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Microsatellite analysis of 20 mulberry varieties preserved in Korea

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Abstract

A total of 20 mulberry varieties preserved in Korea were typed for eight polymorphic microsatellite loci. We obtained 6 to 15 alleles per locus with an average value of 10.6, per-locus observed heterozygosity ranging from 0.35 to 1.00, and per-locus polymorphic information content (PIC) ranging from 0.61 to 0.87, indicating that most loci are highly variable. Phylogenetic analysis using the eight microsatellite loci was sufficiently suitable for classifying 20 mulberry varieties preserved in Korea. A total of 160 variety-specific apomorphic alleles were obtained from eight loci discriminated 20 mulberry varieties. These variety-specific alleles from this analysis are expected to be useful for the discrimination of other mulberry varieties. Furthermore, a substantial number of homozygote loci, represented by 60 among 180 alleles in eight loci were found. These results collectively suggest that these microsatellite locus primers are potentially crucial molecular markers for the eventual classification of mulberry varieties that are preserved as hundreds in Korea.

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Introduction

Conventional plant identification has been based on biochemical characteristics in addition to phenotypic morphological features using traditional Mendel's law, but this method has a low variability and is limited in testing the purity of seeds and classifying seeds. Therefore, the need for the introduction of effective techniques that can be directly applied to variety identification has emerged, and interest in characteristic investigation of DNA markers is increasing.

Since DNA markers reflect not only the genetic characteristics of the variety but also the universal characteristics of the plant, it is easy to quantify the distinction between varieties. The genotyping technologies include various technologies such as restriction fragment length polymorphism (RFLP), random amplified polymorphism DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), and Internal transcribed spacer (ITS) *etc*. Recently, various studies have been published to investigate the genetic diversity of mulberry trees through genetic approaches using RAPD (Venkateswarlu *et al*., 2004), ISSR (Park *et al*., 2020; Venkateswarlu *et al*., 2004; Vijayan, 2005), and ITS (Kwon *et al*., 2018). Microsatellites are one to six base pairs simple sequence repeats (SSR), which are abundantly present both in coding and non-coding sequences of all eukaryotic and some prokaryotic genomes (Tautz and Renz, 1984). Hypervariability of allele, flanking sequence conservation, codominance of inheritance, and the advances in

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Table 1. Information of the 8 microsatellite loci using analysis

Primer name		Primer sequence	Primer length	Repeat motif	Expected Size (bp)	Ta (℃)
19	F	CCCCACTAAAAGCCATCAAG	20	$(CTCA)_6$	265	54
	R	CCTACCTCTTCCGAAACGTG	20			56
28	F	TTG GCG CCC AAT GAG AAT	18	$(TC)_{17}$	244	51
	R	GAGGCTACCCCAATCCTGTT	20			56
B2-3	F	CATTGGTCCTATCACTGACACA	22	$(GA)_{27}$	133	55
	R	CAGGAAGAGATTTTGGCACCT	21			55
B2-26	F	GCCCTACTAACTGCACGGATT	21	$(CT)_{17}$	145	57
	R	CATGCGCAACTTGACCTTTA	20			52
B2-28	F	GCTTCAGAAAGCTAGAGAGAGAAA	24	$(GA)_{17}$	175	56
	R	CATCTCAGAAACCAGCAGCA	20			54
B2-38	F	CACTTCTTTTTCGGGCTCAG	20	$(CT)_{23}$	264	54
	R	GAATACATGCGCGACAAATG	20			52
B2-42	F	CAACAACCATGGGTCAAACA	20	$(CT)_{15}$	152	52
	R	CACATGTGTGCCGCTCTACT	20			56
B2-44	F	ATGAGTCGGTGCTGGCTATT	20	$(GA)_{49}$	226	54
	R	ACAACCAAGACGCCCAAAT	19			52
B2-38	F	TCTTGACTGCTTGTTCTCTTGG	22	$(CT)_{21}$	163	55
	R	TTCATCGATGCCATTTTCAA	20			48
B2-55	F	CGATCAAGGGCTAAATGGAA	20	$(TTC)_{14}$	234	52
	R	TATGCCAACCTGACAAGCAC	20			54

molecular biotechnology made these short molecules as useful markers for several fields of sciences, where detection of variability is required (Li *et al*., 2002; Meglécz *et al*., 2007; Weber and May, 1989).

The mulberry tree is a plant belonging to the *Moraceae* family and the Morus genus, which is widely distributed from tropical to temperate regions (Awasthi *et al*., 2004). There are about 10 species, but in Korea, *Morus bombycis*, *M. cathayana*, and *M. mongolica* mainly inhabit. In June, female and male flowers bloom separately in one tree or in a different tree, and the fruit ripens in black purple. Mulberry leaves are also used to grow silkworms importantly. As research on the development of new biological materials has been actively conducted, mulberry trees, which are mainly used for silkworm breeding, have recently been used as biopharmaceutical materials. In addition, studies on antioxidant effects of mulberry leaves (Kim, 2005; Katsube *et al*., 2006; Park *et al*., 2008) and the decreasing blood sugar level of mulberry leaves (Lee *et al*., 1998; Kang *et al*., 2006) have been published. Furthermore, the possibility of mulberry leaves as new materials for functional foods, cosmetics, and medicines has been recognized. Therefore, it is necessary to maximize industrial value by identifying excellent mulberry varieties and evaluating genetic characteristics.

In this study, the eight available microsatellite markers were selected and genotyped 20 mulberry varieties preserved in Korea, and was suggested the utility of the microsatellite loci in detecting DNA polymorphism and to assess their potential for use in variety classification.

Materials and Methods

Preparation of genomic DNAs

Approximately 100 mg of each *mulberry* varieties was grinded by a mortar in liquid nitrogen, and genomic DNA of each was extracted using the DNA Extraction Kit (Qiagen, USA) in accordance with the manufacturer's instruction.

Design of microsatellite primers

Eight primers (19, 28, B2-3, B2-26, B2-28, B2-42, B2-42, B2-48, B2-48, and B2-55) were designed by referring to the genetic sequence of the microsatellite DNA library derived from mulberry BR 60 leaves (Table 1). Among them, the forward primer was synthesized by labeling the 5'-terminal with a fluorescent substance FAM (SAFC, USA). Using Mermade

Table 2. Summary of the 8 microsatellite loci analyzed in 20 mulberry varieties

Marker	Frequency of Major Allele	Genotype No.	Sample Size	No. of Obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
19	0.43	7	20	20	6	1	0.7088	0.35	0.6648
28	0.23	16	20	20	12	1	0.8638	1	0.8495
B2-3	0.20	13	20	20	11	1	0.8613	0.45	0.8460
B2-26	0.38	13	20	20	11	1	0.8025	0.6	0.7840
B2-28	0.23	15	20	20	15	1	0.8838	0.75	0.8739
B2-42	0.23	13	20	20	13	1	0.8725	0.45	0.8601
B2-48	0.33	12	20	20	10	1	0.8125	0.65	0.7905
B2-55	0.58	8	20	20	7	1	0.6313	0.75	0.6050
Mean	0.32	12.1	20	20	10.6	1	0.8045	0.63	0.7842

192 (Bio automation corporation, Sweden) and Polygen (Gmbh, Germany), the synthesis of these primers was carried out from a detritylation (deblocking) step, an activation step, a coupling step, oxidation step, and capping step according to manufacturer's instructions.

Analysis of variation and phylogenetic tree

980 µl Hi-Di formamide (Applied Biosystems) and 40 µl GeneScanTM 500 LIZ SIZE Standard (Applied Biosystems) were mixed to prepare a gel loading buffer for genotyping. Meanwhile, 5 µl of the PCR product and 10 µl of the gel loading buffer for genotyping were added into each well of 96 well plate, spin-down, and then thermally denatured at 95°C for 3 minutes, and immediately cooled in ice for 5 minutes. Each of the cooled samples was transferred to each well of the Microam optical 96well plate (Axygen), spin-down, and then the 96-well plate base (Applied Biosystems), septa (Applied Biosystems), and 96-well plate retainer (Applied Biosystems) assembly together, and was transferred to ABI 3730xl genetic analyzer (Applied Biosystems) to obtain genotyping results.

According to the results derived from electrophoresis, the number, size of alleles of samples amplified with each primer were determined. Heterozygosity, Polymorphic Information Content (PIC), and frequencies of allele and genotype at per-locus were calculated using PowerMarker ver. 3.25 (Bostein *et al*., 1980; Liu and Muse, 2005). To calculate the allelic PIC, the following formula was used: $PIC = 1 - \sum (P_i)^2$, where P_i is the proportion of the strain carrying i^{th} allele, calculated for each microsatellite locus (Bostein *et al*., 1980). The relationships among mulberry varieties were described by Neighboring-Joining (NJ) method as a distance matrix in basis of the shared allelic methods (Jin and Chakraborty, 1993) using PowerMarker ver. 3.25 (Liu and Muse, 2005).

Results and Discussion

Characteristics of alleles in 20 mulberries

In this study, the analysis was conducted to determine the relationship between 16 mulberry varieties grown mainly in Korea and four foreign varieties among more than 600 mulberry varieties preserved in Korea. Fragment analysis of the 20 mulberry varieties in eight microsatellite loci were successful. Thus, a total of 73 alleles at eight loci were detected and the average allele number of the eight loci was 10.6, ranging in number from six (the locus 19) to 15 (the locus B2-28) (Table 2). 28, B2-3, B2-26, B2-28, B2-42, and B2-48, which are supposed to be the dinucleotide repeats, provided high allele number. The B2-28 exhibited the highest 15 alleles among those. On the other hand, the locus 19, which is supposed to be consisted of trinucleotide repeats, provided the lowest allele number as six. In general, dinucleotide repeats identify many alleles, but trinucleotide repeats provide relatively few alleles in microsatellite analysis (Reddy *et al*., 1999; Itoh *et al*., 1991). This study shows similar results.

Heterozygosity of 20 mulberries preserved in Korea

According to the allelic diversity, the number of genotypes also is roughly proportional to the number of alleles (Table 2). For example, the locus 19, which provided only six alleles, only

Table 3. Genotypes of 20 mulberry preserved in Korea at each microsatellite locus

Sample	Microsatellite locus (bp)							
	19	28	B2-3	B2-26	B2-28	B2-42	B2-48	B2-55
Turkey D	265 265	220 242	123 135	119 123	177 177	178 178	169 169	213 219
Cheongilppong	265 265	222 242	115 133	123 145	175 185	154 154	155 165	234 330
Gaeryangppong	265 265	222 248	115 115	147 147	157 175	154 154	155 165	219 330
Gugoksang	261 265	238 260	109 109	123 141	175 175	162 162	151 165	219 234
Kkorippong	253 253	250 268	107 107	135 145	169 177	156 158	165 165	219 219
Suseongppong	261 261	238 254	109 109	123 139	175 179	180 180	165 171	219 222
BR 60	265 265	220 238	121 121	123 153	157 199	172 198	171 171	219 330
Daeseongppong	273 273	240 242	105 105	123 123	179 179	162 178	153 165	213 219
Hasusang	261 261	240 248	113 113	123 123	165 179	152 168	165 165	219 234
Daebungppong (Gyeongnam)	261 261	220 254	109 109	125 125	175 175	162 162	171 171	219 219
Daechukppong	261 261	222 238	115 115	123 147	155 183	152 152	155 165	219 219
Cheongsuppong	249 261	222 240	105 105	145 157	157 175	144 168	149 159	210 219
Okinawa	249 261	222 238	103 129	147 147	167 183	152 152	155 155	207 219
Jeolgokjosaeng	261 261	234 248	113 113	125 125	167 185	164 164	143 165	210 219
Mujadaesip	245 261	238 242	107 113	123 157	193 197	154 172	159 171	219 219
Gwasang 2ho	245 261	238 242	105 113	123 157	193 197	154 172	159 171	219 219
Daebungppong	265 265	222 242	115 133	123 145	175 185	154 154	155 165	234 330
Iksuppong	273 273	240 244	109 115	123 123	179 179	162 178	153 165	213 219
Cheonggangsang	261 265	222 238	105 113	145 157	159 179	178 178	155 161	219 234
America 13	245 261	238 240	109 123	143 143	161 177	154 194	169 169	210 219

resulted in seven genotypes in 20 mulberries, whereas the locus B2-28, which provided 15 alleles resulted in 15 genotypes in 20 mulberries. The frequency of the most common allele in per-locus ranged from 0.20 (Locus B2-3) to 0.58 (Locus B2-55). Thus, some loci provided high frequency of particular alleles, although other loci were not. For example, the allele 261 found in the locus 19 occurred in 12 varieties either as homozygote or heterozygote, but the allele 253 in the locus 19 was found in only one variety as a homozygote. The allele 273 in the locus 19, allele 121 in the locus B2-3, allele 143 in the locus B2-26, and alleles 164 and 180 in the locus B2-42 were also found in only one strain as a homozygote.

The gene diversity and the observed heterozygosity over all microsatellite loci ranged from 0.63 to 0.88 and 0.35 to 1.00, respectively (Table 2). The loci 28 and B2-55 revealed somewhat higher estimates of heterozygosity compared to gene diversity but the remaining loci 16 (0.71 vs. 0.35), B2-3 (0.86 vs. 0.45), B2-26 (0.80 vs. 0.6), B2-28 (0.88 vs. 0.75), B2-42 (0.87 vs. 0.45) and B2-48 (0.81 vs. 0.65) have shown substantially lower estimates of heterozygosity, indicating long-held inbreeding within varieties. The PIC value ranged from 0.61 to 0.87 with an average of 0.78 per locus (Table 2). The locus B2-28, which provided the high allele number as 15, recorded the highest value of 0.87, and all of the remaining loci recorded the PIC values higher than 0.50, indicating the high quality of variety discrimination in those loci.

Strain discrimination

The eight microsatellite loci revealed the presence of variety-specific alleles (Table 3). The locus 19 provided the allele 253 which is unique to variety Kkorippong (Table 3). In the same manner, the locus 28 provided the alleles 234, 244, 250, 260 and 268 unique to the variety Jeolgokjosaeng, Iksuppong, Kkorippong, Gugoksang, and Kkorippong, respectively; the locus B2-3 provided the allele 103, 121, and 129 unique to Okinawa, BR 60, and Okinawa, repectively; the locus B2-26 provided the allele 119, 135, 139, 141, 143, and 153 unique to Turkey D, Kkorippong, Suseongppong, Gugoksang, America 13, and BR 60, respectively; and the locus B2-28 provided the allele 155 unique to Daechukppong, 159 unique to Cheonggangsang, 161 unique to America 13, 165 unique to Hasusang, 169 unique to Kkorippong, and 199 unique to BR 60; and the locus B2-42 provided the allele 144 unique to Cheongsuppong, 156 and 158 unique to Kkorippong, 164 unique to Jeolgokjosaeng, 180 unique to Suseongppong, 194 unique to America 13, and 198 unique to BR60; and the locus B2-48 provided the allele 143 unique to Jeolgokjosaeng, 149 unique to Cheongsuppong, 151 unique to Gugoksang, and 161 unique to Cheonggangsang; and the locus B2-55 provided the allele 207 and 222 unique to Okinawa and Suseongppong, respectively (Table 3). Resultantly, a total of 34 apomorphic alleles, which discriminated 14 among 20 mulberry varieties, were obtained. Therefore, these variety-specific alleles can be easily used to identify applicable mulberry varieties without additional typing from other loci.

Genotyping results have shown a substantial number of strains to possess homozygotes (Table 3). The locus 19 provided one variety with the homozygote of allele 253, 5 varieties with the homozygote of allele 261, 5 varieties with the homozygote of allele 265, and 2 strains with the homozygote of allele 273 (Table 3). Likewise, the locus B2-3 provided a total of 11 varieties possessing homozygote in six alleles; the locus B2-26 provided a total of 8 varieties possessing homozygote in four alleles; the locus B2-28 provided a total of 5 varieties possessing homozygote in three alleles; the locus B2-42 provided a total of 11 varieties possessing homozygote in six alleles; the locus B2-48 provided a total of 7 varieties possessing homozygote in four alleles; and the locus B2-55 provided a total of 5 varieties possessing homozygote in one allele (Table 3). Consequently, a total of 60 among 180 genotypes in eight loci were typed into homozygote. Thus, these results indicate that these microsatellite loci will powerfully be utilized for the discrimination of

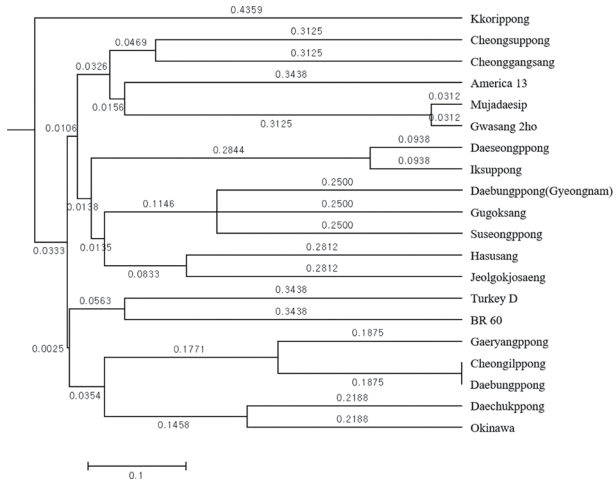

Fig. 1. Phylogenetic tree of 20 mulberry varieties preserved in Korea. According to microsatellite analysis, the phylogenetic tree was described. As a distance matrix based on the alleles, the relationships between 20 mulberry varieties were constructed by Neighboring-Joining method.

mulberry varieties. In conclusion, based on the results of microsatellite analysis, a phylogenetic tree of 20 varieties of mulberry varieties preserved in Korea was described (Fig. 1). In the case of Kkorippong, it showed a distinct relationship with the rest of the mulberry varieties. Mujadaeship and Gwasang 2ho, Daeseongppong and Iksuppong showed close relationships, respectively. Unusually, Cheongilppong and Daebungppong were found to be the same variety by present microsatellite analysis. Although Daebungppong (Gyeongnam) and Daebungppong have the same name, the relationship was not close depending on the region. This shows the limitations of phylogeny using only one DNA-based marker. In some cases, it is difficult to reveal the phylogeny with one analysis method. In some papers, the results of SSR markers for phylogenetic analysis showed many differences from other PCR-based markers. Although they accepted powerful value of SSR markers in phylogenetic analysis, previous studies were analyzed the classification and origin of varieties by comprehensively comparing SSR, ISSR, and RAPD. Not only in plant phylogeny such as the mastic trees (Abuduli *et al*., 2016) and buffalograsses (Budak *et al*., 2004), but also in the classification of Fusarium oxysporum causing chickpea wilt (Dubey and Singh, 2008) and insects such as Bombyx mori (Nagaraju *et al*., 2001), various markers were used in cross-analysis to compensate for the shortcomings and limitations of each analysis marker. In order to overcome for the limitations of present study using only microsatellite marker,

further research using other markers such as ISSR and RAPD will be conducted, and the phylogenetic relationship between Daebungppong (Gyeongnam) and Daebungppong should be comprehensively analyzed using those markers.

Still more microsatellite loci should be developed for casual discrimination of more than 600 mulberry varieties that are currently under preservation in Korea. These are always exposed to accidental mixing between varieties, but proper information that allows for casual morphological features of mulberry varieties has not yet been available. Nevertheless, considering the current results, the microsatellite loci are expected continuously to be used for the strains discrimination for mulberry varieties that are kept in Korea.

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