Microsatellite Loci Transferability in Chestnut

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ABSTRACT. Cross-species amplification of 55 microsatellite loci developed in european chestnut (*Castanea sativa* Mill.) and Japanese chestnut (*C. crenata* Sieb & Zucc.) was tested in three chestnut species from China [*C. mollissima* Blume, *C. seguinii* Dode, and *C. henryi* (Skan.) Rehder & Wilson]. Among all the tested loci, 47 (85.5%), 47 (85.5%), and 44 (80%) were successfully amplified in each of the three Chinese species, respectively. All microsatellite loci tested from *C. crenata* successfully amplified in the Chinese species, while only 80.5%, 80.5%, and 73.2% of the loci originating from *C. sativa* amplified in the three Chinese species. The level of polymorphism and mean number of alleles was 58.2% and 4.12 for *C. mollissima*, 60% and 4.64 for *C. seguinii*, and 60% and 4.76 for *C. henryi*, with mean observed heterozygosity ranging from 0.440 to 0.549 and mean expected heterozygosity ranging from 0.506 to 0.615. Transferability of *Castanea* Mill. microsatellites provides a powerful tool for chestnut breeding programs and conservation genetic studies of *Castanea* species.

The genus *Castanea* is comprised of seven economically and ecologically important timber tree taxa that are widely distributed in the temperate forests of the Northern Hemisphere (Johnson, 1988). The center of origin of *Castanea* is located in China where three endemic chestnuts occur: *C. mollissima*, *C. seguinii*, and *C. henryi* (Lang, 2004; Lang and Huang, 1999). *C. mollissima*, *C. crenata*, and *C. sativa* are economically important for chestnut nut production. *C. mollissima* is valued for its resistance to chestnut blight fungus [*Cryphonectria parasitica* (Murrill) Barr] and has been used as a source of blight resistance genes in chestnut breeding (Rutter et al., 1990). A closely related species, *C. seguinii*, has little value for nut production due to its small nut size, but it is highly valuable for chestnut breeding because of its dwarf stature, precocity, and heavy yielding of nuts that provide a food source for wildlife (Jaynes, 1979). *Castanea henryi* is a keystone forest plant and a good timber tree. Many Chinese chestnut cultivars and landraces have been cultivated for centuries in China. However, development of a modern chestnut industry is hampered in China because of cultivar mixing and lack of international standards for chestnut marketing. Therefore, there is an urgent need for cultivar identification and fingerprinting of traditional cultivars and landraces using molecular markers. Moreover, due to overharvesting and deforestation, natural resources and wild populations of Chinese native chestnut species have been seriously damaged in recent decades. Thus, for the formulation of a long-term conservation strategy, the inventory of natural genetic diversity of Chinese *Castanea* populations is critical, and a set of molecular markers is also required.

Microsatellites or simple sequence repeats (SSRs) are DNA segments containing short sequence repeats (1–6 nucleotides) and have been widely recognized as powerful and informative markers due to their abundance, codominance, high polymorphism, and ease of use with the polymerase chain reaction (PCR) (Kuleung et al., 2004; Peakall et al., 1998). In plants, SSRs are useful for cultivar identification, genetic linkage mapping, and marker-assisted selection in breeding and population genetic studies (Barreneche et al., 1998; Butcher et al., 2000; Steinkellner et al., 1997). Microsatellites have been developed in *Castanea* species, for example, in *C. crenata* and *C. sativa* (Buck et al., 2003; Marinoni et al., 2003; Yamamoto et al., 2003), and also have recently been applied in the genetic studies of *C. crenata* and American chestnut [*C. dentata* (Marsh.) Borkh] (Kubisiak and Roberds, 2006; Tanaka et al., 2005). However, few studies have been reported on Chinese *Castanea* species using microsatellite markers due to the lack of efforts in development of microsatellite markers for Chinese chestnuts. Nevertheless, the lack of microsatellite markers directly developed from Chinese chestnuts could be largely solved by considering the great conservation of SSR flanking regions in closely related species (Bushar et al., 2001; Schnell et al., 2005; Steinkellner et al., 1997). Thus, screening primers from different sources can offer a potential chance for the development of SSR markers for species without prior sequence information (reviewed by Barbará et al., 2007). In the present study, we analyzed the transferability of 55 microsatellite loci originally developed from *C. sativa* and *C. crenata* in three Chinese endemic *Castanea* species (*C. mollissima*, *C. seguinii*, and *C. henryi*), and tested this set of SSR loci for robust use in a genetic study of Chinese *Castanea* species.

Materials and Methods

PLANT MATERIALS AND DNA EXTRACTION. To test the transferability of microsatellite loci, 30 individuals of each of the three Chinese species were randomly sampled as a test population

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from Jishou city of Hunan province (*C. mollissima*), Pingba county of Guizhou province (*C. seguinii*) and Zigui county of Hubei province (*C. henryi*). Young leaves were collected and stored with silica gel until DNA extraction. About 0.25 g of dry leaf tissues were ground into powder in Eppendorf tube containing 5 × 5-mm magnetic bead, using a FastPrep FP 2000 mill (Thermo Electron, Waltham, MA). DNA was extracted following a modified protocol from Doyle and Doyle (1987) with some modifications: the extraction buffer contained 10% polyvinylpyrrolidone and 2% Na₂S₂O₅, and the lysis buffer contained 3% cetyltrimethyl ammonium bromide and 2% Na₂S₂O₅. DNA was dissolved in 200 µL of TE solution (10 mM Tris-HCl buffer solution, pH 8.3, and 1 mM EDTA, pH 8.0); 20 µg of RNase was added to the solution, which was then incubated at 37 °C for 1 h. DNA concentration was quantified by spectrophotometry (GENEQUANT; Eppendorf, Hamburg, Germany).

PCR AMPLIFICATION AND DATA ANALYSIS. Fifty-five pairs of SSR primers were tested (Table 1), 14 pairs from *C. crenata* (Yamamoto et al., 2003) and 41 from *C. sativa* (eight were reported by Buck et al., 2003 and 33 by Marinoni et al., 2003). For transferability analyses, each locus was first amplified using four individuals of each species, randomly chosen from the 30 individuals sampled. PCR reactions were performed in a final volume of 10 µL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.5 µM each primer, 0.2 mM dNTPs, 25 ng of genomic DNA, and 0.25 U of *Taq* polymerase (Shenergy Biocolors, Shanghai, China). The PCR amplification was conducted in a Mastercycler gradient thermocycler (Eppendorf) using the following thermal profile: 5 min of denaturation at 95 °C, 35 cycles of 1 min at 94 °C, 45 s at the annealing temperature of 45 °C to 67 °C depending on the different primers and different species (Buck et al., 2003; Marinoni et al., 2003; Yamamoto et al., 2003), 1 min and 30 s at 72 °C, with a final extension at 72 °C for 7 min. The amplification products combined with three-quarters volume of formamide loading buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.25% bromophenol blue, and 0.25% xylene cyanol) were denatured at 94 °C for 5 min and were electrophoresed in 6% denaturing polyacrylamide gels using Sequi-Gen GT System (Bio-Rad, Hercules, CA). Electrophoresis was carried out with 1× Tris-borate-EDTA buffer for 1.5 h at 55 W. Silver staining was conducted according to the protocol of Sanguinetti et al. (1994).

For each microsatellite locus, sizes of the alleles were estimated by comparison with standard size DNA markers (25-bp DNA ladder; Promega, Madison, WI) and were scored across all of the samples manually. Control samples were used to ensure that the same alleles were obtained whatever the method used. For those primers that produced clear bands and reproducible amplification, 30 individuals of each species were genotyped. GENEPOP software (version 3.4; Raymond and Rousset, 1995) was used to perform exact tests for the deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) within populations using the Markov chain method (Guo and Thompson, 1992). The number of alleles per locus (N_a), observed heterozygosity (H_O), and expected heterozygosity (H_E) (Nei, 1978) were estimated using software GenAlEx, version 6.0 (Peakall and Smouse, 2006). Total exclusion power of the first [$Pr(Ex_1)$] and second parent [$Pr(Ex_2)$] were estimated using CERVUS, version 2.0 (Marshall et al., 1998).

Results and Discussion

Of the 55 microsatellite loci assayed, 90.9% (including 14 loci from *C. crenata* and 36 from *C. sativa*) were amplified in at least one of the species tested, and 74.5% (14 from *C. crenata* and 27 from *C. sativa*) could be amplified in all three Chinese species (Table 1). Figure 1 shows transferability of two microsatellite loci, one locus from *C. sativa* (Fig. 1A), the other from *C. crenata* (Fig. 1B), successfully amplified in the Chinese species. A total of 85.5% loci (14 loci from *C. crenata* and 33 from *C. sativa*) can be cross-species amplified in *C. mollissima* and *C. seguinii* and 80% (14 from *C. crenata* and 30 from *C. sativa*) for *C. henryi* (Table 2). Among the successfully amplified loci, 32 (58.2%), 33 (60%), and 33 (60%) loci were found to be polymorphic in *C. mollissima*, *C. seguinii*, and *C. henryi*, and the other 15 (27.3%), 14 (25.5%), and 11 (20%) were monomorphic, respectively (Table 1). A high percentage of *C. crenata* markers revealed polymorphism in *C. mollissima* (13 of 14 loci, 92.9%), *C. seguinii*, and *C. henryi* (14 of 14 loci, 100%), which is in good agreement with the results of Yamamoto et al. (2003), who claimed that all the SSR loci developed in the *C. crenata* could be applicable to *C. mollissima* and *C. seguinii*. However, they found two loci (KT006a and KT015a) that failed to amplify in *C. henryi*, which is more distant from the other species. Also, our results show a higher number of alleles at some loci and a larger size range than those reported by Yamamoto et al. (2003). For example, KT002a showed larger allele size range (243–252 bp) in *C. seguinii* than that in their study (243–249 bp). This difference may be the result of a larger sample size assayed in this study. In contrast, only 19 loci (46.3%) developed from *C. sativa* showed polymorphism in the three Chinese species. This is in agreement with results that *C. sativa* and *C. dentata* are more closely related to each other than to the Asian species (Lang et al., 2006, 2007). The increase of evolutionary distance might result in a decline of cross-species amplification success rate. In addition, China and Japan are geographically close, thus interspecific introgression might have been taken place between Chinese *Castanea* species and *C. crenata*. This also could have led to a higher transferability between Japanese and Chinese chestnuts.

Among the polymorphic loci, a total of 132, 153, and 157 alleles were detected from each of the three random populations containing 30 individuals of *C. mollissima*, *C. seguinii*, and *C. henryi*, with the average value of 4.12 (from 2 to 7), 4.64 (from 2 to 8), and 4.76 (from 2 to 13) alleles per locus (Table 1). A high level of genetic diversity was observed in the three Chinese chestnut populations, with the mean observed heterozygosity (H_O) of 0.440 (from 0.065 to 1.000) in *C. mollissima*, 0.549 (from 0.100 to 0.950) in *C. seguinii*, and 0.508 (from 0.065 to 1.000) in *C. henryi*, and the average expected heterozygosity (H_E) was 0.506 (from 0.062 to 0.778) in *C. mollissima*, 0.615 (from 0.375 to 0.815) in *C. seguinii*, and 0.605 (from 0.063 to 0.882) in *C. henryi*, respectively (Table 1). A high level of heterozygosity is commonly observed in *Castanea* species (e.g., *C. dentata*: H_O = 0.755, H_E = 0.818, Kubisiak and Roberds, 2006; *C. crenata*: H_O = 0.727, H_E = 0.780, Tanaka et al., 2005), which can be explained by the outbreeding nature of species in the genus.

The combined values (overall loci) for the first parent total exclusionary power [$Pr(Ex_1)$] were estimated to be 0.9894, 0.9989, and 0.9905 for *C. mollissima*, *C. seguinii*, and *C.*

Table 1. Characterization of 55 tested *Castanea* primer pairs and cross-species transferability in three *Castanea* species from China.

Locus ^c	Repeat motif	Sequence 5'-3'	S ₁ (bp) ^y	Species	N _a ^x	S ₂ (bp) ^w	H _O ^v	H _E ^u	P ^t	Pr (Ex ₁) ^s	Pr (Ex ₂) ^r
KT001b	(AG) _{8.5}	F: TATTGGTTCTTCCTTGCTG	194	<i>C. mollissima</i>	5	191–211	0.464	0.582	0.718	0.138	0.275
		R: CACGAAACTTGTGGTAAAA		<i>C. seguinii</i>	5	191–211	0.417	0.740	0.001	0.328	0.503
KT002a	(GA) ₁₀	F: TACACATGAAAAGAGGAAGGG	236	<i>C. henryi</i>	4	191–207	0.105	0.518	0.000	0.131	0.268
		R: CAAAAGGATGAAAACAATGAGA		<i>C. mollissima</i>	4	236–250	0.893	0.692	0.000	0.143	0.293
KT004a	(AG) _{12.5}	F: CGTACCCCTTGTGATTATGTTT	109	<i>C. seguinii</i>	4	243–252	0.917	0.674	0.000	0.242	0.401
		R: CGAGAAAGGAGTGGATTACG		<i>C. henryi</i>	7	243–261	0.895	0.802	0.000	0.391	0.569
KT005a	(GA) ₁₈	F: GTAGAGAAAGAGTCGTGCATGAG	195	<i>C. mollissima</i>	6	112–127	0.679	0.778	0.527	0.272	0.442
		R: ATAGTTATTCGATTGTTTGGTCT		<i>C. seguinii</i>	8	110–139	0.875	0.815	0.593	0.467	0.642
KT006a	(GA) ₉	F: GCATAAAAGGCTCCAATCAA	131	<i>C. henryi</i>	4	110–120	1.000	0.565	0.000	0.153	0.252
		R: TATGGGCGGAGCACAA		<i>C. mollissima</i>	5	175–391	0.100	0.546	0.000	0.117	0.216
KT007b	(AG) _{7.5}	F: TAGCCTCTTGGACTGGTATG	193	<i>C. seguinii</i>	7	170–391	0.667	0.684	0.174	0.082	0.209
		R: TTGGTGTGAAACTTAGCCT		<i>C. henryi</i>	5	175–394	0.455	0.554	0.377	0.042	0.124
KT008a	(AG) ₅ A(AG) _{7.5}	F: AATTTGTTTGGTGTGCTCA	200	<i>C. mollissima</i>	4	125–138	0.125	0.293	0.000	0.044	0.154
		R: CTAGGGGCAAATACTTAGAGA		<i>C. seguinii</i>	5	125–141	0.625	0.715	0.012	0.307	0.486
KT009a	(AG) ₁₀	F: AATTCGACAAACATTTTGGAA	54	<i>C. henryi</i>	4	125–138	0.333	0.467	0.534	0.105	0.221
		R: TAACAGATTCTTTTCTCTC		<i>C. mollissima</i>	2	187–225	0.100	0.095	0.608	0.005	0.045
KT010a	(GA) ₁₀	F: TAATGTGAAAGAGAGAGGGG	84	<i>C. seguinii</i>	3	192–201	0.250	0.531	0.244	0.141	0.277
		R: ACCGGCCACAAAGAATCAAG		<i>C. henryi</i>	3	192–201	0.263	0.653	0.004	0.202	0.348
KT014a	(GA) ₇	F: AAAAGAGGAAGAAGGTGGGA	148	<i>C. seguinii</i>	5	190–220	0.231	0.586	0.001	0.114	0.254
		R: CATGCACACGCACATCATAG		<i>C. henryi</i>	3	198–212	0.550	0.509	0.833	0.129	0.214
KT015a	(GA) ₈ (GT) ₉	F: TTCTTGTAGTGGCATGATCT	168	<i>C. mollissima</i>	6	190–214	0.600	0.666	0.102	0.244	0.400
		R: GAAAAACCTAAATCAACCC		<i>C. seguinii</i>	7	160–210	0.409	0.607	0.007	0.218	0.397
KT019a	(GA) _{8.5}	F: AGAATTGAAGTCCGTGCTG	120	<i>C. seguinii</i>	5	160–190	0.444	0.547	0.201	0.121	0.186
		R: TTCGGTGGGATAGAGTAGA		<i>C. henryi</i>	7	160–210	0.435	0.863	0.000	0.002	0.030
KT020a	(GA) ₁₀	F: GGTTTCTCAAAGCGCAGGC	88	<i>C. mollissima</i>	4	80–86	0.667	0.609	0.682	0.201	0.364
		R: TGTGAGGTGATTGGTAGGCA		<i>C. seguinii</i>	4	80–86	0.667	0.653	0.631	0.234	0.400
KT024a	(GA) _{12.5}	F: CACCCACTACCAGTAGTTGT	97	<i>C. henryi</i>	4	80–86	0.128	0.258	0.000	0.033	0.137
		R: AACATCAACCCAACTAATCTT		<i>C. mollissima</i>	3	148–152	0.893	0.612	0.000	0.404	0.582
EMCs2	(CGG) ₇	F: GCTGATATGGCAATGCTTTTCCTC	172–178	<i>C. seguinii</i>	2	148–150	0.950	0.499	0.000	0.124	0.187
		R: GCCCTCCAGCCTCACCTTCATCAG		<i>C. henryi</i>	4	146–152	1.000	0.659	0.000	0.224	0.378
				<i>C. mollissima</i>	7	163–210	0.409	0.607	0.000	0.002	0.033
				<i>C. seguinii</i>	5	160–190	0.444	0.547	0.808	0.050	0.164
				<i>C. henryi</i>	13	160–225	0.435	0.882	0.006	0.573	0.729
				<i>C. seguinii</i>	1	125	0.000	0.000	—	—	—
				<i>C. mollissima</i>	3	125–132	0.176	0.590	0.002	0.087	0.226
				<i>C. seguinii</i>	6	125–160	0.500	0.576	0.000	0.108	0.227
				<i>C. henryi</i>	6	90–100	0.500	0.695	0.000	0.245	0.404
				<i>C. mollissima</i>	4	83–97	0.600	0.380	0.103	0.183	0.346
				<i>C. seguinii</i>	5	85–100	0.176	0.667	0.000	0.239	0.394
				<i>C. henryi</i>	4	89–104	0.097	0.181	0.000	0.017	0.095
				<i>C. mollissima</i>	6	89–125	0.500	0.798	0.000	0.402	0.582
				<i>C. seguinii</i>	4	95–104	0.944	0.567	0.023	0.154	0.255
				<i>C. henryi</i>	1	125	0.000	0.000	—	—	—
				<i>C. mollissima</i>	3	157–167	0.784	0.655	0.199	0.214	0.361
				<i>C. seguinii</i>	3	160–167	0.579	0.579	0.018	0.159	0.294
				<i>C. henryi</i>							

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Table 1. Continued.

Locus ^c	Repeat motif	Sequence 5'-3'	S ₁ (bp) ^y	Species	N _a ^x	S ₂ (bp) ^w	H _o ^v	H _E ^u	P ^s	Pr (Ex ₁) ^s	Pr (Ex ₂) ^r
EMCs4	(GGC) ₇	F: CGCCGAACTCACCGACCTC R: GCCAAAAACGACACCCAAATCC	170-179	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	2 2 2	170-175 170-175 170-175	0.214 0.100 0.273	0.459 0.375 0.645	0.004 0.001 0.002	0.100 0.051 0.202	0.200 0.134 0.361
EMCs10	(CA) ₈	F: GTCTCCCCAATCATAAGTAGGTC R: TCAAGGGAACATTAGGTCATTTT	218-230	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	2 1 1	122-125 125 125	0.115 0.000 0.000	0.440 0.000 0.000	0.000 — —	0.097 — —	0.172 — —
EMCs11	(GGA) ₇ (GGC) ₆	F: ATCCACTCCACCAACAACAACC R: GGAGGGAGATGGGACGACG	96-111	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	3 4 4	125-128 125-136 125-136	0.375 0.400 0.158	0.320 0.640 0.609	0.934 0.521 0.000	0.005 0.216 0.188	0.047 0.373 0.348
EMCs13	(GCA) ₈	F: TAGTCGGAGTACGGGCACAG R: TGATATGAGCATTTGACTTTGATT	158-164	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA ^q 1 1	— ^p 155 155	— 0.000 0.000	— 0.000 0.000	— — —	— — —	— — —
EMCs14	(GAG) ₇	F: GTGCTTCAGGGACCTTTCTTCTC R: GCCGCCGCTCTCTGCTGCTC	144-153	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 NA NA	137 — —	0.000 — —	0.000 — —	— — —	— — —	— — —
EMCs15	(CAC) ₉	F: CTCTTAGACTCCTTCGCCAATC R: CAGAATCAAAGAAGAGAAAGGTC	83-95	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 3	83 83 83-95	0.000 0.000 0.286	0.000 0.000 0.659	— — 1.000	— — 0.187	— — 0.320
EMCs17	(AGC) ₄ (CCA) ₅	F: CGCCACGATTAGCTCATTTTCA R: GAGGTAGGGTCTTCTTCGGTCATC	210-222	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 2 1	217 210-220 210	0.000 0.348 0.000	0.000 0.423 0.000	— 0.014 —	— 0.090 —	— 0.167 —
CsCAT1	(TG) ₅ TA(TG) ₂₄	F: GAGAAATGCCACTTTTGCA R: GCTCCCTTATGCTCTCG	220	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 6 4	196-203 190-203 190-203	0.222 0.375 0.667	0.403 0.450 0.716	0.173 0.860 0.000	0.085 0.113 0.289	0.227 0.273 0.459
CsCAT2	(AG) ₁₆	F: GTAACCTGAAGCAGTGTGAAC R: CGCATCATAGTAGTGACAG	206	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA 4 4	— 186-215 186-217	— 0.909 0.600	— 0.551 0.652	— 0.018 0.000	— 0.154 0.222	— 0.265 0.381
CsCAT3	(AG) ₂₀	F: CACTATTTTATCATGGACGG R: CGAATTGAGAGTTCATACTC	224	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	6 4 5	197-215 197-210 197-215	0.375 0.583 0.611	0.590 0.707 0.576	0.456 0.521 0.526	0.200 0.278 0.186	0.375 0.445 0.354
CsCAT4	(CA) ₂₃	F: CATAGGTCAAACCATACCCGTG R: CTCATCTTTGTAGGGTATAATACC	243	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 7 7	225-257 205-269 200-240	0.808 0.900 0.500	0.612 0.720 0.679	0.285 0.666 0.868	0.201 0.321 0.256	0.355 0.498 0.426
CsCAT5	(GA) ₂₀	F: CATTTTCTCATTTGGCTGC R: CACTTGCACATCCAATTAGG	242	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	5 7 5	225-250 215-242 225-250	0.750 0.607 0.579	0.750 0.753 0.718	0.738 0.002 0.261	0.303 0.276 0.294	0.477 0.454 0.475
CsCAT6	(AC) ₂₄ AT(AC) ₄	F: AGTGCTCGTGTGTCAGTGAG R: CAACTCTGCATGATAAC	180	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA 4 NA	— 152-160 —	— 0.839 —	— 0.613 —	— 0.560 —	— 0.193 —	— 0.326 —
CsCAT7	(TG) ₈ CG(TG) ₁₄	F: GAACATGATGATTGGCCTC R: CCAAACATGACATATGTCCC	186	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 6 6	192-217 190-217 190-217	0.250 0.538 0.789	0.609 0.761 0.822	0.850 0.012 0.648	0.285 0.333 0.423	0.455 0.510 0.601
CsCAT8	(GT) ₇ (GA) ₂₀	F: CTGCAAGACAAGAATTACAC R: GAATAACCTGCAGAAAGGC	210	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	5 6 5	195-218 195-220 187-204	0.370 0.333 0.579	0.377 0.393 0.557	0.725 0.017 0.001	0.074 0.085 0.160	0.210 0.234 0.279

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Table 1. Continued.

Locus ^c	Repeat motif	Sequence 5'-3'	S ₁ (bp) ^y	Species	N _a ^x	S ₂ (bp) ^w	H ₀ ^v	H _E ^u	P ^t	Pr (Ex ₁) ^s	Pr (Ex ₂) ^r
CsCAT12	(TG) ₁₉	F: GGGTTGTATTAGGTATATCATGCC R: CAGGATTTCCACGGGAAAATCCG	304	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	275 275 275	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —
CsCAT14	(CA) ₂₂	F: CGAGGTGTGTTGTCATCATTTAC R: GATCTCAAGTCAAAAAGGTGTC	164	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 7 5	136–154 136–166 140–157	0.474 0.543 0.500	0.514 0.573 0.698	0.185 0.382 0.001	0.072 0.123 0.286	0.193 0.274 0.464
CsCAT15	(TC) ₁₂	F: TTCTGCGACCTCGAAACCGA R: GCTAGGGTTTTCATTTCTAG	136	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 5 3	125–140 127–142 125–140	0.375 0.355 0.462	0.555 0.790 0.489	0.452 0.001 0.802	0.072 0.222 0.111	0.193 0.396 0.246
CsCAT16	(TC) ₂₀	F: CTCCTTGACTTTTGAAGTTGC R: CTGATCGAGAGTAATAAG	143	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	5 NA 7	132–160 — 127–168	0.167 — 0.520	0.556 — 0.736	0.006 — 0.003	0.169 — 0.331	0.330 — 0.515
CsCAT17	(CA) ₁₀ A(CA) ₂ AA(CA) ₃	F: TTGGCTATACTTGTCTGCAAG R: GCCCCATGTTTCTTCCATGG	147	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 NA	140 142 —	0.000 0.000 —	0.000 0.000 —	— — —	— — —	— — —
CsCAT18	(CA) ₁₂ CG(CA) ₁₀	F: GCTTGATTGCACTGATAACC R: CACATGAGCGGTGCTCAGAAAG	108	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	2 NA NA	144–147 — —	0.250 — —	0.500 — —	0.186 — —	0.110 — —	0.179 — —
CsCAT19	(TG) ₁₀ CG(TG) ₁ TT(TG) ₄	F: GTTAATGTATACCCACTAAACCGTC R: GCTACTCAACCAATGATCCAAAC	180	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA NA NA	— — —	— — —	— — —	— — —	— — —	— — —
CsCAT20	(AC) ₄ (AT) ₃ A (AT) ₃	F: GAATTAAAGCAGTCCTCAATG R: CAAGTCTCACCTTCAGTTAG	143	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	143 143 143	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —
CsCAT21	(TG) ₁₀ TA(TG) ₁₂	F: GCCATACCTTTAGGACATTGAC R: TCCTAAACATGAGAGTCCATC	174	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 NA NA	157 — —	0.000 — —	0.000 — —	— — —	— — —	— — —
CsCAT22	(AC) ₂₁	F: GCTGTCTGATCTAGGATCAATC R: CTAGCTTTAATATGAGCAAGGATGG	240	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	3 3 3	225–245 215–245 215–230	0.875 0.500 0.667	0.656 0.531 0.667	0.313 0.081 0.538	0.215 0.141 0.187	0.362 0.277 0.326
CsCAT23	(CA) ₂ CT(CA) ₄ CT(CA) ₁ CT(CA) ₃	F: GCATGCTACTCAACCAAGATCC R: GATTGGGCCATCACAAAAGTTTAC	207	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	197 197 197	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —
CsCAT24	(AG) ₁₇	F: AAAACGAAAGATATAAGAGAGAG R: CATTAGGCCAAATAGGCACC	214	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	2 6 5	175–197 188–235 175–220	0.250 0.684 0.750	0.219 0.705 0.718	0.686 0.844 0.721	0.032 0.308 0.284	0.121 0.490 0.462
CsCAT25	(AC) ₄ TT(TA) ₅	F: CTCATGGGACAGGCTTGATTTTG R: GTCTCACCTTCAGTTAGTCTGC	127	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	127 127 127	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —
CsCAT26	(CT) ₂₅	F: GAGACTTGAGATTGCAAGG R: CTCACATTCAGTTAACAC	116	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 6 NA	105–122 100–122 —	0.486 0.571 —	0.641 0.689 —	0.000 0.001 —	0.215 0.275 —	0.378 0.450 —
CsCAT27	(AC) ₄	F: CGAATCTTTTGTGTGATCTAG R: CTCCTCAAAAGGATTAAG	118	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	134 134 134	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —

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Table 1. Continued.

Locus ^z	Repeat motif (TG) ₄ (TG) ₁₇	Sequence 5'-3'	S ₁ (bp) ^y	Species	N _a ^x	S ₂ (bp) ^w	H _O ^v	H _E ^u	P ^t	Pr (Ex ₁) ^s	Pr (Ex ₂) ^r
CsCAT31		F: CCCTTTAAATACTGTGTG R: CTACAGGAACACTCTGAATAG	91	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA NA NA	— — —	— — —	— — —	— — —	— — —	— — —
CsCAT32	(AC) ₄ TC(AC) ₃	F: CAATTTTAAAGCACAGGGATC R: CAATGTGAATTGGCCATCAC	139	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	139 139 139	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —
CsCAT33	(GA) ₅ GG(GA) ₁₁	F: CTCGGAAACCAACATGAATC R: CGTTTTTGCTCTTAGATTCC	153	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA NA NA	— — —	— — —	— — —	— — —	— — —	— — —
CsCAT34	(GT) ₂₃	F: TGAGCAAGGATGGATGATGAG R: GGTGTCATCATGACTGCATC	187	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	3 4 3	155–170 155–170 155–170	0.700 0.567 0.710	0.529 0.709 0.538	0.026 0.000 0.253	0.196 0.278 0.140	0.339 0.447 0.239
CsCAT35	(AC) ₅	F: GAGAGGTGTCAAGAAAGCTG R: GTATGCTCACTAAACTGTCT	239	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	NA NA NA	— — —	— — —	— — —	— — —	— — —	— — —
CsCAT37	(CA) ₁₂	F: CAGTGAGCATGTAGTGTGCG R: CACCTCTCTTCGTAATTTTCATG	192	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	2 3 3	200–207 200–213 200–213	0.594 0.452 0.375	0.500 0.632 0.430	0.285 0.000 0.544	0.125 0.205 0.089	0.187 0.350 0.208
CsCAT38	(CT) ₇	F: CTAGAAATGAAAACCCCTAGC R: CGTGAACCCACGTATCTACC	160	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 1 1	160–170 170 —	0.500 0.000 —	0.680 0.000 —	0.501 — —	0.251 — —	0.411 — —
CsCAT39	(AG) ₃₉	F: TGAGTTCTTAGCTTATCAAAGAGTGG R: ACTAGGAATAGAGGCATACATCAATAGG	146	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	84 84 84	0.000 0.000 0.000	0.000 0.000 0.000	— — —	— — —	— — —
CsCAT40	(GT) ₆ G(GT) ₃	F: CCCTTGATAATTCTGTTCTG R: GTCGCGGCATATTACTACC	198	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	1 1 1	198 198 —	0.000 0.000 —	0.000 0.000 —	— — —	— — —	— — —
CsCAT41	(AG) ₂₀	F: AAGTCAGCAACACCATATGC R: CCCACTGTTTCATGAGTTTCT	224	<i>C. mollissima</i> <i>C. seguinii</i> <i>C. henryi</i>	4 1 1	180–250 200 200	1.000 0.000 0.000	0.633 0.000 0.000	0.238 — —	0.215 — —	0.369 — —

^zLoci KT001b-KT024a were originally developed for *Castanea crenata* (Yamamoto et al., 2003), and EMCs2-EMCs17 and CsCAT1-CsCAT41 for *C. sativa* (Buck et al., 2003; Marinoni et al., 2003).

^yPredicted product size (for KT001b-KT024a and CsCAT1-CsCAT41) or size range alleles (for EMCs2-EMCs17) of loci in originated species.

^wNumber of alleles per locus found in each of Chinese *Castanea* species.

^vSize range of alleles in each of Chinese *Castanea* species.

^uObserved heterozygosity (Nei, 1978).

^tExpected heterozygosity (Nei, 1978).

^sProbability of exact tests for deviation from Hardy–Weinberg equilibrium (Guo and Thompson, 1992).

^rThe first estimate of paternity exclusion probability.

^tThe second estimate of paternity exclusion probability.

^uNo amplification in Chinese chestnuts.

^vInformation not available.

Fig. 1. Transferability of two microsatellite loci in three Chinese *Castanea* species: (A) CsCAT8 from *C. sativa* and (B) KT001b from *C. crenata*.

henryi, respectively, whereas if the genotype of one parent is known, the combined values for the total exclusionary power for the second parent [$Pr(Ex_2)$] were 0.9999, 0.9999, and 0.9998 for three chestnuts, indicating that these microsatellite loci are suitable for paternity analysis. High polymorphism and the codominant nature of microsatellite alleles in most cases permits the exclusion of all potential pollen donors only with the use of small number of loci (Dow and Ashley, 1996; Kameyama et al., 2000; Streiff et al., 1999; Wang et al., 2008). Gene flow analysis using microsatellites polymorphism has been conducted on many wind-pollinated plants (e.g., Nakamishi et al., 2005; Streiff et al., 1999). The high resolution of this set of microsatellites tested in our study should provide a suitable tool for parentage analysis in breeding programs and pollen and/or seed-mediated gene flow studies for this monoecious and wind-pollinated genus.

Of the 55 loci analyzed, 14, 16, and 19 loci deviated from HWE in *C. mollissima*, *C. seguinii*, and *C. henryi*, respectively, after a Bonferroni correction for multiple tests ($P < 0.05$, Table 1). This might be due to the limitation of sample size or the existence of null alleles. In addition, significant LD were discovered in four pairs of loci (KT001b/KT004a, KT008a/KT010a, KT005a/CsCAT26, and CsCAT3/EMCs4) in *C. mollissima*, eight pairs of loci (KT002a/KT004a, KT008a/KT019a, KT019a/KT024a, KT002a/CsCAT2, KT006a/CsCAT2, KT009a/CsCAT3, KT008a/CsCAT8, KT019a/CsCAT8, and CsCAT3/EMCs11) in *C. seguinii* and two pairs of loci (KT005a/KT007b and KT002a/CsCAT4) in *C. henryi*, respectively ($P < 0.05$ after Bonferroni's correction). This is probably due to the small sample size and should be considered before analyses using these loci. However, a difference of

microsatellite transferability between the three chestnuts was revealed. Several primer pairs were found to be specific in one or two species, such as EMCs14 and CsCAT21 were amplified only in *C. mollissima*, while CsCAT38 was amplified only in *C. mollissima* and *C. seguinii*. Also, some loci showed monomorphism in one species, but polymorphism in the other species. For example, KT019a and EMCs2 identified monomorphic loci in *C. mollissima*, while polymorphic loci were found in *C. seguinii* and *C. henryi* (Table 1). Most alleles are shared, which may be due to the shared ancestral variation and/or the recent gene flow resulting from inter-specific hybridization. The allele number of loci was found to vary

among the species, but species-specific alleles were detected. KT007b showed the 225-bp allele only in *C. mollissima*, CsCAT12 identified the 300-bp allele only in *C. seguinii*, and CsCAT16 showed the 168-bp allele only in *C. henryi*. Although SSR allele specificity needs to be further verified among Chinese chestnut species, these alleles that were found in only one species may provide a potential tool for studying interspecific hybridization and for facilitating the analysis of hybridization among these three closely related species for marker-assisted selection. Artificial interspecific crosses among chestnuts have been used to introgress disease and/or pest resistance traits in chestnut breeding programs (e.g., Clapper, 1954; Diller and Clapper, 1969). Molecular markers such as allozymes also have been applied in chestnut breeding (Huang et al., 1994). Therefore, these highly polymorphic SSR loci would be more favorable to breeding programs in genus *Castanea*.

It was suggested that the successful cross-species transferability of microsatellites in plants is largely restricted to congeners and tends to decrease proportionally to phylogenetic divergence between target species and the "source species" of microsatellites (Barbará et al., 2007; Peakall et al., 1998; Roa et al., 2000; Varshney et al., 2005;). The Chinese chestnut species are considered the most ancestral species in genus *Castanea* (Lang and Huang 1999; Lang et al., 2006), and the high transferability (>80.0%) of microsatellite loci revealed a close relationship among these five species used in the present study. *Castanea henryi* belongs to the section *Hypocastanon* Dode of the genus, but the other two Chinese chestnut species (*C. mollissima* and *C. seguinii*) and the two "source species" of microsatellites (*C. crenata* and *C. sativa*) are included in section *Eucastanon* Dode. *Castanea mollissima* has a closer

Table 2. Transferable microsatellite loci among *Castanea* species.

Source species	Loci tested (no.)	Target species					
		<i>C. mollissima</i>		<i>C. seguinii</i>		<i>C. henryi</i>	
		Amplification	Polymorphism	Amplification	Polymorphism	Amplification	Polymorphism
<i>Castanea crenata</i>	14	14 (100%) ^a	13 (92.9%)	14 (100%)	14 (100%)	14 (100%)	14 (100%)
<i>C. sativa</i>	41	33 (80.5%)	19 (46.3%)	33 (80.5%)	19 (46.3%)	30 (73.2%)	19 (46.3%)
Total	55	47 (85.5%)	32 (58.2%)	47 (85.5%)	33 (60.0%)	44 (80.0%)	33 (60.0%)

^aPercentages of transferable loci.

relationship to *C. seguinii* than *C. henryi* (Lang and Huang, 1999), which is well supported by molecular phylogeny analysis (Lang et al., 2007). The percentage of transferability among species belonging to the same section was high (*C. mollissima* and *C. seguinii*, 85.5%), whereas that belonging to different sections was slightly lower (*C. henryi*, 80.0%). Therefore, the transferability of microsatellite primers in *Castanea* was in good agreement with currently accepted phylogeny of the genus.

This article demonstrates cross-species amplification of a set of microsatellite primers in the genus *Castanea*. The fact that over half of microsatellite primers designed for *C. sativa* and *C. crenata* can be applied in the three Chinese chestnut species suggests that these microsatellites are relatively conserved in the genus. Likewise, these markers can be widely applicable to other members of *Castanea*. Yamamoto et al. (2003) have proved the successful transferability of the microsatellite primers of *C. crenata* to other *Castanea* species (including the three Chinese chestnut species, *C. sativa*, and *C. dentata*). Kubisiak and Roberds (2006) studied the population structure of *C. dentata* using six microsatellite markers from *C. sativa*. Furthermore, Barreneche et al. (2004) tested the transferability of microsatellites of *Castanea* to *Quercus* L., a genus of the Fagaceae. Therefore, these microsatellite loci should provide a powerful tool for chestnut breeding programs and for conservation genetic studies of Chinese endemic *Castanea* species and other species of this genus.

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