable polymorphism of ALP markers is no greater than that of CAPS markers. Second, the detection of polymorphism of ALP markers is potentially more demanding than that of CAPS markers, since the possible presence of heteroduplexes makes the scoring of ALPs difficult and, in some cases, sequence analysis is needed to confirm the observed polymorphism (Perry and Bousquet 1998). Although CAPS markers will also be affected by the presence of heteroduplexes, the influence is not so large that makes the scoring of banding patterns difficult. This is because the scoring of CAPS markers basically depends on whether STS fragments, each of which shows a single band in electrophoresis before enzyme digestion, have sequence-specific cuts or not.

The linkage map constructed in this study showed the location of 59 CAPS markers, the position of which could be confirmed in 46 cases. Together with the CAPS markers developed by Tsumura et al. (1997) and Nikaido et al. (2000), the positions of 72 loci of CAPS markers were confirmed on these maps. Together with RFLP markers developed by Mukai et al. (1995), 158 EST loci were determined. These markers should provide good anchor points for integrating maps derived from different families.

The linkage map contained 220 markers in total. However, the number of linkage groups detected (15) has not yet coverged to half the number of chromosomes in sugi (i.e. 11; 2n=22). One cause of this failure could be attributed to the segregating population used in the mapping, which was derived from the self-pollination of an F_1 individual, because this type of population is expected to be heavily influenced by deleterious or lethal factors. For constructing a completely converged linkage map of sugi, it will be essential to integrate the information contained in different maps, such as that developed by Nikaido et al. (2000), based on AFLP and CAPS markers.

The mapping population used in this study was also used to map QTLs associated with juvenile growth, flower-bearing and rooting ability (Yoshimaru et al. 1998). The detected QTLs could be compared with those detected in other populations based on the integrated map. Moreover, the integrated map and its EST loci will make it possible to find homologous regions of a chromosome and, also, homologous QTLs that have been conserved among related species, such as hinoki. For the above purposes, CAPS markers are very useful and convenient, especially for allogamous plants with large genomes like conifers (the genome size of *C. japonica* is 6×10^9 bp, Sasaki et al. 1997).

The newly developed CAPS markers should be effective not only in genome mapping but also in population analysis. The polymorphisms of CAPS markers are mostly co-dominant and will be mainly selectively neutral. Hence, they should be just as suitable for population analysis as isozyme markers. Moreover, CAPS markers have the advantage that the number of available markers is virtually unlimited. The potential of CAPS markers in population analysis has already been shown in a study by Tsumura and Tomaru (1999), in which they evaluated the genetic diversity among natural populations of sugi using several CAPS markers. The newly developed CAPS markers and the information about their positions on the linkage map will provide a useful way to analyze genetic diversity at the chromosome level.

Data concerning the markers developed in this study should be valuable for researchers studying related species, and will be made available on the net, at http://www.ffpri.affrc.go.jp/labs/cjgenome/database/cjdatae.html.

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Appendix Description of CAPS and ALP markers in sugi (*C. japonica*).

Locus	Forward primer	Reverse primer	An. temp.[a]	Cycle	Size[b]	LG[c]	CAPS and ALP[d]
CC0257	GCCCTCGACGATGTGTGGAC	CACGAAACCTTCCGCCAGTG	55	40	400		*Xho*I (5, NA)
CC0283	TGGGATCATGAAGGGCAAAG	AGGCACTCTCGCAGGCACTA	55	40	400	L5	*Eco*RI (1, 0.37), *Hha*I (1, 020)
CC0285	TTGGATAAACTCTCGCAGAC	GAGGAGCCTGGCATCA	55	40	400		*Nci*I (1, 0.24), *Scr*FI (1, 0.36)
CC0288	TTTGTGATTTCATGCCTGCTA	GAAATCCAAGCTTGCCCTAC	60	40	550		*Dra*I (1, 0.17), *Rsa*I (2, 0.12)
CC0292	AGGCGGCGTTCAAGC	TGCCAAGCAATCTGAGAGAG	55	40	700		*Sph*I (3, 0.31)
CC0293	GGCGAGCTTAGCGTCTG	AATTCATTTCCTGGGCACAT	60	40	1,600		*AluI (3, 0.17)*, *Eco*T38I *(3, 0.12)*
CC0294	GGCCTCACCCTAAGAATGTT	ATGATGTCAAACCGCACTG	55	40	1,100		*Scr*FI (3, 0.06)
CC0300	ATGGCTGCTTGCGTAA	GCTCGCGTTTATTCTATCAG	60	40	700	L14	*Scr*FI (3, 0.17)
CC0303**	AGAAGAGAAACCCTGAGAAA	CCCATCGAAGTATAAACCA	50	40	700	K1	*Hha*I (3, 0.06)
CC0317	GAAAGCCAGGTTGAGG	GCAGCAATTAGTAACCAGAT	60	40	1,700		*Dra*I (1, 0.29)
CC0318	TGAGTTGTGAGGCGTGAGA	AACAGGGAGCTTAGGCAATG	60	40	600		*Alu*I (3, 0.34)
CC0323	TGCATGTCCTGTAATCCCATA	GCCCTGTGTTTGCTGCTA	50	40	450		*Bcl*I (1, 0.32)
CC0336	ATCAGCTGCGCTCCAGT	TCTCATCCCACTCCATCTCTC	50	40	550		*Eco*RI (3, 0.36)
CC0337	ATTTACCCGTTTGCAAAGATT	CAGGATCGCATAGGGTTCTA	50	40	400		*Eco*T38I (1, 0.35), *Hae*III (1, 0.24)
CC0341	TGCTGAGAATTGCGGGAGTC	CGGGCTTTCACACCACTGTC	60	40	1,200		*Hha*I (3, 0.29)
CC0342	GGCAGCAGAGCAGAAGGTT	GACAAGCCAACAGGACTCCA	65	40	600		*Hha*I (1, 0.20)
CC0343	GACTCTTCCGTCGGCATAAT	GCTGGATCGGCATCATC	60	40	1,600	L7	*Hha*I (1, 0.24), *Nsi*I (1, 0.33), *Bgl*II (3, 0.12)
CC0344	TTATAGTAATGGCGGATTCA	TCTCCGCCATTACAAGA	55	40	500	L14	*Alu*I (3, 0.34), *Hha*I (3, 0.32), *Rsa*I (3, 0.36)
CC0346	GCAGATCGATTGTGGG	AGCAGCAATTAGTAACCAGA	60	40	1,800		*Dra*I (1, 0.27), *Eco*T38I (1, 0.31), *Bcl*I (3, 0.06)
CC0349	GCGGATGCCTGATGTTCTTGA	CAGGACGCCGGTCTGTTGAT	60	40	400		*Hinc*II (3, 0.06)
CC0356	AGAAATTTGCAGCGGAGATT	GAAAGACTGAGAAGGGCATTG	60	40	1,500		*Nsi*I (1, 0.35)
CC0374	AGGTGGTGGCGTTCAAGA	TATTGCCCACGTTTCTCTCTG	55	40	1,000	L2	*Hin*dIII (1, 0.20), *Msp*I (1, 0.29)
CC0380	GAATAGGCGAGCCACAGTCAA	CAGCTCACCGAGGCAAGAAT	65	36	1,900		*Hin*fI (1, 0.27), *Ase*I (3, 0.25), *Msp*I (3, 0.25), *Rsa*I (3, 0.12)
CC0381**	TGGCATCAAAGAAACGGTTAG	GGGTTCGAATGGCATAAGAAG	60	36	1,300	H12	*Scr*FI (3, 0.06)
CC0388	GCCAGGCCAGATAGAATGC	CTACACCTTGTGCTGCGTCTC	50	40	400		*Dra*I (5, NA)
CC0390	CATAAAGTCTAAACGCTGATA	TCTTAGAAACCGAATCTCTT	50	40	1,600		*Rsa*I (1, 0.27)
CC0392	CGCGATAAACCTAAGCTACAA	AATGGACCAGAAGACAGGAG	55	40	400	L14	*Sty*I (1, 0.20), *Mbo*II (3, 0.06)
CC0393	CAGAGAAATTGCCCTTCACTT	CAACCCTAAGAGACCCAACC	55	36	400	L1	*Sty*I (3, 0.22)
CC0404	GTGGGCTGTTCGGCTTGT	GGCCCTTCTTCTCACCCTCTT	65	32	1,100		ALP (1, 0.16), *Scr*FI (1, 0.37)
CC0405	GCGATGGTGAAAGTGGAG	CAAACAAATTCTTGCGGAGAT	60	40	450		*Msp*I (3, 0.37)
CC0406-1	GCCGCCTCTTCTTTCCTTC	CTCTAAGCCGTCTCCCTGGTA	60	36	1,100		*Sal*I (2, 0.12), *Bgl*I (3, 0.06), *Eco*O109I (3, 0.06), *Sty*I (3, 0.06)
CC0411	GATGAGCCGAGTATTGGTTAT	GATTTCATCGCCTTGGTT	50	40	400	L3	*Nsp*V (1, 0.36, *Hin*fI (3, 0.12)
CC0415	CTCTTCCTTTGCACCGAGTTG	TCATGGCAGGAAAGAGATTGG	60	36	<2,000		*Hinc*II (1, 0.29)
CC0421-1	TGCAAGGCCTGTATCATTA	ATATATTTACCCGACAAGCAA	55	40	600		*Sty*I (1, 0.37)
CC0428	CAATTCCAAAGGACGCTGCTT	TTGCGCTTGTTCTGCCACTTA	60	40	550		*Ssp*I (1, 0.35)
CC0432	AGCTCTTGGCCATCCTACTTA	TTTGGTGGCCTCATAAACA	55	40	1,600		*Bst*XI (1, 0.37), *Bcl*I (3, 0.06)
CC0445	GGCAAATAGCGGAAATCAAAT	GGCCGCAAACTCTCTGAATA	50	40	300		*Mbo*II (3, 0.22)
CC0446	GTAATCGCCGCCAACTATCAT	TGAGTGGGCGTGTTTCCTC	55	36	500		*Dra*I (1, 0.36)
CC0456	TGCAGAAAGCCAGGTT	TTATTCTATCTGCTGCTCACA	55	40	1,500		*Dra*I (1, 0.29), *Eco*T38I (1, 0.33)
CC0460	AATCTCACCACGGGCTTCTC	TACATTGAGGTAGCGGGTTCC	55	36	1,100		*Hae*II (1, 0.37)
CC0462	CTTCAAAGCGCAGAGACAAGT	TTCCCAAGCCCACACAATA	55	36	1,900	L4	*Nci*I (1, 0.36), *Eco*O109I (3, 0.29)
CC0463	CAGGCCAAGAAGCATCTC	TTTAATAGTGTGTCCGCATCC	60	36	>2,000		*Rsa*I (1, 0.37)
CC0471	CTTTGCCCTGCTGTTCCTATT	CGCATGAGCTCAGATTTGTTT	65	40	1,400		*Eco*RI (3, 0.06), *Msp*I (3, 0.12)
CC0472**	AGTGTCATCCGAGCTTCCT	TCAACCGGATGTATATTTGT	55	40	500	K18	*Hha*I (3, 0.25)
CC0481	TATATTTGGTAAGCCGTCAGA	TGGTTAAAGATGCGGTCAT	55	36	1,000		*Xho*I (3, 0.37), *Msp*I (5, NA)
CC0482	TGGAACGTCACTCTGCGAAAC	TTGTAATTAGCCGCCCATCCT	65	40	900	L7	ALP (3, 0.25), *Ssp*I (3, 0.12), *Dra*I (4, 0.50)

Appendix (continued)

Locus	Forward primer	Reverse primer	An. temp.[a]	Cycle	Size[b]	LG[c]	CAPS and ALP[d]
CC0484	CGCCATGGATTCAAACAGA	AAATTAAACCGACTGCCAGAA	55	40	650		*Sty*I (1, 0.24)
CC0489	TGGGCTTGAGCTATTCC	TGTGTAACACAATTTGAATGC	60	40	1,500	L1	ALP (3, 0.17), *Hin*fI (4, 0.49)
CC0493	TTATAACATATTGGCAAAGCA	CCCGTCGAACAGAGAAG	55	40	1,500	L5	*Bgl*II (1, 0.33), *Hha*I (1, 0.33)
CC0500	CTAAGCCAAAGCCTCCTCCAT	GGCGCTTGATCTATCCACCTC	55	40	800		ALP (3, 0.12)
CC0501	AGCAAGGGCAGGGTTTC	ATATGGCCATGGAATCAACAT	55	40	1,200	L7	*Bgl*II (1, 0.36)
CC0504	GCAGGTTTCGATACTCAGAC	CAAATTTACTTCTCGGCTCAC	60	36	>2,000		*Bst*XI (3, 0.17)
CC0507	GCAGATCGATTGGAAGGAA	GCAGTAATTACATCCGCTGAA	55	40	700		*Hae*III (1, 0.33)
CC0514**	GACCAGCGATGAGGAAGAA	GGAGAGATCGACCCGAAATAC	60	40	1,000	H3	*Ssp*I (3, 0.29)
CC0520	TGAGGGCTACGGCGTGCTT	TTCCTTGCCTCGTTTGCTTCC	60	36	2,000	L5	*Mlu*I (1, 0.21), *Alu*I (3, 0.17), *Scr*FI (5, NA)
CC0530	GGCATGCTGTAGTGAATGTC	TAGACCTCAGGTTCCCAAGTA	55	40	2,000		*Hinc*II (1, 0.37)
CC0538	ATCCGGTATTGAGCACTCT	AATCCGGAAATAACACACATA	60	40	700		*Ssp*I (2, 0.20)
CC0539	AACCCTCAGCCTGGCAGAT	CCCAACATTAAGCCCAAGTCC	60	36	1,800	L8	*Hae*III (4, 0.37)
CC0541	GCGAGCGAACAGAAAGTGA	AATCTCAAGACGGCATTCCAT	55	40	650	L10	*Eco*T38I (1, 0.37)
CC0544	CTTCGGGAGCAGTCACAGAGT	CGTCGATAGAAAGGCGGAGAA	55	36	600		*Hha*I (3, 0.25)
CC0546	AATAGCGTTTCGGGTTTCTGG	GAGATCAATGCGGCTGCTG	65	36	1,100		*Msp*I (1, 0.28), *Nci*I (1, 0.31), *Alu*I (3, 0.32)
CC0547	GTCGCATGGAAATGGAC	TGGAAACTGTTTGGCACTTAT	55	40	2,000		*Bam*HI (1, 0.33), *Msp*I (1, 0.33), *Bgl*II (3, 0.12)
CC0551	GGGCTCTTGTAGTTTGTTCAT	GGGAAATTTGGCTGGTTTA	55	40	2,000		*Hin*dIII (1, 0.38), *Bcl*I (3, 0.06)
CC0560	GCTAAGGAAGAAGGCAAGGA	GCCCTGCTGCTACAGAGAATA	55	40	1,800		*Hin*dIII (3, 0.12)
CC0576**	AAGCCCAATTTCTTCATTTC	TTACCACCCAAGTATGTAGAC	55	40	1,500		*Scr*FI (3, 0.34)
CC0588	AAACTCACGCATACAAACACA	GCTCATTCGAGAAAGGAAC	55	40	>2,000		*Nsi*I (1, 0.36)
CC0590	AGTTTCGAAACCCTAGCATCA	AACCATAAACTCGCTCCTTCA	55	40	500	L7	*Xho*I (1, 0.36), *Hha*I (3, 0.06)
CC0608	TTTAATCCATCCCGTTCAAAC	GGAAACAATTCAGGGCTCAT	55	40	800		*Hae*III (3, 0.06)
CC0613**	GGAACCAACACCGCTGCTCTG	GAGGGTGCCAATGTCGGAATC	55	40	700	H19	*Hin*fI (1, 0.36)
CC0616**	CTTTGGTTGTAGTGGGCATTC	GAAGACGCGCATTTGAGAA	55	40	1,100	K2	*Bcl*I (3, 0.12), *Hha*I (5, NA)
CC0622	GGCTTGTTTGGGAAGAAG	AGATAAACACACCACGCATAC	55	40	1,300		*Scr*FI (1, 0.37), ALP (3, 0.06)
CC0625	CCCAAGCCTCCTAACAATAGA	GCCACATCCCTGTAGACCA	55	40	1,400		*Hha*I (1, 0.37)
CC0673**	CCGGCAGAACCTTCCAAA	AAGACCATGGCCTCCTATCGT	55	40	1,500	H7	*Alu*I (3, 0.22), *Rsa*I (5, NA)
CC0676	AAAGTGCCGATTGTTGTGGAT	TTGGCTGCAGTTTCTTCCTCT	55	40	1,600		*Scr*FI (3, 0.06)
CC0680	ATCACAGCGATCTTCTTACAG	AAACATCATGCCAGACAAA	55	40	>2,000	L11	*Hin*fI (1, 0.33), *Dra*I (3, 0.17)
CC0682	CGCAGCTGCTCTCAATCC	TGAAACAAACACCGCTCATTC	55	40	1,100		*Hha*I (3, 0.29), *Rsa*I (5, NA)
CC0706	GGCGGTCTATCACCTTGTA	ACTAGAGAGTCCAACGCATAA	55	40	>2,000		*Sph*I (1, 0.33)
CC0707	AGCCCTGGTTTCAGTCTAA	ACCAGTTAAATTATGCCATCA	55	40	>2,000	L5	*Alu*I (1, 0.36), *Hin*fI (3, 0.36), *Dra*I (4, 0.41)
CC0708	TCCTGGGGAGAAGATCATCA	AATGTCTGGTGTCACTGGAGC	55	40	500		*Rsa*I (3, 0.31)
CC0718**	GCGGAAAGATGGGAGCACTTG	GGCCATCGCACTACGCAGAG	55	40	1,300	L10, K18	*Alu*I (1, 0.24), *Hin*fI (3, 0.31)
CC0719	GGTGGAAGAAGAGCCGCTTGT	AAACTGGCGCCCATTGAGTC	55	40	800	L7	*Alu*I (1, 0.24), *Hin*fI (1, 0.24), *Hae*III (3, 0.12)
CC0731	TGCGCGCTTTCGGAGTTT	GTCCACGCAGACGACCAGTTC	55	40	700	L7	*Hin*fI (1, 0.20), *Hha*I (3, 0.36)
CC0734	TCTGCGCCCTGAACCAT	ACAGTCCACCAATACCCAATA	55	40	700		*Hinc*II (1, 0.33)
CC0737**	TCTCTAATGTGGCAGCTCTTT	TGGAACTTATGCTACATCCCT	55	40	600	K16	*Eco*T38I (1, 0.31)
CC0738	GGGCTCTCCTTCTCTCA	TAAACCCAATTACAACTTGCT	55	40	1,400		*Sau*96I (1, 0.37)
CC0741	GGGAACGGCTGTTATGC	CGGCTACCATTTCAAGTGATA	60	40	1,000		*Hae*III (3, 0.25), *Hin*dIII (3, 0.25)
CC0747	GTACCCAAGATGCAGATTTTT	GGCCTACGAACATTGAACATA	55	36	1,500	L8	*Sal*I (1, 0.16), *Rsa*I (3, 0.06)
CC0757	GGTTAGGAAAGCCAGAAGAAG	CACGTGGGATCACTAAACAC	55	40	750		*Bgl*I (1, 0.20), *Alu*I (3, 0.12)
CC0776	AATTGCGATACTTACGAGTTT	AACCTCCTCTGCCACAT	55	36	1,500		*Hha*I (3, 022)
CC0787	CGCCACTTCAAACCCTTCAAA	CAGATTGCAGAGCCGACGAA	55	40	1,300		*Hin*fI (1, 0.37), *Nsi*I (4, 0.55), *Ssp*I (4, 0.49)
CC0796	AGCGGTTTGTCTCATAGTTCC	CGCTTGATCCCACCTTCTC	55	40	1,400	L6	*Eco*RV (1, 0.35), *Alu*I (3, 0.32), *Hinc*II (3, 0.12), *Msp*I (4, 0.54)
CC0797	CGGTGATCTGCTAAGCGAGAC	TTACAACGGGATGAGGCAATA	55	40	1,500	L2	*Bst*XI (3, 0.34), *Hha*I (3, 0.29)

Appendix (continued)

Locus	Forward primer	Reverse primer	An. temp.[a]	Cycle	Size[b]	LG[c]	CAPS and ALP[d]
CC0798	CTCAGCGCAGCTACCCATAAC	CACTCGCGTGTTGGTCTTGT	55	40	1,200		*Spe*I (3, 0.22)
CC0801	CGGATGAATAGCCTTGAAAC	CACCTCTATTGGGACACTTGT	55	40	1,500		*Hin*fI (3, 0.37)
CC0802	GGCAACTTTCCTCTTCCTCTA	TACCTAAAGGGCAGCCATTA	55	40	1,400	L9	*Bc*II (3, 0.17), *Bst*XI (3, 0.12)
CC0803	TGATCAGAGCGCAGAGGAAGG	TGTCCACGCAGACGACCAGT	60	40	700	L7	*Eco*T381I (1, 0.37), *Hae*III (1, 0.37), *Hha*I (3, 0.36)
CC0820	GTTCTGGACTTGGGTGGAA	TGCTCAGATACATCGCCATTA	55	40	800		*Hha*I (3, 0.17)
CC0832	AAAGATCAAGCGTATCAATGC	CAGAAGATCCTCCTGCGTAAT	55	40	1,300		*Ssp*I (1, 0.32)
CC0846	CCCTCACCGGTAAGACCATCA	GAGTTCGCCCATCCTCAAGC	64	40	1,800	L3	*Scr*FI (5, NA)
CC0850	CTTCACCTGGTTCTGCGTCTA	GTAATGGTCTTGCCCGTCAG	60	40	500		*Hin*fI (3, 0.31)
CC0860	AACAGCGCGCAAGTCTACA	CTAAGCCCTTTCACCACGAAT	55	40	1,300		*Hin*dIII (1, 0.29), *Hin*fI (1, 0.20)
CC0864	GTGAGTTCGGTGGCTCTG	CCCAAACAGCATAATCCTTTC	55	40	700	L3	*Alu*I (3, 0.25), *Mlu*I (3, 0.25)
CC0906	AATTTAAAGCGTGCTCTGGTG	AGGTTGTCAACAGCCAAGATT	60	40	>2,000	L1	*Eco*RV (1, 0.37), *Hinc*II (3, 0.12)
CC0924	CGAGCCCGAATGGAGAATC	ACGAACACCGTCCAGATGAAC	55	40	1,500	L8	*Bst*XI (1, 0.13), *Sty*I (1, 0.32), *Bg*II (3, 0.07)
CC0941	CAGAAGCGCAGGAGAAGT	TAGATCTAGCACAGCCCAGTA	60	40	2,000		*Xho*I (1, 0.27), *Rsa*I (3, 0.12)
CC0942	CCTTCAGAATGGCAACTACTG	GCTCTCTAAGGTGGCATACAT	55	40	1,100		*Ase*I (1, 0.27)
CC0958**	CTCAATGGCCGCTATTTCACT	GAAGCCAAACGAGAAGCAATG	55	40	500	K6	*Eco*T38I (3, 0.25)
CC0959	TTTGCCAAGAAGGGTTTGA	TCCACTTGTAGAATGCCACAC	55	40	800		*Rsa*I (1, 0.20)
CC0970	GACCTGCCAGGCTGAGATGAA	AGGCTCGCAGGAAACAAACAA	55	40	1,000		*Alu*I (5, NA), *Hae*III (5, NA), *Hin*FI (5, NA), *Ssp*I (5, NA)
CC0973	CCGGTGTGGTTTACAGG	AGTACACAGCTATGGCAGACT	55	40	600		*Hin*dIII (3, 0.25), *Hin*fI (3, 0.12)
CC0975	GCTGGCCTGCTGAGTAAAC	GAATAATCGCCATCAATGAAC	55	40	650		*Msp*I (3, 0.06)
CC0985	GACGCATTCGGTTCTTTAGG	AAATGCATCCGCTCACATAAT	55	40	600		*Bst*XI (3, 0.34)
CC0990**	CGTCAGAGTTTGGCATTG	AAGTAACCGGTTCACATCCAT	60	40	1,800	K22	*Spe*I (3, 0.29)
CC0991	ATGCCAGGCTGATTTCTACAA	GACACAAGCCAAACATTCACA	55	40	1,600		*Rsa*I (2, 0.12), *Sau*96I (3, 0.06)
CC1012	ACATGCGAATGACAATGGATT	CGCGACTTTGAGTGTTGGAA	55	40	1,400		*Sau*96I (3, 0.06)
CC1014	GGACATGTAGGCGAGATTC	AAGTACACTACCAGCCCAAAG	60	40	2,000	L14	*Eco*T38I (1, 0.35), *Hha*I (1, 0.24), *Hae*III (4, 0.42)
CC1015	GGAGAAATTGGAGGGCATAG	AAGTCTTAAGCAACCGAATGG	60	40	1,200	L7	*Hha*I (3, 0.12), *Hin*fI (3, 0.17), *Spe*I (3, 0.37), *Rsa*I (5, NA)
CC1032	TGGCGACAGAGAAGAAATCAA	AAATAACCATGCCCTCCATCA	55	40	750		*Bg*II (3, 0.06)
CC1112**	GGGACACTAAAGAGAAGAAAC	ATTCAGAGTCCAGCTATCAA	55	40	800	L8, K1	*Bg*III (1, 0.24)
CC1113	TCCCAAGGATTTGAGGTTCAC	CTGAGGGTGCGGATTGC	55	40	450		*Hae*III (3, 0.12)
CC1125**	GTTTCAGATTTCACCCGAAGC	AGCACAACATCCCAAGCAAT	55	40	800		*Dra*I (1, 0.33)
CC1127	AGGAATACTGTGCGAAGAAGA	ATTGACCTTGCCATCCC	60	40	1,100		*Eco*T38I (1, 0.38), *Hha*I (3, 0.25)
CC1148	CTCCTCCTTGTTGGGTTTG	AATACATTGGATTGGGCATCT	55	40	850		*Msp*I (3, 0.12)
CC1158	AAATGCTATGCTTTGGCTCTT	CGACGGTGGTGACAGGA	55	40	1,300		*Msp*I (3, 0.37)
CC1172	GCGCGCTTTCGTAGTTTGGAC	GGAGCATCACGACGCACTGTA	55	40	600	L7	*Hha*I (3, 0.36)
CC1176	TGGCCTGTAGTCTGAAGC	TAGTTAAGATCGTGGCAAAGA	55	40	700		*Hin*fI (3, 0.25)
CC1187	CAGATCCTGCACCATGTGGAA	CAAACTGCCATGGCCAGACT	55	40	450		*Alu*I (3, 0.17)
CC1189	GGATCTGAACGGCACTG	GATTATGAAGATTGGATGGT	55	40	750		*Hae*III (3, 0.31), *Rsa*I (3, 0.31), *Scr*FI (3, 0.36)
CC1190	CTCCTCTCTGGCTTTCC	TCTTATTCTATCTGCTGCTCA	55	45	>2,000		*Eco*T38I (1, 0.31)
CC1204	CGAGCGCTCTGGAACATT	TCTGATTGATTGCCTTACGAG	55	40	1,300	L7	*Rsa*I (3, 0.12), *Spe*I (3, 0.37)
CC1244	ACATATATGGCCTGCTACATT	GCGCGAATCAACACTT	55	40	1,800	L12	*Hin*fI (1, 0.27), *Sty*I (1, 0.29)
CC1262	GAGAAACGGGCATAGTGAG	ACATATTCTTTCCCAACTCCA	55	40	1,300		*Hae*III (3, 0.32)
CC1263	TTTGATTAACGCAGACTTCTC	GCAGAGTATGGTTCGCTTAT	55	40	1,400		*Sty*I (4, 0.41)
CC1266	CTGCATAAATCGGCTCATT	ATTTAAATACCCTGCCCTGTT	55	40	600		*Dra*I (3, 0.12)
CC1271	GCAGCTACGTTTGATGTCC	TTTACTGCAATTACGGCTTAG	55	40	1,900		*Ssp*I (3, 0.06)
CC1274	GTCAACTACTACTGGGCATCT	GCGCATCTCCAAACACTA	55	40	1,700	L4	*Hae*III (3, 0.12)

Appendix (continued)

Locus	Forward primer	Reverse primer	An. temp.[a]	Cycle	Size[b]	LG[c]	CAPS and ALP[d]
CC1287	TCAACTACTACTGGGCATCTG	ACCACTTGGCTCCTTCTT	55	40	1,500	L4	*Hae*III (3, 0.12)
CC1298	CATGGCGGACGCAGATTTG	CCAGGCGGCTTGTAATGCTTG	60	40	1,600		*Rsa*I (1, 0.36)
CC1319	ATAGTAATGGCGGATTCAAAT	CGCCATTACAAGAACATAAGC	55	40	400	L14	*Alu*I (3, 0.37), *Rsa*I (3, 0.36)
CC1367	GAGTGTTGTACATCTGCGTAG	AAATCGCTTAAACCCTCTAA	55	40	500	L4	*Hae*III (1, 0.37)
CC1389	CACCTGCTCGGCGAATC	TGTGAACAGCTGGCAAGAGTG	55	40	900		*Hae*III (1, 0.36)
CC1390	AGGACTTCTGAGATGCGGTAA	GCAGCGACTGGGTATTTGT	60	40	700		*Hin*fI (3, 0.37)
CC1395	ACACTCACTCACGGGTTCCTC	GCTGAGCTTCACCGCAGAAT	60	40	2,000	L7	*Bgl*II (3, 0.25), *Hha*I (3, 0.37), *Hin*fI (5, NA)
CC1417	AGTTTGCGATGGCTTTCC	CAGAAGATCCTCCTGCGTAAT	60	40	1,300		*Hin*cII (3, 0.17)
CC1429	GCCTCTCTTGTCTGTGTCC	GGTGATGATTCCCAATAATGT	60	40	1,100		*Hin*dIII (3, 0.17)
CC1432	TGGTGATGTACACGCCTGGTT	CGTTGAAATTCTCGGGAGCAT	60	40	1,500		*Msp*I (3, 0.25), *Nsi*I (3, 0.06)
CC1439	GCATGGGAGTGGCGAATG	AGCTGAGCTTCACCGCAGAAT	60	40	1,300		*Bgl*II (3, 0.25)
CC1440	GCGATGGCGTTTACAGAC	CGCCTAATCATCAGAGAGACC	60	40	1,100		*Nsi*I (3, 0.12)
CC1444	CAATTGGGATTTATCAGAAGC	TACCATCCCGATTATCAACA	60	40	700		*Rsa*I (1, 0.27), *Alu*I (3, 0.06)
CC1454	GCCAAGCCGACAAAGGAG	CTAACCAATTCCCAGCGAACA	60	40	1,200		*Msp*I (1, 0.24), *Scr*FI (1, 0.27), *Sty*I (3, 0.06)
CC1456	CTAGCGCTGGTGGTTTGC	AACTCTTCTTGCCCTTGCTGT	60	40	1,800		*Msp*I (3, 0.25)
CC1462	ACATGGCAAACGCTTAC	AAATCTCACTGACCCAATCTA	60	40	>2,000		*Hha*I (5, NA)
CC1497	CAGCGGAAAGATGGGAGTA	TCATTCAGTGATTCGTCGTTG	60	40	1,200	L10	*Hae*III (1, 0.24), *Hin*fI (3, 0.31), *Eco*T38I (5, NA)
CC1527	AGATCCCTACTATCGCCAAAC	GGCTGCAGGAAATGAAGTT	60	40	650		*Alu*I (3, 0.12)
CC1531	AAACGCAGTCGAGAGGATGAG	CACCGGCAGATTCCAGTCTAC	60	40	650		*Hin*fI (3, 0.06)
CC1545	TTGTGATCGGCACCATT	TTCGAAGCCAATAAATACAGC	60	40	700	L6	*Hha*I (3, 0.17)
CC1551	ATTCCCAGCTTCTTCGGTAGG	ACGTTTGGCTTCGGTTCC	60	40	650		ALP (3, 0.17)
CC1606	TAACCAGCTTTGCCCTCAG	ATACAATTCGCGGCTACCATA	60	40	900		*Sty*I (3, 0.06)
CC1613	AAACGCCGAAATCAATAGAGG	AAGCACTACAACGCAACGGTA	60	40	1,500		*Sty*I (2, 0.12), *Bg*II (3, 0.22)
CC1615	GGCACCCAAGGCAGAG	CAATAATGGCACCAAACCCTA	60	40	500		*Alu*I (3, 0.25)
CC1620	GATATGGCAGAAGGAAGGAAA	CAAGACGTCGCCTGAGTAAC	62	40	2,000		*Eco*T38I (1, 0.35), *Hin*fI (1, 0.36)
CC1625	CTCCAGACAGCGGTTCTTATG	TGCCATGGACTTCCTCTTCT	60	40	1,100		*Hae*III (3, 0.29)
CC1627	GGAGCGCCAAGCAGTATTCAG	CCGCCAAGATCTGTCCTTTCA	60	40	600		*Alu*I (1, 0.29)
CC1629	TGCTGGCCTTACAGGTA	AACTTCGGAATAACTGAATGA	60	40	>2,000		*Hin*cII (1, 0.35)
CC1641	TGGTCCGTGCGGTGAA	TCCCAGCCGATCATTTGACTA	60	40	1,800	L1	*Scr*FI (1, 0.36)
CC1642	AAACGAGGTGTATGGCACAGT	GCCTGATAGTACCCAAATGGA	60	40	1,000		*Rsa*I (1, 0.35)
CC1652	AAAGTGAGAGACCCAAGTTGA	TCAGGTGAAGGGCAGTAAA	60	40	1,500		*Msp*I (3, 0.22)
CC1655	TATTTCACAGCAATGGCAGAT	AGCGGTATAAACGAATCAGGA	60	40	900		*Hin*fI (1, 0.33), *Hae*III (3, 0.36)
CC1689	CAGGAAGCAACTCGCCACTA	ATTCCCGGTACCCACCATTTA	60	40	1,100	L8	*Bgl*II (3, 0.22), *Hae*III (3, 0.22), *Hin*fl (3, 0.25), *Sty*I (3, 0.06)
CC1695	GCAGTGACTCTGGCGAGGATT	CCGGACGGAAGGCACCTA	60	40	700		*Hin*cII (1, 0.31), *Msp*I (3, 0.17)
CC1698	ATATTGCGACAGGATTTGG	ATTTCCGCAATAAAGATTTGA	60	40	700	L3	*Rsa*I (3, 0.17)
CC1704	CGCCATTCATCAGTGACAA	GCACCGAATGAAATACTTCCA	60	40	1,500	L3	*Bam*HI (3, 0.36)
CC1717	GCCATGGCACACAATCA	TGTAATCATGAAACCGCAGAA	60	40	>2,000		*Ase*I (3, 0.17), *Sty*I (3, 0.12)
CC1721	GCCTGTCAGTTGGAAAGA	GGTACACAATTTGCCATAGAG	60	40	>2,000		*Rsa*I (1, 0.35)
CC1727	CGCGGGACTGGTTGGAGTT	GCAGAGACCCGCAGAAGGAAA	60	40	1,300	L11	ALP (1, 0.36), *Hha*I (1, 0.36), *Alu*I 3, 0.37), *Dra*I (3, 0.12), *Hin*dIII (4, 0.31), *Rsa*I (4, 0.59), *Scr*FI (4, 0.57)
CC1730	ATGTTTATGGGATGGGCTGTA	GAATTCGATTCCGTCTCCTC	60	40	1,100	L3	*Hin*fI (3, 0.36), *Sph*I (3, 0.32)
CC1731	CTCTGCGAGAGGCTATGTGT	GGAAAGAGCTGCCAATCTAAA	60	40	2,000		*Hae*III (3, 0.22)
CC1737	CTCGCCGGATTCCTCAAAGC	GGGCGGTCTGCAATGAAGAAG	60	40	1,100		*Bam*HI (3, 0.31), *Hin*fI (3, 0.30)
CC1748	GAAGGTAGATTATTGGGAACA	CATGCGCATTAGTGAGATA	60	40	1,800	L3	*Hha*I (1, 0.37)
CC1752	CGAGGACCAACGAGAAGC	AAGGACTGATAGCAGCCAATG	55	40	600		*Scr*FI (3, 0.12)

Appendix (continued)

Locus	Forward primer	Reverse primer	An. temp.[a]	Cycle	Size[b]	LG[c]	CAPS and ALP[d]
CC1754	TGTACCCAGGGCATTTGTAGC	TGGCCCGGTTGACTTCATA	60	40	1,700		*Scr*FI (1, 0.27), *Hae*III (3, 0.06)
CC1756	GAGGTGTTCCGTTGTAGTTCT	GGACGGGCTTCTCTGTTT	60	40	1,300		*Rsa*I (3, 0.22)
CC1762	ACTCTGGATCGGAAATCTTTG	ATGACCTACGCATACGCTGTA	60	40	900		*Scr*FI (1, 0.29), *Msp*I (4, 0.38)
CC1784	TGAAGCAAGGAATCGCCAGTT	AGCTGAGCTTCACCGCAGAAT	60	40	1,500		*Bgl*II (3, 0.25), *Hin*fI (5, NA)
CC1785	CGCGCTTAACAGACATTATGC	CCATTTACAGCGGCTACCATT	60	40	1,100		*Msp*I (3, 0.06)
CC1790	GCAACCATACCCATCACTGT	TATAGGCAGGTTCTGGCATAA	60	40	2,000		*Hae*III (1, 0.24), *Scr*FI (1, 0.37)
CC1798	GGCGGCGGAGATTACTGTA	TAGAAGACGCGCATTTGAGAA	60	40	800		*Xho*I (3, 0.25)
CC1806	GAGCCGAAGGAGCCAATAC	AGAAGCCGATCAAACACTCA	60	40	900	L2	*Msp*I (4, 0.46)
CC1811	TGCTGGCAATAATGGCGGTAT	ACAGCACACGGGCACACATA	60	40	1,300	L1	ALP (1, 0.36), *Scr*FI (3, 0.36)
CC1827	CCCAGCTGCCATTGTTC	GATTTCATCCCAAGACGAGAA	55	40	1,200		*Nsi*I (3, 0.22)
CC1835	GGAAGCTGCACCCAAGAGAGT	GATAGGGCCTTCAACACACCA	62	40	1,800		*Bgl*II (4, 0.39)
CC1846	TTGGGCCTCTTTCTACGC	CGTCGTTGGATCATTAGCATA	60	40	800	L2	*Hae*III (5, NA)
CC1856	AGAGACACCGTTCGGGAGACA	CCGCATGCATACACCACCAAT	60	40	1,100		*Dra*I (1, 0.36), *Scr*FI (1, 0.35), *Rsa*I (3, 0.07)
CC1864	CACTTTCAAGGATGCCACAT	ATACTGACCAAGAACCCAACC	60	40	650		*Hae*III (3, 0.12), *Nci*I (3, 0.12), *Alu*I (5, NA), *Dra*I (5, NA)
CD3	GCTGCCTGTTACTTCTGCTA	CTAAGCAAGGAACGAAGAAA	62	40	1,400		*Sau*96I (1, 0.37), *Bgl*II (3, 0.25), *Ssp*I (3, 0.17), *Dra*I (4, 0.56), *Hin*fI (5, NA)
CD4*	AGGAAGAAAATGCTCCAGAC	CATCTTGCACCCCTTATTTA	58†	40	1,300	H19	*Bam*HI (3, 0.12), *Hae*III (3, 0.29)
CD15	CAGAGCAACCGCCACAAGAG	TGAGGCGGCGATAACTTGTA	60	36	500		ALP (3, 0.34)
CD19	TCAAACAGCGTAGTCAACCA	TGGAAGGAAAGGAGGAAGAT	58	36	1,400		*Eco*O109I (3, 0.12)
CD470*	GGCCTACGAACATTGAACAT	ACCCTTACCGGAAAGACCAT	58	36	1,500	L6	*Bgl*II (1, 0.20), *Sal*I (3, 0.06)
CD482	GGGGTTTCGTCCAAGGTTAT	TCCACAGGGTTTTTGCTTCA	62†	36	600	L6	*Eco*T38I (1, 0.29)
CD568	CAAGAGCGAGAAGAGCAGAA	ATTGGAATCAGGAGGAGAAG	60†	36	700		*Bst*XI (3, 0.17)
CD657**	TCCTGATACTGTGGGCAACT	CCCCGATATGCTCTTCAACT	60	36	800		*Hae*III (1, 0.37), *Hha*I (1, 0.35), *Msp*I (3, 0.29)
CD674	TTCCGCTATTTTGTCAGGAG	AGCAGGGCTTACAGCAGTTG	52	36	650		*Sty*I (1, 0.24), *Nsi*I (3, 0.06)
CD810	CCGCTTTAGGAGTTCATTTC	TCACAAAAGTCCAATGACCA	60	36	1,200		*Rsa*I (3, 0.22)
CD1071	GATCGTACCCAGCCCTTTTT	CAGAGCTTCTGGGGTCTTTA	58†	36	600		*Bst*XI (3, 0.37), *Hae*III (3, 0.06)
CD1111-1	GGGCCAAACTAATATCTAAAC	TGCCGAGCTGTCTGTG	55	35	500		*Bst*XI (3, 0.29)
CD1179	TGGGTTTGGGCATAAGTCTG	TTGCCCCTGTTGTTTTATCC	58†	36	850	L7	*Alu*I (1, 0.31), *Eco*RI (1, 0.24), *Bst*XI (3, 0.06), *Hin*fI (3, 0.22)
CD1291	ATGCTAATGTAATCAAATCCTG	ATCACAAGGACCCCAACTAC	55	35	400		ALP (1, 0.36)
CD1310-1	CGCCTTGACATTTCTTTTCC	ATGTAATGCATCCTGACTGTC	56	36	1,800		*Hin*fI (1, 0.29)
CD1410-2	GTGTAATAACAAGGCTGCTC	GCAACATGGAAAAGACTGAG	56	35	200		*Rsa*I (3, 0.29)
CD1569	AATATCGCCTCCCTTGAC	TAGGCAGATACATAGTTACC	55	35	400		*Alu*I (3, 0.12)
CD1617	TGCCAACTCCACCTACTTCA	TATGGCCCTCAAAACAGTTC	58†	36	1,800		*Rsa*I (3, 0.25)
CD1712	CCAGGTCAGCAGCAGACTAA	CATTGCTGGGCTGGTGAATA	62	36	800	L1	*Eco*O109I (3, 0.37)
CD1761*	AGTCAACTCAATGCCCTCAA	TAAACAGGTCGCTCCCAGAT	58†	36	800	H3	*Ase*I (1, 0.24)
CD1802-2	CATCCAAAAACAACCTAACC	CAGTACCCATTTGAAAGTGC	58	35	1,050		*Mbo*II (2, 0.20), *Hae*III (5, NA)
CD1875	GAGTCGCAAGGAGAACAGC	GAATGCCATGTGAGTATCC	56	35	400		*Rsa*I (3, 0.25), *Sph*I (3, 0.29)
CD1943	GGGGACAGATCCAACTAACA	GCCATTTTATTTGCCACAGA	60	36	900		*Dra*I (3, 0.34)
CD1950	CCAACTGGTACAGATTCTC	CTACAATCCTGCTCTCAATG	56	45	500		*Alu*I (3, 0.17)

Appendix (continued)

Locus	Forward primer	Reverse primer	An. temp.[a]	Cycle	Size[b]	LG[c]	CAPS and ALP[d]
CD1951-1	CTCTGTCAAAGGTGTTAGTG	GCATCACACTTTCTCTTAGG	56	35	1,300		*Mbo*II (1, 0.35), *Bcl*I (3, 0.37), *Scr*FI (3, 0.36)
CD1960-2	CTGGCTAAACTTGGTCCTAC	ACCATATTCATCCCCTCCTG	56	35	1,400		*Msp*I (1, 0.27)
CD2039-1	ACTCTGCTGATAGGCGACTG	TCCCATGTTTCTGACCTTAG	55	35	>2,000		*Hha*I (3, 0.37), *Rsa*I (5, NA)

* Tsumura et al. (1997), ** Nikaido et al. (2000)

[r] Concentration of $MgCl_2$ in the PCR reaction mixture was 2.0 mM

[a] Annealing temperature (°C)

[b] STS fragment size (bp)

[c] Linkage group 'L' indicates markers located on the map developed in this study. 'K' and 'H' indicate markers on the maps of 'Kumotooshi' and 'Haara4' in Nikaido et al. (2000), respectively. The numbers following the letters indicate linkage group numbers (see also Fig. 3)

[d] Detected polymorphisms. Restriction site polymorphisms (CAPS) are indicated with the name of the restriction enzymes that detected the polymorphisms. Amplicon length polymorphisms (ALPs) are shown as 'ALP'. The first and second numbers in the parentheses indicate the type of polymorphism (see text) and polymorphic information content (PIC), respectively. 'NA' indicates that PIC was not available because the allelic relationship between polymorphic bands has not been identified

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