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Original article

Development and characteristics of microsatellite markers for sugi (*Cryptomeria japonica* D. Don) derived from microsatellite-enriched libraries

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Abstract – We have developed a series of microsatellite markers for *C. japonica*. First, DNA fragments including microsatellite sequences were isolated from two GA-enriched genomic libraries using magnetic beads. After eliminating redundant clones and clones in which the tandem repeats were located too close to the cloning site to allow primers to be constructed, the remaining sequences could be examined for their suitability for primer design. Primer sets were designed from each conserved sequence flanking the microsatellites. We found 1 479 unique sequences in the enriched genomic libraries, of which 962 contained a tandem repeat motif, and we have been able to design 196 primer pairs using these sequences to date. The potential of these primers to amplify single fragment, and the polymorphism of the sequences they amplify, were investigated using a panel of 28 plus trees selected from *Cryptomeria* plantations covering the wide distributional range of the species in Japan. Forty-two of the microsatellite markers displayed a polymorphic nature throughout this panel of 28 DNA samples. The polymorphic information coefficients (PICs) ranged from 0.156 to 0.919. There was a significant correlation, between the number of repeats and the size of the PICs, according to Kendall's τ rank correlation coefficient analyses.

taxodiaceae / conifer / simple sequence repeat / enrichment / primer

Résumé – Développement et caractéristiques de marqueurs microsatellites pour le sugi (*Cryptomeria japonica* D. Don) trouvés dans des banques microsatellites enrichies. Nous avons développé une série de marqueurs microsatellites pour *Cryptomeria japonica*. Dans un premier temps, des fragments d'ADN comportant des séquences microsatellites ont été isolées à partir de 2 banques de séquences génomiques enrichies en GA, grâce à l'utilisation de billes magnétiques. Puis, après avoir éliminé les clones redondants et les clones pour lesquels les séquences en tandem étaient trop proches du site de clonage pour permettre aux amorces d'être construites, les séquences restantes ont été examinées afin de determiner si elles convenaient pour la construction d'amorces. Des jeux d'amorces ont été conçus à partir de chaque séquence conservée flanquant les microsattelites. Nous avons trouvé 1479 séquences uniques dans les banques génomiques enrichies, parmi lesquelles 962 contenaient 1 motif répété en tandem, et à ce jour, nous avons pu concevoir 196 paires d'amorces en utilisant ces séquences. Les capacités de ces amorces à amplifier un fragment unique, ainsi que le polymorphisme des séquences que nous avons amplifiées, ont été étudiées à partir d'un échantillon de 28 arbres « plus » sélectionnés à partir de plantations de *Cryptomeria* couvrant la totalité de l'aire de répartition au Japon. Quarante-deux de ces marqueurs microsatellites se sont révélés polymorphes au sein de cet échantillon. Les coefficients d'information sur le polymorphisme (PIC : Polymorphism Information Coefficient) varient de 0.156 à 0.919. Les analyses de coefficients de corrélation de rangs de Kendall ont mis en évidence une corrélation significative entre le nombre de répétitions et la valeur des PIC.

taxodiacée / conifère / répétition de séquence simple / enrichissement / amorce

1. INTRODUCTION

Microsatellites, also known as single sequence repeats (SSRs), occur as tandem arrays of mono-, di-, tri-, tetra- or penta-nucleotide repeat units in many plant and animal species [30]. The variability of the number of repeat units at a particular locus and the conservation of the sequences flanking the tandem repeat make microsatellites valuable, codominant genetic markers [30]. When microsatellite markers for a particular species are developed, their capacity to be amplified by PCR allows large-scale genotyping on automated DNA analyzers for the construction of genetic linkage maps, and facilitates studies of population genetics and reproduction ecology. For these reasons, microsatellite markers have been developed for

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use in analyses of a number of coniferous species (see, for instance, [1, 3, 9, 10, 18, 20, 34, 36]).

Sugi (*Cryptomeria japonica*) is the most important forest tree species in Japanese forestry. Plantations of the species are widely distributed in Japan, from the southern part of Hokkaido to Yaku-shima island off the coast of Kyushu. *C. japonica* has been subjected to intensive genetic investigations, including the construction of genetic linkage maps [14, 19, 22, 24, 28], analysis of its genetic population structure and reproductive systems [21, 27, 29, 31, 33], and development of genetic markers [14, 20, 32]. In a recent study, Moriguchi et al. [20] developed 34 microsatellite markers from a microsatellite-enriched library and cDNA libraries for use in paternity analyses within seed orchards of *C. japonica*. However, the number of microsatellite markers was insufficient to construct genetic linkage maps, or for population genetic studies covering desired proportions of the *C. japonica* genome. Use of multiple pedigrees is an efficient approach for constructing genetic linkage maps for species with allogamous characteristics, such as coniferous species, and microsatellite markers can provide valuable bridges when integrating independent genetic linkage maps derived from different pedigrees. Therefore, we have continued to develop additional microsatellite markers.

2. MATERIALS AND METHODS

2.1. Construction of microsatellite enrichment libraries

We successfully constructed or acquired two microsatellite-enriched libraries. One, designated CS, was constructed by Genetic Identification Service Inc. (Chatsworth, USA), and we constructed the other, named CJS, as follows. Five micrograms of genomic DNA was extracted from needles of a *C. japonica* tree growing in a nursery of the Forestry and Forest Products Research Institute by the modified CTAB method [23]. It was then purified by equilibrium centrifugation in CsCl-ethidium bromide gradients [26] to construct an enriched microsatellite library according to modified methods published by Armour et al. [2], Fleischer and Loew [12] and Fischer and Bachmann [11]. The genomic DNA was digested with the restriction enzyme *Nde*II, and fragments ranging from 300 to 1,000bp in size were ligated into *Sau*3AI linkers (TaKaRa, Kyoto, Japan). DNA fragments with linkers were resolved in binding buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) and hybridized to 5' biotin-labeled oligonucleotide probes (5'biotin$(CT)_{15}$3') after denaturation. The DNA molecules bound to the biotin-labeled probes were subsequently isolated by binding them to streptavidin-coupled (M-280) Dynabeads® (Dynal Biotech, Oslo, Norway). After rinsing the beads in two kinds of washing buffer (2× SSC, 0.1% SDS and 1× SSC, 0.1% SDS), target DNAs were recovered by denaturing them in boiled water. The resulting fragments were then amplified by PCR and digested with *Nde*II to remove the linkers. The enriched fragments selected in this way were ligated into pUC118/*Bam*HI (TaKaRa, Kyoto, Japan) and cloned into competent cells (*Escherichia coli* DH5). Plasmids from these clones were prepared using the Wizard® SV96 system (Promega, Madison, USA) and sequenced using a 3100 DNA sequencer with a BigDye Terminator kit (PE Applied Biosystems, Foster, USA).

2.2. Primer design, PCR and electrophoresis

Primer pairs were designed using OLIGO 5.0 software (Molecular Biology Insights, Inc., Cascade, USA). Subsequent PCR amplification was performed in 20 µL reaction volumes containing 0.2 µM of each primer, 0.2 mM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 U of *Taq* DNA polymerase and 0.5–3 ng of template DNA using a PTC200 DNA Engine Thermal Cycler with gradient temperature control (MJ Research, Inc., Waltham, USA). The thermal program was as follows: 4 min at 94 °C, then 30–35 cycles of 45 s at 94 °C, a 45 s gradient from 45 to 65 °C and 45 s at 72 °C, finishing with 5 min at 72 °C. The fragments resulting from the PCR amplifications were electrophoretically separated in 7.5% polyacrylamide gels and stained by ethidium bromide. They were then examined to identify primer pairs yielding clear single bands and to optimize the annealing temperature and number of PCR cycles for each pair of primers selected.

2.3. Plant materials, polymorphism and inheritance of microsatellite markers in *C. japonica*

Microsatellite sequences detected from the two enriched libraries were classified into three categories (perfect, imperfect and compound repeats), as defined by Weber [35]. The potential value of these primers for use as microsatellite markers and for evaluating polymorphism was investigated using a panel of DNAs from 28 plus trees (see Fig. 1) selected from *Cryptomeria japonica* plantations covering the species' wide distributional range in Japan. The segregation of alleles at 42 microsatellite loci was compared with expected Mendelian ratios by χ^2 tests. For this, a segregating population of 150 trees was produced from a cross between two full-sib trees originating from a cross between 'Iwao (female)' and 'Yabukuguri (male)', which are local cultivars of *C. japonica*. The DNAs were extracted from needle tissue using a modified CTAB method [22]. PCR amplifications were carried out using a GeneAmp PCR System Model 9700 (Applied Biosystems) in a total volume of 20 µL including 0.2 µM of each primer, 0.2 mM of each dNTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 U of *Taq* DNA polymerase and 0.5–3 ng of template DNA, with the following temperature profile: 4 min at 94 °C then 30–35 cycles of 45 s at 94 °C, 45 s at 55–60 °C and 45 s at 72 °C, followed by 5 min at 72 °C. PCR fragments amplified from these sample DNAs using the microsatellite primers were electrophoretically separated on 7.5% polyacrylamide gels, stained by ethidium bromide, and visualized under a UV illuminator (Fig. 1).

2.4. Data analysis

From the genotype data of the 28 trees comprising the screening panel, the number of alleles per locus (NA), and polymorphism information content, PIC, [4], were obtained for each locus using the program G-DIVERSE developed by H. Iwata. The PIC was calculated as follows:

$$\mathrm{PIC} = 2\sum_{i=2}^{l}\sum_{j=1}^{i-1}[P_iP_j(1-P_iP_j)],$$

where, p_i and p_j refer to the frequency of alleles A_i and A_j, respectively, and summation extends over l alleles.

The relationships between polymorphic parameters (NA and PIC) and characteristics of the microsatellite sequences, such as the number of repeats (NOR), the number of nucleotides per repeat (NNR), the total number of nucleotides (TNN) and the number of nucleotides in flanking regions of the microsatellites (NNF) were examined using JMP 4 software (SAS Institute) to calculate Kendall's rank correlation coefficients [16].

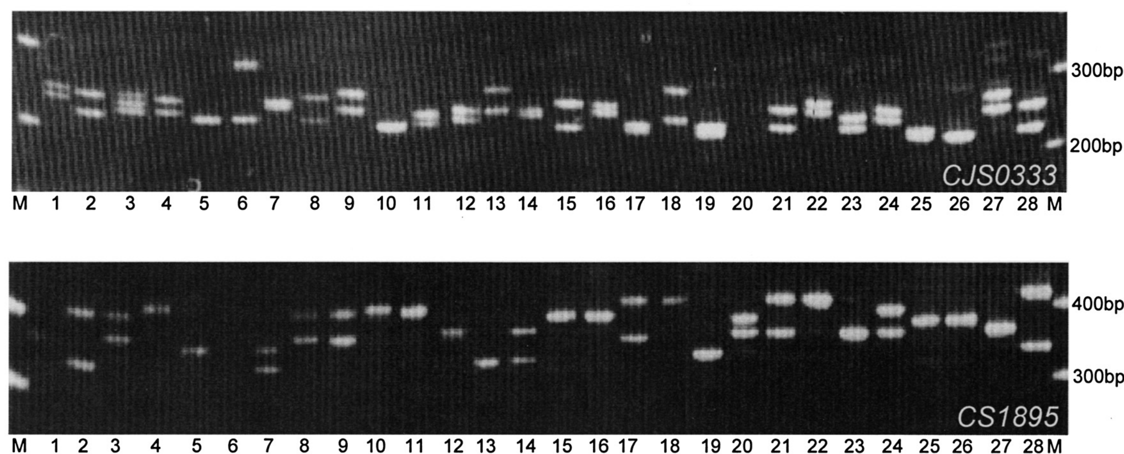

Figure 1. Microsatellite markers, CJS0333 and CS1895, developed for surveying levels of polymorphism in the panel of 28 plus trees, electrophoretically separated in 7.5% polyacrylamide gels. M stands for marker lanes. Lanes 1 to 28, numbered from right to left, correspond to: 1, Hamamatsu 1; 2, Nishikawa 16; 3, Gifu 2; 4, Higashiusuki 14; 5, Gujyo 5; 6, Kusu 12; 7, Fukuokasho 2; 8, Satsuma 3; 9, Ishikawa 5; 10, Syochiku 6; 11, Tone 6; 12, Haara; 13, Kumotoshi; 14, F1 of Kumotoshi × Haara; 15, Minaminasu 3; 16, Takasaki 4; 17, Numata 4; 18, Kuji 3; 19, Inashiki 2; 20, Hiki 13; 21, Nagano 2; 22, Ohi 2; 23, Tenryu 6; 24, Minamiaizu 1; 25, Imaichi 2; 26, Ohtsuki 5; 27, Higashikamo 8; 28, Nukata 3, respectively.

3. RESULTS AND DISCUSSION

3.1. Sequences of clones from the two microsatellite-enriched libraries

We sequenced 1079 clones from the CS library. The data showed 413 (38.3%) of these clones to be redundant and 665 to be unique, 202 of which included microsatellite sequences. By contrast, the CJS library showed a low redundancy ratio, 15.8%, and it contained 760 (out of a total of 814) unique sequences that included microsatellite sequences. Thus, in total, we obtained 962 unique sequences including microsatellite motif sequences (Tab. I). In addition, we found di-nucleotide and tri-nucleotide repeat types of microsatellite motif permutations in both of our libraries. The microsatellite sequences were classified into microsatellite motif permutations according to Echt and May-Marquardt [8]. 46% (CS) and 87% (CJS) of the microsatellite sequences were assigned into the poly(AG)n category of microsatellite sequence permutations, which we expected to find, since we used a (CT)n repeat oligo-nucleotide probe for the enrichment of microsatellite fragments. Nevertheless, despite using the (CT)n probe, 74 and 142 clones including microsatellite sequences with (AC) permutations were detected in the CS and CJS libraries, respectively. Most of the poly(AC)n sequences (21 in CS and 104 in CJS) were accompanied with poly(CT)n sequences, explaining why they were captured in the microsatellite-enriched libraries by the (CT)n oligo-nucleotide probe. However, the other clones with poly(AC)n sequences did not include any other microsatellites with different types of motif. A large-scale survey of microsatellite sequences in a rice genomic library found an estimated 1360 poly(GA)n and 1230 poly(GT)n microsatellites in the rice genome [25]. If *C. japonica* genome also possesses abundant poly(AC)n microsatellites, as the rice genome appears to do, it is possible that we detected microsatellites with this type of motif by chance. We also detected two other di-nucleotide repeat types of motif and seven tri-nucleotide repeat types of motif, but the number of microsatellites involved in these cases was very small (Tab. II).

Table I. Results of sequencing SSR-enriched genomic libraries from *Cryptomeria japonica.*

Designation of genomic library	Number of clones sequenced	Number of clones with unique sequences	Number of clones with unique sequence and SSRs
CS	1 079	665	202
CJS	967	814	760
Total	2 046	1 479	962

Microsatellite markers have been developed for various coniferous species using microsatellite-enrichment methods (see, for instance, [1, 10, 37]. Our successful construction of microsatellite-enriched genomic libraries also showed that enrichment using magnetic particles can promote the efficiency of the development of large amounts of microsatellite markers for coniferous species.

3.2. Characterization and polymorphisms of microsatellite markers

PCR primer pairs were able to design for 196 clones, which showed clear sequence and have enough sequence length for the flanking region of SSR. Forty-two new primers that performed as microsatellite markers were chosen out of the 196 primer pairs we constructed because they detected polymorphisms and gave clear banding patterns when subjected to 7.5%

Table II. Summary of numbers of SSRs found in two genomic libraries enrichied by the CT repeat probe. Roman numbers showed a kind of repeat motif of dinucleotide or trinucleotide. The dinucleotide and trinucleotide repeats have four and six kinds of motif, respectively, thus, the roman numers are corresponding to their motifs.

Dinucleotide repeat		CS							CJS						
I II III IV	Abbreviation	I	II	III	IV			Total	I	II	III	IV			Total
AC CA or GT TG	(AC)	18	12	32	12			74	15	22	62	43			142
AG GA or CT TC	(AG)	66	43	7	11			127	604	410	134	132			1 280
AT TA (self complementary)	(AT)	1	2					3	12	4					16
CG GC (self complementary)	(CG)	0	0					0	4	4					8
Sub-total number of SSRs								204							1 446
Trinucleotide repeat															
I II III IV V VI		I	II	III	IV	V	VI	Total	I	II	III	IV	V	VI	Total
AAC CAA ACA or GTT TTG TGT	(AAC)							0				1			1
AAG AGA GAA or CTT TCT TTC	(AAG)	5	4	5	3	3	1	21	1	4	9	3	3		20
AAT ATA TAA or ATT TAT TTA	(AAT)							0							0
ACC CCA CAC or GGT TGG GTG	(ACC)	1						1							0
ACG CGA GAC or CGT TCG GTC	(ACG)							0							0
ACT CTA TAC or AGT TAG GTA	(ACT)	12	1					13	1	1					2
AGC GCA CAG or GCT TGC CTG	(AGC)							0	2		1				3
AGG GGA GAG or CCT TCC CTC	(AGG)	9	4	11				24	8	1	3				12
ATC TCA CAT or GAT TGA ATG	(ATC)				1	12		13			1	1	1		3
CCG CGC GCC or CGG GCG GGC	(CCG)							0							0
Sub total number of SSRs								72							41
Total number of SSRs								276							1 487

polyacrylamide electrophoresis (Fig. 1). The primer sequences and PCR conditions for these loci are listed in Table III. The panel of 28 plus trees allowed the polymorphic levels at these loci to be evaluated using two statistics: the number of alleles per locus (NA) and the polymorphism information content (PIC). NA and PIC values generated from the 28 plus trees ranged from 3 to 20 with an average of 7.38, and from 0.156 to 0.919 with an average of 0.620, respectively (Tab. IV). We then examined the correlations between two measures of polymorphic levels (NA and PIC) on one hand, and two measures of the length of repeat unit (NOR and NNR), total number of nucleotides (TNN) and a measure of the number of nucleotides in flanking regions of microsatellites (NNF) on the other by Kendall's rank order tests. The degree of polymorphism, according to the derived PIC and NA values, was strongly correlated to the length of the repeat units (NOR and NNR). However, there was no correlation between the polymorphic level and both the total number of nucleotides (TNN) and the length of the flanking region (NNF; Tab. V). In some previous characterizations of microsatellite sequences, evidence for not only nucleotide substitutions, but also indels has been detected in flanking sequences of microsatellites [6, 17]. However, mutations in the flanking regions of the microsatellites do not appear to have affected the degree of polymorphism amongst the microsatellite markers we studied at a statistically significant level according to the Kendall's correlation analysis. Most of the length variation of the PCR products from microsatellite markers might depend upon slippage mutations of the microsatellite sequences.

3.3. Segregation analysis

Segregation in the sib-crossed pedigree was assessed at 42 microsatellite loci (Tab. IV). Twenty-eight and 14 loci were found to be polymorphic and monomorphic in the investigated pedigree, respectively. According to the results of the χ^2 tests, no statistically significant deviations were detected at 26 microsatellite loci. However, we detected statistically significant deviations (at the 5% probability level) at two microsatellite loci, CS2260 and CS2294. The expected segregation ratio at the CS2260 locus was 1:1, because one of the parents was a heterozygote and the other was a homozygote. The expected segregation ratio at the CS2294 locus was 1:2:1, because both parents were heterozygous, with the same genotype. For both of the microsatellite loci showing evidence of segregation distortion, we detected heterozygote excess in 150 individuals of the segregation generation. Inbreeding depression due to the sib-cross or to random chance may have been responsible for this segregation distortion. We detected null alleles (non-amplifying alleles) that may have been due to mutation at the priming sequence at only four loci [5, 15]. By contrast, Moriguchi et al. [20] detected null alleles at 12 out of 34 loci developed (35.3%) in a previous segregation analysis using the same pedigree as in this study, and deduced that the high rate of null allele detection was caused by a high mutation rate at

Table III. Description of *sugi* microsatellite markers.

Locus	Forward primer 5' to 3'	Reverse primer 5' to 3'	Anneal temp.	PCR cycle	DDBJ accession No.	Motif	Putative size (bp)[a]	Repeat status
CJS0002	CTTTTTTCAAATTTAGTGATGT	CCCATGCCCCACTGTCCACC	55	30	AB161634	(TC)12(TC)17	237	Imperfect
CJS0091	GAGAGATAAGAGGGTAGAGGT	CAATGCCAACTTAGAAGAC	60	30	AB161635	(GA)43	298	Perfect
CJS0268	CCTTAGAAAGCTATGCCAC	GCAACGCATCCATAATACC	60	30	AB161636	(AC)53	352	Perfect
CJS0331	GGAGAGATAGACGACAAAAGAG	CCATCTTGCTAATCTGTCC	60	30	AB161637	(GA)6	245	Perfect
CJS0333	AGGAGATTAGGATGGTGGG	GGTTTGCCTCTTCTATGAG	60	30	AB161638	(GA)26	264	Perfect
CJS0336	CAGGGAGTGGTTAAGGGAG	CTTCCATCTCTTCCCATCTC	60	30	AB161639	(GA)11(GA)40	259	Compound
CJS0356	CTAAAGAATAGATGACTCCAC	TATAACGCTTTTGCCCTCA	60	30	AB161640	(GA)64	337	Perfect
CJS0401	GATCTAAACTTGAGCATAAC	CAATCCTGTCTCCATACCC	55	30	AB161641	(CG)8(GA)54	222	Compound
CJS0455	GTTACTTTGAAAAATGAGCC	AACATCAAGATTAAAGGGAC	58	30	AB161642	(CT)20	166	Perfect
CJS0485	CATATCTAATATCTAATACCTTG	TCTCCCTATCTAGCCCTCTG	50	35	AB161643	(GA)9(GA)30(GA)27	331	Compound
CJS0520	TCCCTTTTGGTATTTTACAC	ACTCAAATTGCGATAATCTC	55	30	AB161644	(TG)18	196	Perfect
CJS0527	ATAGAAGAAGAGAAGTAGGG	TCATATCGTGTCATGTGTCC	55	30	AB161645	(GA)18	103	Perfect
CJS0537	ATGAAGGGAATGATTGATGG	TCTCTCACTTGGGTTCTCTC	55	30	AB161646	(GA)34(AG)6	163	Compound
CJS0584	TGGTTTGCCTTTGGTTGCTC	GGACTTTCTATTTACCTCTTGG	60	30	AB161647	(AG)80	329	Perfect
CJS0665	CCAAGCATAGGGAAAAAGAG	GGGGAGTAAGGATGACATTT	60	30	AB161648	(GA)45(GA)29	367	Imperfect
CJS0686	ACATGCAAATATAAGTTCACCC	TCCACCTCTTTTTCATTCTC	55	30	AB161649	(GA)52	275	Perfect
CJS0838	TATGTAGAAGCGTGTGATGT	GATAATTGCCTTTGTTGTCC	58	30	AB161650	(GT)23	170	Perfect
CJS0955	CACACTCCCCGTCTCCGACAG	ACCCTGATTCCCCATACACC	58	30	AB161651	(TCT)4(GA)29	137	Compound
CS1218	CATCACATACAAATAGCACC	GAAGATTGTCTCACGCACTC	60	30	AB161652	(GT)13	332	Perfect
CS1219	AAGGTGTTGTTTTAAGGAGG	CAGCCATCTATTATTTGTGC	60	30	AB161653	(GT)10	103	Perfect
CS1226	CTCTAGTCCTCAATGGTGGT	TATTAAGCATTTTCCCTCTC	60	35	AB161654	(CA)14	139	Perfect
CS1281	CCCCCTCTCATTAGTTACCA	CAAAAATCAACAAGCCAACC	60	30	AB161655	(CT)15	233	Perfect
CS1289	CATCCACCACTAAATACAAC	TCGCTATCCCTTGCCTATCC	60	35	AB161656	(AC)26(A)26	147	Compound
CS1364	TGATTATGGTCGGTGGTCTT	GTGATGTGGTGTTATCTTGT	62	30	AB161657	(AC)7	297	Perfect
CS1450	GGCATTAAACCATCAAGACA	AGTTGGGCAGAGATCATAAG	62	35	AB161658	(TG)9	401	Perfect
CS1522	AAAGTTTGATTAGGGCAGGG	AAACGTGGGTGCTATCCTTC	62	30	AB161659	(AC)16	222	Perfect
CS1525	ATGAAGTGCCCTTGGTTTGT	ATCGCCTCCTCTTTTATCCT	60	30	AB161660	(CA)18	200	Perfect
CS1579	ACTCTAGCAGCATTTCTCAC	CAGATTTTGTATGAGTGGTT	60	30	AB161661	(TG)11	291	Perfect
CS1671	ACTTGTCCGCTTTTGTTGTT	GCCTCAAGGTAGGAGAAGAA	60	30	AB161662	(TG)16	280	Perfect
CS1737	TACCCTCAACCCTTCACCCT	TTACCCACCTCTCTTTCCTC	60	30	AB161663	(AG)40	248	Perfect
CS1895	TGAGAGAGGGAGGGAGGGTT	GAGTCCTTGTCCCGTTTTGT	60	30	AB161664	(TG)10	405	Perfect
CS1906	AGTCATTCCCAGGCAGTGTC	ATCCCTCCACCTCTCCTACC	60	30	AB161665	(TGA)6	346	Perfect
CS2024	AGTAATACAAGATAAGGGAG	TCCACCTCTATACCTCTACA	55	30	AB161666	(AG)15(AG)4(AG)10	314	Imperfect
CS2048	CCCTCTATCTTCATCTCTTC	AGGGATAGATATAGGGGTAG	60	30	AB161667	(CT)7	225	Perfect
CS2056	GAGAGACATGGGGGAAGAGG	GGTTCTAACACATGAATGGC	60	30	AB161668	(GA)20(GA)7	295	Compound
CS2165	GAGAGAGGTTTGAAGAGAGA	CCCTCATCTTCTATCAACTC	60	35	AB161669	(AG)6(GA)30(AG)40(GA)7(GA)25	395	Compound
CS2169	GTAGAGGAGGGATATAGAGT	TCCTTGTCCATCTCTCTTTA	55	30	AB161670	(GA)9	141	Perfect
CS2230	AGACATAAAGAGGGAGGTAGAG	TACTCTTGCTGACTGGTCCG	60	30	AB161671	(GA)9	119	Perfect
CS2245	GAGGCAAAGGTAGAGGTGAA	CCCTCCCAAGTTCTAAGTAA	60	30	AB161672	(GA)9	167	Perfect
CS2260	GGAGGGTAGATAGAGAAAATAG	TCTACCTACCTCTCTTCCCA	60	30	AB161673	(GA)39	206	Perfect
CS2294	TTTCCTCTTCCATCTCACCC	TCATGCTCCATTACGAATCT	60	30	AB161674	(CT)30	129	Perfect
CS2484	TGAGAAAGGGAGAGAGGGAT	CCCCCTTCTCTTTTTCACTC	60	30	AB161675	(GA)13	158	Perfect

[a] Putative PCR fragment sizes were deduced from sequences of genomic clones between forward and reverse primers.

Table IV. Polymorphic level and deviation from Hardy-Weinberg expectation of microsatellite markers with more than two alleles in 28 screening panel samples.

Marker	NA	PIC	Segregation	Expected	χ^2	Probability
CJS0002	10	0.779	38:28:39:39	1:1:1:1	2.389	0.496
CJS0091	9	0.807	41:35:30:37	1:1:1:1	1.755	0.625
CJS0268	11	0.591	74:74	1:1	0.000	1.000
CJS0331	3	0.160	76:71	1:1	0.170	0.680
CJS0333	10	0.850	39:45:34:31	1:1:1:1	3.027	0.388
CJS0336	9	0.734	Invariant	–	–	–
CJS0356	8	0.754	41:39:39:27	1:1:1:1	3.370	0.338
CJS0401	9	0.817	42:34:32:27	1:1:1:1	3.459	0.326
CJS0455	6	0.540	80:70	1:1	0.667	0.414
CJS0485	9	0.825	31:86:31	1:2:1	3.892	0.143
CJS0520	5	0.408	76:72	1:1	0.108	0.742
CJS0527	3	0.526	Invariant	–	–	–
CJS0537	10	0.837	Invariant	–	–	–
CJS0584	9	0.794	28:40:38:41	1:1:1:1	2.905	0.407
CJS0665	8	0.733	33:32:43:39	1:1:1:1	2.197	0.532
CJS0686	6	0.729	72:78	1:1	0.240	0.624
CJS0838	8	0.736	73:73	1:1	0.000	1.000
CJS0955	5	0.664	72:75	1:1	0.061	0.805
CS1218	3	0.181	Invariant	–	–	–
CS1219	6	0.351	Invariant	–	–	–
CS1226	6	0.731	77:66	1:1	0.846	0.358
CS1281	8	0.768	31:44:40:35	1:1:1:1	2.587	0.460
CS1289	10	0.834	Invariant	–	–	–
CS1364	4	0.433	Invariant	–	–	–
CS1450	3	0.234	Invariant	–	–	–
CS1522	9	0.744	63:87	1:1	3.840	0.050
CS1525	8	0.461	30:75:34	1:2:1	1.101	0.577
CS1579	4	0.438	84:66	1:1	2.160	0.142
CS1671	6	0.394	Invariant	–	–	–
CS1737	10	0.864	42:40:35:31	1:1:1:1	2.000	0.572
CS1895	10	0.801	41:39:29:36	1:1:1:1	2.283	0.516
CS1906	3	0.156	Invariant	–	–	–
CS2024	20	0.919	38:33:37:39	1:1:1:1	0.565	0.904
CS2048	4	0.246	Invariant	–	–	–
CS2056	6	0.727	83:66	1:1	1.940	0.164
CS2165	15	0.908	Invariant	–	–	–
CS2169	7	0.636	65:85	1:1	2.667	0.102
CS2230	3	0.334	Invariant	–	–	–
CS2245	3	0.308	Invariant	–	–	–
CS2260	10	0.859	57:94	1:1	9.066	0.003
CS2284	10	0.823	78:70	1:1	0.432	0.511
CS2294	4	0.601	44:99:8	1:2:1	31.795	0.000

Table V. Rank correlation coefficients [Kendall's (Kendall 1970)], between two measures of variation (the number of alleles (NA) and polymorphism information content (PIC)) on one hand, and four measures of length [the number of repeats (NOR), the number of nucleotides per repeat (NNR), total number of nucleotides (TNN) and the number of nucleotides in the microsatellites' flanking regions (NNF)] on the other.

	NOR	NNR	TNN	NNF
NA	0.3973***	0.4987***	0.0570	–0.0655
PIC	0.4447***	0.5230***	0.1357	–0.1468

the priming sequences in *C. japonica*. Our low rate of null allele detection suggests that the high mutation rate at priming sequences is not pandemic in this species. Null alleles can cause a number of problems, such as underestimations of the number of heterozygotes in population genetic studies of natural populations, overestimates of the inbreeding rate in mating system analysis using open-pollinated seeds, and underestimates of pollen dispersal distance in paternity analyses. Our newly developed microsatellite markers revealed a lower rate of null allele detection than the microsatellite markers previously developed by Moriguchi et al. [20]. Therefore, these markers are likely to be valuable tools, not only for genetic mapping, but also for analyses of population genetics and reproduction ecology in natural populations [7, 13].

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