Development of Simple Sequence Repeat Markers in Chinese Chestnut and Their Characterization in Diverse Chestnut Cultivars

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ABSTRACT. To develop and characterize valid simple sequence repeat (SSR) markers in chestnut (*Castanea* spp.), we used a selective hybridization method to perform SSR sequence enrichment in the genomic library of chinese chestnut (*Castanea mollissima*). From 47 sequences of the enriched library, we designed 24 primer pairs for SSR polymerase chain reaction (PCR). SSR PCR was performed for 23 chinese chestnut cultivars. Among the 24 primers, 22 primers amplified their respective SSR loci; 21 primer pairs yielded polymorphic fragments across the cultivars. Among these 21 primer pairs, 17 primer pairs amplified single SSR loci with polymorphisms. Multilocus amplification patterns were observed in the other four primers. For the corresponding 17 SSR loci, the number of alleles per locus was two to 13, and the average number of alleles was 7.19. The observed heterozygosity (number of genotypes of the heterozygote/total number of genotypes scored at that locus) ranged from 0.00 to 0.87 (average = 0.68), and the expected heterozygosity ranged from 0.00 to 0.87 (average = 0.68). One of the SSR loci—ICMA014—was a unique locus, because the primer pair for this locus did not amplify any fragment in any of the cultivars, except in Qianchi, which was used to construct the SSR-enriched genomic library. Cross-species amplifications using the 17 primer pairs were also successful in the other species of genus *Castanea*. A dendrogram depicting the relationships among the genotypes on the basis of genetic distances was generated using the unweighted pair group method with arithmetic mean cluster analysis. The genotypes were clearly separated into three large groups, which reflected the three cultivated species, namely, *C. mollissima*, *C. crenata*, and *C. sativa*.

Chestnut is one of the most important nut trees in the world and has been cultivated for more than 2700 years in Asia (Adua, 1999). *Castanea* species have a diploid genome ($n = 12$) and are distributed in the temperate regions. The genus *Castanea* is a member of the Fagaceae family and comprises 10 species, including three cultivated species, namely, chinese chestnut (*C. mollissima*), japanese chestnut (*C. crenata*), and european chestnut (*C. sativa*) (U.S. Department of Agriculture, 2007). Among these, chinese chestnut and japanese chestnut are the two most economically important *Castanea* species (Tanaka and Kotobuki, 1992). Chinese chestnut originated and is mainly cultivated in China.

Several molecular markers, including isozyme markers (Casasoli et al., 2001; Dane et al., 2003; Sawano et al., 1984), chloroplast sequences (Fineschi et al., 2000), random amplified polymorphic DNA markers (Casasoli et al., 2001; Galderisi et al., 1998; Inoue et al., 2003; Morimoto et al., 1997), inter-simple sequence repeat markers (Casasoli et al., 2001), restriction fragment length polymorphism markers (Morimoto et al., 1997), and amplified fragment length polymorphism markers (Yamamoto et al., 1998), have been used to study the genetic

diversity and relationships in chestnut cultivars. Simple sequence repeat (SSR), which is one such marker, is a short tandem repeat sequence that is distributed throughout the genome. They are used to develop molecular markers for several organisms such as horticultural crops (Clarke and Tobutt, 2003, 2006; Inoue et al., 2006; James et al., 2003; Yamamoto et al., 2002; Zane et al., 2002). Recently, SSR markers have been developed (Buck et al., 2003; Yamamoto et al., 2003) and used not only for genetic differentiation, which is used in phylogenetic research and cultivar identification, but also for advanced genetic mapping, including comparative mapping (Barreneche et al., 2003) and quantitative trait locus (QTL) analysis (Casasoli et al., 2004, 2006), in chestnut plants of different origins. SSRs afford a high level of polymorphism and codominance. Although the development of SSR markers is difficult, they can be easily detected using polymerase chain reaction (PCR) and electrophoresis. Microsatellite markers for japanese chestnuts and european chestnuts have been developed and used (Buck et al., 2003; Yamamoto et al., 2003); however, there have been no studies on the development of microsatellite markers for chinese chestnuts. Furthermore, the number of available markers is insufficient for breeding selection or advanced QTL analysis in chestnut, and the available types of repeat motifs are limited.

Therefore, in this study, to develop an analysis tool and obtain information regarding the chestnut genome, we attempted

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to develop useful microsatellite markers from an enriched genomic library of chinese chestnut that was constructed by performing selective hybridization with eight types of microsatellite repeat sequences (Zane et al., 2002).

We also investigated the use of these markers in cultivar identification and parentage analysis. Furthermore, we characterized the microsatellite markers in *Castanea* and investigated their cross-species transferability to determine whether the markers were effective in characterizing other members of the Fagaceae family.

Materials and Methods

PLANT MATERIALS. A microsatellite-enriched genomic library was constructed from the genomic DNA of the chinese chestnut 'Qianchi'. 'Qianchi' is an old cultivar grown in Hubei Province, China. To survey the SSR loci in *Castanea*, 23

chinese chestnut accessions, 42 japanese chestnut accessions, one european chestnut accession, and five interspecific hybrids were used as the plant materials (Table 1). To investigate the cross-species transferability of the SSR primers developed in this experiment, we used related woody plants of the Fagaceae family, including 17 species belonging to four different genera.

DNA EXTRACTION. Mature leaves of each genotype were frozen in liquid nitrogen and ground with tungsten beads by using a tissue grinder (SH-48; Kurabo, Osaka, Japan). Genomic DNA was isolated from the ground tissue by performing a modified protocol of the cetyltrimethylammonium bromide method (Bousquet et al., 1990). The extracted DNA was quantified by ultraviolet spectrophotometry with the Molecular Probes™ PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA).

DEVELOPMENT OF SIMPLE SEQUENCE REPEAT MARKERS. SSR sequences were obtained from the 'Qianchi' genomic library

Table 1. Chestnut accessions used for characterizing the newly developed simple sequence repeat (SSR) loci in *Castanea*.^z

No.	Accessions	<i>Castanea</i> species	Origin	No.	Accessions	<i>Castanea</i> species	Origin
1	Chushuhong	<i>C. mollissima</i>	China	36	Shougatu	<i>C. crenata</i>	Japan
2	Duanzhatou	<i>C. mollissima</i>	China	37	Bonguri	<i>C. crenata</i>	Japan
3	Ershuizhao	<i>C. mollissima</i>	China	38	Arima	<i>C. crenata</i>	Japan
4	Ershuizhao2-11	<i>C. mollissima</i>	China	39	Chuutana	<i>C. crenata</i>	Japan
5	Hongguang	<i>C. mollissima</i>	China	40	Ginrei	<i>C. crenata</i>	Japan
6	Hongli	<i>C. mollissima</i>	China	41	Kanotsune	<i>C. crenata</i>	Japan
7	Hongli1hao	<i>C. mollissima</i>	China	42	Hashikami	<i>C. crenata</i>	Japan
8	Houzhuang2hao	<i>C. mollissima</i>	China	43	Ninomiya	<i>C. crenata</i>	Japan
9	Jinpingduanchui	<i>C. mollissima</i>	China	44	Obuse2gou	<i>C. crenata</i>	Japan
10	Linfen	<i>C. mollissima</i>	China	45	Wasechoubei	<i>C. crenata</i>	Japan
11	Niandiban	<i>C. mollissima</i>	China	46	Ishiduka	<i>C. crenata</i>	Japan
12	Qianchi	<i>C. mollissima</i>	China	47	Kasaharawase	<i>C. crenata</i>	Japan
13	Shandongzicui	<i>C. mollissima</i>	China	48	Choukouji	<i>C. crenata</i>	Japan
14	Shandongzicui3	<i>C. mollissima</i>	China	49	Obuse3gou	<i>C. crenata</i>	Japan
15	Taianaisheng	<i>C. mollissima</i>	China	50	Oharaguri	<i>C. crenata</i>	Japan
16	Taibo	<i>C. mollissima</i>	China	51	Tamanishiki	<i>C. crenata</i>	Japan
17	Wuhua	<i>C. mollissima</i>	China	52	Yamatowase	<i>C. crenata</i>	Japan
18	Xiazuang2hao	<i>C. mollissima</i>	China	53	Choubei	<i>C. crenata</i>	Japan
19	Xinlong9hao	<i>C. mollissima</i>	China	54	Osaya	<i>C. crenata</i>	Japan
20	Yanchen207	<i>C. mollissima</i>	China	55	Imakita	<i>C. crenata</i>	Japan
21	Yebanli	<i>C. mollissima</i>	China	56	Ibuki	<i>C. crenata</i>	Japan
22	Yebianzha	<i>C. mollissima</i>	China	57	Autumn koron	<i>C. crenata</i>	Japan
23	Yelichang	<i>C. mollissima</i>	China	58	Autumn poron	<i>C. crenata</i>	Japan
24	Idumo	<i>C. crenata</i>	Japan	59	Togenasiguri	<i>C. crenata</i>	Japan
25	Isiduti	<i>C. crenata</i>	Japan	60	Shibaguri1	<i>C. crenata</i>	Japan
26	Kinka	<i>C. crenata</i>	Japan	61	Shibaguri2	<i>C. crenata</i>	Japan
27	Moriwase	<i>C. crenata</i>	Japan	62	Shibaguri3	<i>C. crenata</i>	Japan
28	Kotai	<i>C. crenata</i>	Japan	63	Kunimi	<i>C. crenata</i>	Japan
29	Baneki	<i>C. crenata</i>	Japan	64	Tanzawa	<i>C. crenata</i>	Japan
30	Nakatetanba	<i>C. crenata</i>	Japan	65	Tsukuba	<i>C. crenata</i>	Japan
31	Ginyose	<i>C. crenata</i>	Japan	66	9113	<i>C. crenata</i> × <i>C. mollissima</i>	China
32	Kanbei	<i>C. crenata</i>	Japan	67	Riheiguri	<i>C. crenata</i> × <i>C. mollissima</i>	Japan
33	Kinseki	<i>C. crenata</i>	Japan	68	Gakuou	<i>C. crenata</i> × (<i>C. crenata</i> × <i>C. mollissima</i>)	Japan
34	Higan	<i>C. crenata</i>	Japan	69	Carpinese	<i>C. sativa</i>	Japan
35	Taishouwase	<i>C. crenata</i>	Japan	70	M-15	<i>C. crenata</i> × <i>C. sativa</i>	Japan
				71	AW-7	<i>C. crenata</i> × <i>C. sativa</i>	Japan

^zTwenty-three chinese chestnut accessions, 42 japanese chestnut accessions, one european chestnut accession, and five interspecific hybrids were used. The genomic DNA for SSR identification was extracted from the mature leaves of the accessions.

that was enriched for eight different types of microsatellites—(GA)_n, (GT)_n, (GC)_n, (AC)_n, (CAA)_n, (GCC)_n, (CTG)_n, and (CAG)_n—by selective hybridization (Zane et al., 2002). The genomic DNA of ‘Qianchi’ was digested with *Rsa*I (New England Biolabs, Ipswich, MA) at 37 °C for ≈12 h. The digested DNA fragments were ligated to the adapter DNA that was constructed using ad1—5′-CTCTTGCTTACGCGTG GACTA-3′ and ad2—5′-TAGTCCACGCGTAAGCAAGAG CACA-3′. The ligation reaction was performed using T4 DNA ligase (Promega, Madison, WI) at room temperature for 2 h. Then, the fragments were incubated at 4 °C overnight. The DNA fragments and the adapters were amplified by PCR with ad1 as the primer. PCR was performed using a 50-μL sample mixture containing 5 μL of the ligated products, 1× PCR buffer (without magnesium), 1.5 mM MgCl₂, 1 μM ad1 primer, 0.2 mM of each dNTP, and 2U Taq DNA polymerase (Invitrogen) in a thermocycler (model 9700; Applied Biosystems, Foster City, CA). The following protocol was used for PCR: 35 cycles of 40 s at 95 °C, 1 min of annealing at 60 °C, and 3 min of extension at 72 °C. Subsequently, the amplified fragments were hybridized with eight types of SSR oligonucleotides: (GA)₁₇, (GT)₁₇, (GC)₁₇, (AC)₁₇, (CAA)₁₀, (GCC)₁₀, (CTG)₁₀, and (CAG)₁₀. The oligonucleotides were bound to a positively charged nylon membrane (Hybond-N+; GE Healthcare, Amersham, UK), and the amplified fragments were enriched for genomic DNA containing the previously mentioned SSR sequences.

The enriched DNA was amplified by PCR and desalted using a Suprec TM-02 centrifuge device (Takara Bio, Madison, WI). Then, the products were ligated with the pGEM-T Easy Vector (Promega) and used to transform *Escherichia coli* DH5α cells. The positive clones, which contained the plasmid DNA with an ≈450-bp-long DNA insert, were selected by direct colony PCR. The plasmids were extracted, and their cloning sites were sequenced using a Big Dye version 3.1 cycle sequencing kit and an ABI3100 genetic analyzer (Applied Biosystems). On the basis of the SSR-containing sequences, the primer pairs for sequences on both sides of the SSR region were constructed using PRIMER3 (Rozen and Skaletsky, 2000). One primer of each pair was labeled using the fluorescent dye 6-FAM or VIC.

SIMPLE SEQUENCE REPEAT POLYMERASE CHAIN REACTION AND ELECTROPHORESIS USING A CAPILLARY SEQUENCER. SSR PCR was performed using 12.5-μL reaction mixtures containing 0.2 mM of each dNTP, 1× PCR buffer, 1.5 mM MgCl₂, 0.25 U Taq DNA polymerase (Invitrogen), 0.2 μM of each primer, and 6.25 ng of genomic DNA. The amplification reactions were performed using the following protocol: denaturation at 94 °C for 3 min; 35 cycles of amplification at 94 °C for 30 s, 45 s of annealing at 55 °C or 60 °C, 1 min of extension at 72 °C, and a final extension step at 72 °C for 10 min. The amplification products were separated using an ABI3100 sequencer with the GENESCAN and GENOTYPER (Applied Biosystems) software packages and a GS500LIZ ladder (Applied Biosystems).

ANALYSIS OF SIMPLE SEQUENCE REPEAT LOCI. The SSR alleles were named according to their length at each locus. The expected heterozygosity (Nei, 1973), observed heterozygosity, and the probability of identity (Paetkau et al., 1995) of the 17 microsatellites that were associated with single loci were calculated using IDENTITY1.0 (Wagner and Sefc, 1999). GENEPOP (Raymond and Rousset, 1995) was used to identify deviations from the Hardy–Weinberg equilibrium (HWE), and the linkage disequilibrium (LD) within populations was analyzed using the Markov chain parameters. Pairwise genetic

distances, which were determined on the basis of the proportion of shared alleles, were calculated for all the genotypes. A dendrogram was generated using the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis method, which uses the PHYLIP3.6 program (Felsenstein, 2005) and the TreeView software (Page, 1996).

Results

DEVELOPMENT OF THE SIMPLE SEQUENCE REPEAT-ENRICHED LIBRARY AND THE GENOMIC SEQUENCES CONTAINING SIMPLE SEQUENCE REPEAT REGIONS. A total of 141 SSR-enriched clones were constructed from the nuclear genome of the ‘Qianchi’ by performing the selective hybridization method, and 105 positive clones were selected from the library by colony direct PCR. Among these, 47 positive clones that contained plasmids with ≈450-bp-long DNA inserts were sequenced. Among the selected clones, 41 clones, including four clones with overlapping sequences, were found to contain SSR sequences.

In the 37 unique sequences, 57 SSR regions were identified: 39 GA/CT regions (68.4%), 14 GT/CA regions (24.6%), two CAG/GTC regions (3.5%), one GCC/CGG region (1.8%), and one AT/TA region (1.8%). To obtain the primers, we designed 24 primer pairs for SSR PCR using the 37 unique clones, excluding the clones that had very short flanking sequences.

EVALUATION OF THE SIMPLE SEQUENCE REPEAT PRIMERS IN THE CHINESE CHESTNUT CULTIVARS. The 24 newly developed microsatellite primer pairs were evaluated using 23 chinese chestnut cultivars (Table 1). The results of this evaluation are shown in Table 2.

Among these pairs, 22 primer pairs amplified their respective SSR loci in the chinese chestnut cultivars. We assessed the occurrence of polymorphic fragments from these 22 primers. Among these primer pairs, 17 amplified single polymorphic SSR loci (Table 2). One primer pair (ICMA001) amplified only a nonpolymorphic fragment in 23 accessions. Four primers—ICMA006, ICMA012, ICMA017, and ICMA024—amplified more than three alleles per locus, thereby generating multiple loci patterns. The analyses with the 17 SSR primers that amplified single loci yielded 129 putative alleles, including one null allele. The number of alleles per locus varied from two to 13, and the average number of alleles was 7.19. The observed heterozygosity, which is the ratio of the number of heterozygotic genotypes to the total number of genotypes scored at the locus, ranged from 0.00 to 0.87 (average, 0.68). The expected heterozygosity ranged from 0.00 to 0.87 (average, 0.68). Although there were no significant deviations from HWE at $P < 0.01$, at $P < 0.05$, there were significant deviations in three loci, namely, ICMA004, ICMA019, and ICMA022 (Table 2). The LD between ICMA008 and ICMA019 was evaluated ($P > 0.001$).

CROSS-SPECIES TRANSFERABILITY. Cross-species amplifications using the 17 primer pairs were successful in other species of genus *Castanea*. Among the 17 SSR loci in the 42 japanese chestnut accessions, the number of alleles per locus was two to 21 (average, 6.91) (Table 2). The observed heterozygosity ranged from 0.17 to 0.93 (average, 0.59), and the expected heterozygosity ranged from 0.18 to 0.93 (average, 0.58). The SSR markers developed from chinese chestnut exhibited high polymorphism. Therefore, they can be used as a reliable tool for differentiating among the japanese chestnut accessions. Among the 42 japanese chestnut accessions, significant deviations from

Table 2. Sequences of the 22 simple sequence repeat (SSR) primers developed from chinese chestnut and their characterizations in 23 chinese chestnut cultivars and 42 japanese chestnut cultivars, including information on allele number, expected heterozygosity (H_E), observed heterozygosity (H_O), and probability of identity (PI).^z

Locus	Dye	Primer sequence (5'-3')	GenBank accession no.	Size (bp) ^y	Repeat	<i>C. mollissima</i>				<i>C. crenata</i>			
						Alleles (no.) ^x	H_E^w	H_O^v	PI^u	Alleles (no.) ^x	H_E^w	H_O^v	PI^u
ICMA001 [†]	6-FAM	F:TCAATGCATTTTCCCATGA R:CAGAGGAAGGGCAAAGCAT	AB271074	230	(CTTTT) ₄	1	—	—	—	1	—	—	—
ICMA003	6-FAM	F:TGTCTGCCTGAAACCATTCT R:GGGACCCACAAGTCTCATGT	AB271075	152–183	(GA) ₁₉	7	0.70	0.61	0.24	4	0.44	0.55	0.50
ICMA004	6-FAM	F:ACCACAGAGAGAGCCACACC R:TTTCATGAGACGAAAAGCTG	AB271076	194–252	(CT) ₃₇	9	0.69	0.48*	0.17	9	0.61	0.57	0.23
ICMA005	6-FAM	F:AAATAAAACCCCTCATCAACACA R:GAACTCAAAACCTCAAAACCTCA	AB271077	142–172	(CT) ₁₈	13	0.87	0.87	0.05	8	0.57	0.52	0.33
ICMA006 [‡]	6-FAM	F:GTGACCTTGGGTCCTTGCTA R:TCATGCTCTGATCAAGTTTCA	AB271078	217–237	(CT) ₁₆	4	—	—	—	4	—	—	—
ICMA007	6-FAM	F:TAGTCACGCCTCTCCGTCTT R:GCCATTGAGGACTGAGGTT	AB271079	201–282	(AG) ₂₂	8	0.79	0.78	0.13	7	0.64	0.67	0.28
ICMA008a	6-FAM	F:CCCAAAATCAAAGTCTGAGCA R:CGTCGACTCTTCTCTATCTCCAA	AB271080	122–140	(AC) ₁₁	9	0.76	0.78	0.16	8	0.69	0.74	0.2
ICMA009a	6-FAM	F:ATTCCAACAGCAGCCAAAAC R:CCACAACACAACAAAAACCA	AB271081	177–196	(AG) ₁₈	5	0.75	0.70	0.19	8	0.50	0.55	0.33
ICMA010	6-FAM	F:GTTGGAGAGGTCGTCTCACG R:ATTGCGAGGAAAAGGAAACA	AB271082	211–240	(CT) ₆ (GT) ₁₇	11	0.82	0.87	0.10	5	0.50	0.52*	0.40
ICMA011	6-FAM	F:TCCTCGTCCCTAACAATTCG R:TGTCTGTTTCTGCATATTGATG	AB271083	221–312	(CT) ₃₅	12	0.83	0.87	0.09	21	0.88	0.88	0.04
ICMA012 [§]	VIC	F:TCCACAGCAAGATCCAAACA R:ATGATTTGGCCATCACAAGA	AB271084	120–210	(AC) ₁₄	8	—	—	—	6	—	—	—
ICMA013	VIC	F:AAAGTTTCAAAAATCAAAATTCG R:ATTGGCTTTCGCGTTCT	AB271085	136–147	(AG) ₁₆	4	0.59	0.65	0.35	7	0.75	0.71	0.18
ICMA014	VIC	F:GGTGATATTTTGGCATCATCA R:GGGTCTCCACCACAATTAG	AB271086	240	(CA) ₁₃	2 ^r	0.00	0.00	1.00	9	0.61	0.69	0.24
ICMA015	VIC	F:TGGACTACATCCCACCACAA R:TCCTTTCCGCATCAAAAATC	AB271087	151–169	(GA) ₂₆	7	0.52	0.52	0.32	21	0.93	0.93	0.02
ICMA016	VIC	F:CAAGACCCACGTTCTCTA R:GGTTTCATGGTGAATGAAGAGAG	AB271088	178–192	(CT) ₂₆	6	0.64	0.78	0.24	4	0.45	0.43	0.52
ICMA017 [§]	VIC	F:CAAGCGGAAATGTTTTCTCA R:CTTTGGATCAAATGGCGTCT	AB271089	176–226	(TG) ₁₇	7	—	—	—	5	—	—	—
ICMA018	6-FAM	F:ACAACGATCCCAGACCAAAG R:CTAGGCGATCGGAGAGAGAC	AB271090	155–207	(CT) ₂₅	11	0.85	0.87	0.07	6	0.71	0.64	0.23
ICMA019	6-FAM	F:GGTCTCGAAGCACCTTTGAA R:CACTCATGCTCCAGACTCCA	AB271091	181–207	(TC) ₂₁	7	0.75	0.78*	0.18	3	0.18	0.17	0.71
ICMA020	6-FAM	F:ACACCACCTCCAAACACAT R:GAAAGTCGTGAGGAGGGAGA	AB271092	186–209	(CT) ₁₆ (AC) ₁₁	10	0.84	0.70	0.08	5	0.52	0.52	0.37
ICMA022	6-FAM	F:CGATGCTGCGTCAAAATCTC R:CTGCTGGGACAGCTGATGTA	AB271093	179–185	(CAG) ₆	3	0.54	0.52*	0.53	2	0.29	0.31	0.63
ICMA023	6-FAM	F:GCATACACAAGAATTACCTTCA R:AGGCGCCAAATGATGTTTC	AB271094	214–222	(AG) ₁₄	5	0.57	0.52	0.32	4	0.60	0.60	0.40
ICMA024 [§]	6-FAM	F:CGCTTTGAATAGAAAGCACCA R:TTTCAGAGCTAACTGGCCTATC	AB271095	135–175	(TC) ₁₁ (AC) ₉	4	—	—	—	5	—	—	—
Mean ^q						7.19	0.68	0.68	0.25	6.91	0.58	0.59	0.33

^zSSR polymerase chain reaction was conducted using a fluorescent-labeled forward primer, and the amplification products were subsequently separated using an ABI3100 genetic analyzer with the GENESCAN and GENOTYPER software and the GS500LIZ ladder (Applied Biosystems, Foster City, CA).

^ySize range of single SSR locus with polymorphisms observed in 23 cultivars of chinese chestnuts.

^xAllele numbers detected in the species.

^wExpected heterozygosity (Nei, 1973) observed in 23 cultivars of chinese chestnuts.

^vObserved heterozygosity (direct count heterozygosity) observed in 23 cultivars of chinese chestnuts. Significant departures from the Hardy–Weinberg equilibrium, * $P < 0.05$.

^uProbability of identity (Paetkau et al., 1995) observed in 23 cultivars of chinese chestnuts.

[†] H_E , H_O , and PI were not calculated because the locus did not segregate.

[‡] H_E , H_O , and PI were not calculated because more than three alleles were observed per locus.

[§]Including a null allele.

^qCalculated by 17 single SSR locus with polymorphisms.

the HWE were detected only in ICMA010 at $P < 0.05$ (Table 2). Linkage disequilibria were detected between ICMA004 and ICMA011, ICMA007 and ICMA015, ICMA009 and ICMA015, and ICMA008 and ICMA018 ($P > 0.001$).

In the european chestnut accessions, 15 primers, except ICMA005 and ICMA014, amplified one or two visible fragments. At seven loci in the european chestnut accessions, one or two alleles were species-specific (Table 3). The 17 primers were able to amplify polymorphic fragments in 17 species belonging to the Fagaceae family (Table 4). ICMA003 and ICMA022 amplified one or two clear bands in all the tested genotypes in the Fagaceae family.

CHARACTERIZATION OF THE ICMA014 LOCUS WITH A NULL ALLELE IN CHINESE CHESTNUT. The SSR locus ICMA014 is considered a unique locus, because it did not amplify any fragment in any of the chinese chestnut cultivars, except in Qianchi, which was used to develop the genomic library. Interestingly, the ICMA014 primer could produce one or two amplified fragments in all the japanese chestnut accessions, and nine putative alleles for this locus were confirmed in the 42 japanese chestnut accessions (Table 3). Furthermore, we used ≈ 100 chinese chestnut accessions to investigate this locus; however, the amplification of the locus was confirmed in only in a few cultivars, which were closely related to 'Qianchi' (data not shown). These results indicated the existence of a null allele in the ICMA014 locus in chinese chestnut. To confirm this hypothesis, we examined the interspecific cultivar Riheiguri and its offspring. Because 'Riheiguri' is an interspecific hybrid of japanese chestnut and chinese chestnut, its genotype on the ICMA014 locus is expected to be 244/244 or 244/n. Furthermore, a cross between the japanese chestnut cultivar Toyotamawase (genotype, 242/242) and 'Riheiguri' yielded an offspring cultivar known as Gakuou. If 'Riheiguri' contains a null allele at the ICMA014 locus inherited from

the chinese parent, its genotype will be 244/n. Consequently, the expected genotype of 'Gakuou' will be 242/244 or 242/n. Because 'Gakuou' exhibits only a single 242 allele, we concluded that its genotype is 242/n, and it possesses a null allele (Fig. 1).

GENETIC CHARACTERIZATION OF THE GENOTYPES IN *CASTANEA*. To depict the relationships among the genotypes, we generated a dendrogram using the genetic distances (Fig. 2). The genotypes were clearly divided into three large groups, which reflected the three species: *C. mollissima*, *C. crenata*, and *C. sativa* (Fig. 2). In the japanese chestnut accessions, the cultivar pairs Nakatetanba and Wasechoubei, Chuutan A and Ginrei, and Ishiduka and Tamanishiki had identical genotypes at all 17 loci.

Discussion

In the present study, we used the selective hybridization method to develop 17 polymorphic microsatellite primers from an enriched genomic library of chinese chestnut. The average observed and expected heterozygosities were higher than 0.6, thereby indicating a high degree of variation in the isolated loci and the absence of null alleles. This is the first report on the development of microsatellite markers for chinese chestnut.

In this study, more than 87% of the clones from the enriched genomic library were found to contain microsatellite sequences. This value for the occurrence of microsatellites in the positive sequences is higher than that reported in previous studies (Buck et al., 2003; Yamamoto et al., 2003). Furthermore, we obtained similar results in our previous study on the development of microsatellite markers in japanese pear (*Pyrus pyrifolia*) (Inoue et al., 2006). Because the selective hybridization method has high concentration efficiency and is

Table 3. Allelic distribution of the 17 simple sequence repeat loci in 23 chinese chestnut accessions, 42 japanese chestnut accessions, and one european chestnut accession.^z

Locus	<i>C. mollissima</i> (23)	<i>C. crenata</i> (42)	<i>C. sativa</i> (1)
ICMA003	152, 169, 173, 177, 179, 181, 183	161, 171, 173, 175	152
ICMA004	194, 230, 232, 234, 236, 240, 244, 250, 252,	210, 212, 220, 224, 228, 232, 236, 240, 242	227, 229
ICMA005	142, 145, 147, 149, 151, 154, 160, 162, 164, 166, 168, 170, 172	136, 138, 140, 142, 149, 154, 160, 164	null
ICMA007	201, 203, 206, 208, 212, 274, 280, 282	199, 208, 210, 212, 214, 220, 222	206, 208
ICMA008a	122, 126, 128, 130, 132, 134, 136, 138, 140	118, 124, 126, 128, 130, 131, 132, 145	147
ICMA009a	177, 185, 187, 194, 196	186, 190, 194, 196, 198, 200, 202, 204	192, 196
ICMA010	211, 213, 214, 220, 223, 228, 230, 232, 236, 238, 240	200, 202, 204, 208, 210	207
ICMA011	221, 269, 271, 281, 283, 291, 294, 297, 298, 299, 303, 312	279, 281, 285, 287, 289, 291, 293, 295, 297, 299, 303, 305, 308, 310, 311, 318, 320, 324, 366, 377, 379	285, 293
ICMA013	136, 138, 144, 147	133, 136, 140, 142, 144, 146, 148	133, 138
ICMA014	null, 240	228, 234, 236, 242, 244, 245, 247, 249, 257	null
ICMA015	151, 153, 155, 161, 163, 165, 169	155, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 191, 195, 197, 199, 201, 203	149, 157
ICMA016	178, 180, 184, 188, 190, 192	168, 174, 176, 182	176
ICMA018	155, 182, 184, 188, 190, 194, 196, 200, 203, 205, 207	172, 182, 184, 186, 190, 198	186, 205
ICMA019	181, 189, 197, 201, 203, 205, 207	177, 189, 191	185, 191
ICMA020	186, 190, 192, 194, 198, 200, 202, 204, 206, 209	176, 177, 190, 192, 194	198, 218
ICMA022	179, 182, 185	179, 182	185
ICMA023	214, 216, 218, 220, 222	216, 218, 220, 222	218, 228

^zThe underlines have been used to indicate the alleles common among *Castanea mollissima*, *C. crenata*, and *C. sativa*.

Table 4. Cross-species transferability analysis of the microsatellite markers in Fagaceae plants demonstrates whether the primer pairs amplified a product (+), failed to amplify a product (-), or produced multiple products (MP).^z

Genus	Species	ICMA 003	ICMA 004	ICMA 005	ICMA 007	ICMA 008a	ICMA 009a	ICMA 010	ICMA 011	ICMA 013	ICMA 014	ICMA 015	ICMA 016	ICMA 018	ICMA 019	ICMA 020	ICMA 022	ICMA 023
<i>Quercus</i>	<i>mongolica</i>	+	-	MP ^y	+	-	+	+	+	-	-	-	+	+	-	MP	+	-
	<i>serrata</i>	+	-	MP	+	-	+	+	+	+	-	-	+	+	-	MP	+	-
	<i>dentata</i>	+	-	+	+	-	+	+	+	+	-	-	+	+	-	MP	+	-
	<i>acutissima</i>	+	-	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+
	<i>palustris</i>	+	-	+	+	-	+	+	+	+	-	-	+	+	-	+	+	+
	<i>robur</i>	+	-	MP	+	+	+	+	+	+	-	-	+	+	-	MP	+	-
	<i>rubra</i>	+	-	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+
<i>Castanopsis</i>	<i>acuta</i>	+	-	MP	-	+	+	+	+	+	-	-	+	+	-	MP	+	+
	<i>myrsinaefolia</i>	+	-	+	-	+	+	+	+	+	-	-	+	+	-	MP	+	+
	<i>salicina</i>	+	-	+	-	+	+	+	+	+	-	-	+	+	-	MP	+	+
	<i>glauca</i>	+	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+
	<i>cuspidata</i>	+	-	+	+	+	+	+	+	+	-	-	+	+	-	MP	+	+
<i>Pasania</i>	<i>cuspidata</i> var. <i>sieboldii</i>	+	-	+	+	+	+	+	+	+	-	-	+	+	-	MP	+	+
	<i>cuspidata</i> var. <i>sieboldii</i>	+	-	+	+	+	+	+	+	+	-	-	+	+	-	MP	+	+
	<i>edulis</i>	+	+	MP	+	+	+	+	+	+	+	+	+	+	-	MP	+	+
<i>Fagus</i>	<i>crenata</i>	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
	<i>japonica</i>	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+

^zLeaf samples of the 17 Fagaceae plants, including four genera and 15 species, were obtained from the plants collected from the College of Agriculture, Ibaraki University, Ibaraki, Japan.

^yMP = multiple products.
^z+ = one or two clear bands.
^w- = no visible bands.

'Riheiguri' is "244/244" or "244/n".

Fig. 1. Confirmation of the existence of a null allele at the ICMA014 locus by studying the interspecific cultivar Riheiguri and its offspring. 'Riheiguri' was obtained by a cross between Japanese chestnut and Chinese chestnut. The genotype of the ICMA014 locus in this cultivar is "244/244" or "244/n." Then, an offspring cultivar, Gakuou, was obtained from the cross between the Japanese chestnut 'Toyotamawase' (genotype "242/242") and 'Riheiguri'. If 'Riheiguri' had a null allele inherited from the Chinese parent, its genotype would certainly be "244/n." Consequently, the expected genotype of 'Gakuou' would be "242/244" or "242/n." Because 'Gakuou' shows only the allele 242, we concluded that the genotype is "242/n" with a null allele.

inexpensive, it is a suitable technique for developing microsatellite libraries.

In this study, 39 of the 57 microsatellite regions consisted of GA/CT sequences (68.4%). Thus, our results indicated a high frequency of GA/CT microsatellites in chestnut genomes. High frequency of GA/CT microsatellites has also been reported in other plant species (Zane et al., 2002).

Except for three pairs of cultivars, all the other accessions used in this study were differentiated by using the allelic information obtained from the 17 microsatellite loci. We concluded that each pair has synonym relationships. Microsatellite analysis has also been shown to be useful in identifying the synonym relationships in European chestnut cultivars (Gobbin et al., 2007).

The ICMA014 locus exhibited a null allele in almost all Chinese chestnut accessions, except in 'Qianchi', which was used to construct the genomic library. Because no null alleles were observed in the ICMA014 locus in the other species, the null allele may have developed from a point mutation that occurred after the differentiation of Chinese chestnut. Loci with null alleles are very useful for identifying Chinese chestnut and for parentage analysis of interspecific hybrid cultivars. Furthermore, these loci are also useful for tracing 'Qianchi'-related cultivars by conventional breeding.

The success of cross-species amplification in Fagaceae suggested that the newly developed 17 primers functioned well in analyses on Japanese and European chestnut and on interspecific hybrids of Chinese and Japanese chestnut. The UPGMA cluster analysis clearly separated the genotypes into three large groups that reflected the conventional classification of *Castanea*. Furthermore, these primers were also efficacious in analysis of some beech species. Intrageneric cross-species amplifications within the genus have been reported using microsatellite primers from Japanese chestnut (Yamamoto et al., 2003) and European chestnut (Casasoli et al., 2006). Because these primers have high transferability, they are useful tools for genetic analysis in both the genus *Castanea* and other members of Fagaceae.

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Fig. 2. Dendrogram generated from the unweighted pair group method with arithmetic mean cluster analysis using PHYLIP3.6 programs (Felsenstein, 2005); the dendrogram is based on the genetic distances of the 17 microsatellite loci in the chestnut genome, including those in 23 chinese chestnut accessions, 42 japanese chestnut accessions, one european chestnut accession, and five interspecific hybrids. The tree was developed using the TreeView software (Page, 1996). Almost all the accessions used in this study were separated in the dendrogram, except for three pairs of cultivars of japanese chestnut. A total of 66 genotypes without five interspecific hybrids were clearly separated into three large groups that reflected the three distinct species: *Castanea mollissima*, *C. crenata*, and *C. sativa*.

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