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Identification of Citrus Varieties Bred in Korea Using Microsatellite Markers

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Abstract

More than 30 citrus varieties have been developed in Korea over almost four decades. Despite the economic importance of citrus fruit production, reliable technologies for the identification of citrus varieties bred in Korea have not been established. We developed 53 polymorphic nuclear microsatellite markers fully covering the nine pseudochromosomes of citrus and applied them to the discrimination of the 32 available citrus varieties bred in Korea. Most Korean citrus varieties were clearly discriminated by 245 alleles derived from the polymorphic microsatellite loci. However, nucellar or bud sport mutant varieties originated from *Citrus* hybrid 'Shiranuhi' could not be discriminated from each other because they share identical genotypes on all of the microsatellite loci investigated. The microsatellite markers developed in this study will be an efficient molecular genetic tool to protect breeders' rights and guarantee the quality of nursery plants in the citrus industry.

Additional key words: breeders' rights, nursery plants, somatic mutants, SSR, UPOV

Introduction

Citrus is a major fruit crop produced in tropical and subtropical regions around the world. Citrus annually produces 146 million tons of fruit from a cultivated area of 7.2 million hectares worldwide. Sweet orange cultivars account for approximately 60% of annual citrus production (FAO, 2018). In 2017, citrus produced over 0.6 million tons of fruit in the arable land of over 20,000 hectares in Korea. The entire production amounted to approximately 0.83 billion USD (www.krei.re.kr). Citrus fruit is an important source of nutrition for humans due to its high contents of vitamin C, citric acid, and other functional compounds, including citrus-specific flavonoids such as hesperidin and tangeretin (Benavente-García and Castillo, 2008; Iranshahi et al., 2015). Despite the tremendous extent

and value of citrus production, most citrus varieties have been selected from natural hybridizations and somatic mutations (e.g., bud sport and nucellar mutants), not from systematic and targeted breeding programs (Talon and Gmitter, 2008). Citrus is considered the most difficult crop for developing new varieties through conventional breeding. Unfavorable factors for conventional breeding in citrus include large plant size, long juvenile stage, self- and cross-incompatibility, pollen and/or ovule sterility, high level of heterozygosity, and apomictic nucellar embryony (Donmez et al., 2013).

Microsatellites, also referred to as simple sequence repeats (SSRs), are highly informative DNA markers due to their high degree of polymorphism, codominant mode of inheritance, and genome-wide distribution (including organelle genomes). The International Union for the Protection of New Varieties of Plants (UPOV) highly recommends the use of molecular markers such as microsatellites to verify new varieties (UPOV, www.upov.int). Microsatellite markers have been widely used for various molecular genetic analyses, such as genetic diversity and population structure studies (Kim et al., 2016; Chung et al., 2019), construction of genetic linkage maps (Chang et al., 2018), identification of zygotic/nucellar individuals (Woo et al., 2019), and cultivar/genotype identification (Kim et al., 2019).

In Korea, systematic citrus breeding programs have been in operation since the Citrus Research Institute, National Institute of Horticultural and Herbal Science, was established in 1992. More than 30 citrus varieties have been developed from bud sport mutants, nucellar mutants, and genetic hybridizations by breeders at governmental institutes and breeding companies as well as private breeders. Development of new citrus varieties will greatly increase in the near future due to citrus breeding programs in government and company sectors. However, reliable technologies for identifying Korean citrus varieties that protect breeders' rights and guarantee the quality of nursery plants are not yet established. Therefore, in this study, we report the development of polymorphic microsatellite makers to identify citrus varieties bred in Korea.

Materials and Methods

Plant Materials and Genomic DNA Extraction

Leaf samples of citrus varieties used in this study were obtained from two public institutes in the Republic of Korea: Agricultural Research and Extension Services, Jeju Special Self-Governing Province, and Citrus Research Institute, National Institute of Horticultural & Herbal Science. Harvested leaf tissues were rinsed with running tap water and then stored at − 80°C until use. Genomic DNA (gDNA) was isolated from the leaf tissue using the Biomedic® Plant gDNA Extraction Kit (Biomedic Co., Ltd., Bucheon, Korea). The isolated gDNA was quantified using the DeNovix DS-11+ spectrophotometer (DeNovix, Wilmington, DE, USA).

Polymorphism Test of Microsatellite Marker Candidates

Putative polymorphic microsatellite markers were selected from routine PCR using gDNAs from 11 varieties belonging to six citrus groups (lemon, mandarin, sour orange, citron, orange, and tangor) as described previously (Woo et al., 2019). The PCR reaction and cycling conditions were described in our previous report (Woo et al., 2019). PCR products were separated on a 2.5% (w/v) agarose gel to confirm PCR amplification and polymorphism among the tested varieties.

Genotyping by M13-tailed PCR

The M13-tailed PCR method was used for genotype analysis using the selected polymorphic microsatellite markers (Schuelke, 2000). The PCR reaction conditions were the same as those previously reported (Woo et al., 2019). The cycling conditions for PCR amplification followed the protocol described previously by Schuelke (2000). Fragment analysis of the PCR products was described previously (Kim et al., 2012). Calling of allele sizes was performed using GeneMapper software (ver. 4.0; Applied Biosystems).

Data Analysis

Genetic parameters such as major allele frequency (MAF), number of alleles (NA), genetic diversity (GD, often referred to as expected heterozygosity), observed heterozygosity (HO), and polymorphic information content (PIC) were measured by calculating the shared allele frequencies using PowerMarker software (v. 3.25) (Liu and Muse, 2005). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram was implemented using MEGA software (v. 7.0) (Kumar, 2016), which is embedded in PowerMarker, with the UPGMA algorithm.

Results and Discussion

Development of Polymorphic Microsatellite Markers

We previously developed 17 polymorphic microsatellite markers and applied them both to analyze the genetic diversity of citrus genetic resources and to identify zygotic/nucellar individuals. However, the markers were not enough to discriminate all citrus genetic resources used in the study (Woo et al., 2019). Genome-wide characterization of microsatellite markers was reported in sweet orange (*Citrus sinensis*) previously (Biswas et al. 2014). We used marker information from the literature to further screen polymorphic microsatellites. To develop high-quality polymorphic markers, the microsatellite markers from sweet orange were applied to 11 citrus varieties belonging to six citrus groups: lemon, mandarin, sour orange, citron, orange, and tangor. We finally selected 36 polymorphic markers out of 74 microsatellite candidates. Table 1 summarizes 53 polymorphic nuclear microsatellite markers, including the 36 microsatellites developed in this study. The physical location of the 53 microsatellite loci on the nine pseudochromosomes was determined by performing a BlastN search against a reference genome of C. *clementina* cv. Clemenules (Wu et al., 2013; https://phytozome.jgi.doe.gov/pz/portal.html). The final microsatellite markers fully covered all of the pseudochromosomes of citrus (Fig. 1).

Table 1. The 53 polymorphic microsatellite markers used in this study

No.	Locus name	Primer sequence (5' → 3')		Repeat motif	Ta[y] (°C)	Size range (bp)	Reference
		Forward[z]	Reverse				
1	BM-CiSSR-012	*TGTAAAACGACGGCCAGT*GGGCTCAGTTCTTC TCTACTC	GCATTAGGCTTCTCTCATACC	(TTA)15	58	283-308	Woo et al., 2019
2	BM-CiSSR-013	*TGTAAAACGACGGCCAGT*GGTGGCATACATA CATACATACA	GCAACATCTGGAACTACTCA	(TA)6	58	127-135	
3	BM-CiSSR-032	*TGTAAAACGACGGCCAGT*GCCTGAGTTTCTTT GTTATG	CATTCCATCGTCTCCTATTGT	(TATG)4	58	136-172	

Table 1. The 53 polymorphic microsatellite markers used in this study (Continued)

No.	Locus name	Primer sequence (5' → 3')		Repeat motif	Ta[y] (°C)	Size range (bp)	Reference
		Forward[z]	Reverse				
4	BM-CiSSR-043	*TGTAAAACGACGGCCAGT*ATTAGTGCGGGTAAGATGAA	AAGGATTTGGTGTAGGAAGTAA	(AAAAT)3	58	286-327	
5	BM-CiSSR-073	*TGTAAAACGACGGCCAGT*CGGACAAGGAGATGAAGATAG	TTCTAACAGCACCAAGCAG	(GA)16	58	319-345	
6	BM-CiSSR-077	*TGTAAAACGACGGCCAGT*TGTATTTATTTCTGACTACGACC	ATGCGTTTGGTGTGTGTT	(AT)12	58	188-207	
7	BM-CiSSR-082	*TGTAAAACGACGGCCAGT*ACCTGAGCCCTTTTTGGTTT	GCCAGATCAAGGCTCAAATC	(TC)13	58	150-155	
8	BM-CiSSR-087	*TGTAAAACGACGGCCAGT*CAGATCCTATTGCAGAGGCA	GCCCATTTGTATTGCCATTT	(CAG)6	58	183-189	
9	BM-CiSSR-093	*TGTAAAACGACGGCCAGT*CCCCCTCTTCTTTCACACAA	GGTGAGCAGCCATCTTCTTC	(TA)6	58	152-156	
10	BM-CiSSR-094	*TGTAAAACGACGGCCAGT*GAATTGGGAGGACGAACTGA	CGAGCCCTAGACAGAGATGG	(AGA)7	58	268-277	Woo et al., 2019
11	BM-CiSSR-100	*TGTAAAACGACGGCCAGT*GTTTTCAGCTGGATTCGAGG	CACGTGTCCTCCTGGAACTT	(GCC)5	58	197-206	
12	BM-CiSSR-111	*TGTAAAACGACGGCCAGT*CCGATACAGCACAAAGCAAA	TGGAAAGAGAGAAGCCAAGC	(AAT)7N15(AGC)7	58	134-143	
13	BM-CiSSR-115b	*TGTAAAACGACGGCCAGT*CGGTGTGTATTGGGTACACG	GCTTTTTCGAAAGCGTCAAG	(TA)17	58	231-250	
14	BM-CiSSR-137	*TGTAAAACGACGGCCAGT*GCAACGTGTACTGACGCTTG	GCTCGTATCTGAAGCTCGCC	(TAT)7	53	301-327	
15	BM-CiSSR-159	*TGTAAAACGACGGCCAGT*ATGACCTCAAACGGTGAGCA	CTTCCACATCCGAACCGACA	(GAGG)5	53	382-404	
16	BM-CiSSR-162	*TGTAAAACGACGGCCAGT*GCTAGGGTTCCAGACTTCCAG	GATTTGGCCGATCGAAAGCC	(AAT)10(CAT)6	53	185-236	
17	BM-CiSSR-165	*TGTAAAACGACGGCCAGT*AGCAACTTAAGGTCCTTCACGA	TTCTCTGCTCTGCTGTGCAT	(AAT)6	53	393-416	
18	BM-CiSSR-201	*TGTAAAACGACGGCCAGT*CAACAGTACCTGATGGTCCG	TTCTGAAATCCAGTCCCCTC	(AAAAGA)6	55	259-270	
19	BM-CiSSR-202	*TGTAAAACGACGGCCAGT*CCCTCTTCAAGAACTGAGCC	CACCAGCTGTTTGCTGTTTT	(AAAT)5	55	281-293	
20	BM-CiSSR-203	*TGTAAAACGACGGCCAGT*ACAACGCACCAAGTCAATGT	GTTGCGTCATCCATTTTGTC	(AGCC)5	55	241-257	
21	BM-CiSSR-204	*TGTAAAACGACGGCCAGT*CCATGACCCACTTTCCCTAC	ATTCGGGTAGGTTGAAATCG	(ACCCG)4	55	326-365	
22	BM-CiSSR-208	*TGTAAAACGACGGCCAGT*GGATGCTTGGCCTGATTTAT	ATTGTCACCGAAGCACCATA	(TAA)7	55	340-352	
23	BM-CiSSR-210	*TGTAAAACGACGGCCAGT*GCCAGGATTGAAGGGTTTTA	TGTGAACAAGGGCAACAGAT	(TTTAT)4	55	333-366	In this study
24	BM-CiSSR-217	*TGTAAAACGACGGCCAGT*ATAATGGAAGCGTCGGATTC	GCCTAACGGCCAGAGTTTAC	(CAG)10	55	313-327	
25	BM-CiSSR-218	*TGTAAAACGACGGCCAGT*TATGTCTACTGGTCGCAGGC	GTTGTCCCCTTGATACCACC	(GGGAT)4	55	236-444	
26	BM-CiSSR-221	*TGTAAAACGACGGCCAGT*GGTCCTTTGGAGAAGGTTGA	CATGACCAAATGTCGGGTTA	(TAAT)5	55	319-332	
27	BM-CiSSR-224	*TGTAAAACGACGGCCAGT*AACCCCTTGTCAAGTGATCC	TCTTCTTCAGTTGGTGCCTG	(CAC)7	55	294-304	
28	BM-CiSSR-225	*TGTAAAACGACGGCCAGT*GTAAGGGGTTGTGAGGCAAT	CAACAGGTTTCGACCATGAC	(GCT)8	55	322-353	

Table 1. The 53 polymorphic microsatellite markers used in this study (Continued)

No.	Locus name	Primer sequence (5' → 3')		Repeat motif	Ta[y] (°C)	Size range (bp)	Reference
		Forward[z]	Reverse				
29	BM-CiSSR-226	*TGTAAAACGACGGCCAGT*ATTAAGGCTGGAAATGCCAC	ATTCTGCTGACGCTTCAATG	(ATT)9	55	389-401	
30	BM-CiSSR-230	*TGTAAAACGACGGCCAGT*TCCATCAGCCATTCCATCTA	ATCTGAACCCTCCAATCCTG	(TTC)9	55	276-302	
31	BM-CiSSR-238	*TGTAAAACGACGGCCAGT*ACTATGCGGCTCGAACTTTT	TCACCTTCACAACCGAACAT	(CTT)11	55	269-277	
32	BM-CiSSR-239	*TGTAAAACGACGGCCAGT*ACATGCCATAGGAAGCAACA	CACCTTCTCATCAATCACGG	(AAG)7	55	395-408	
33	BM-CiSSR-240	*TGTAAAACGACGGCCAGT*GCTGCTGCTGCTAGTTTGTC	AAAGATGGCAATGGGTTAGG	(TCA)7	55	429-432	
34	BM-CiSSR-241	*TGTAAAACGACGGCCAGT*GGTCCTTTGGAGAAGGTTGA	CATGACCAAATGTCGGGTTA	(TAAT)5	55	296-301	
35	BM-CiSSR-243	*TGTAAAACGACGGCCAGT*CCATCCCTGTAAATTCCACC	ATTGGTCGTTTCCTTTCCTG	(AAAAT)4	55	319-323	
36	BM-CiSSR-245	*TGTAAAACGACGGCCAGT*TTTCCCAGGAGCTTACCAAG	TGCGTTCCATGGTCAGTATT	(AAC)6	55	338-347	
37	BM-CiSSR-246	*TGTAAAACGACGGCCAGT*CCCTAGGGAAATTTGGGAAT	GCACTCGAGAGTTCTCGTTAAG	(CAT)11	55	310-375	
38	BM-CiSSR-247	*TGTAAAACGACGGCCAGT*ATCTGTGTTTGGTCGCATGT	GGAAGATTACCGGACTTGGA	(GAA)6	55	409-427	
39	BM-CiSSR-248	*TGTAAAACGACGGCCAGT*GCTCGGTTCTTGCATACTGA	GTCTGCAAACCCTGTTGATG	(TGA)6	55	316-327	
40	BM-CiSSR-249	*TGTAAAACGACGGCCAGT*ATGGGCAAGAACAGGAAATC	CCATAGGATTTGCATGAGGA	(GGA)6	55	321-331	
41	BM-CiSSR-253	*TGTAAAACGACGGCCAGT*AATTTCCTGCTCCAAACCAG	TCCAACAACTTGAACACGGT	(TAA)14	55	319-355	In this study
42	BM-CiSSR-254	*TGTAAAACGACGGCCAGT*TAAAATCCCTCGGAAACAGG	CTTTGCATGTTCAACGTTCC	(ATC)6	55	234-313	
43	BM-CiSSR-256	*TGTAAAACGACGGCCAGT*CTCTCTGAACCTGACACCGA	TTTTCTCCACCCTTTCAACC	(GAT)7	55	290-322	
44	BM-CiSSR-258	*TGTAAAACGACGGCCAGT*GGTAAGCACCTGCAAACTGA	ATTATGCAATTCCTCCTGGC	(TATG)6	55	350-374	
45	BM-CiSSR-260	*TGTAAAACGACGGCCAGT*TCATCTGAACGGACCACAAT	TAACTGCACTTGCTTCCCTG	(TTC)6	55	381-384	
46	BM-CiSSR-262	*TGTAAAACGACGGCCAGT*CAGTTTCATCCCACTGATGC	ACCAAGCGTCCTTAACAACA	(ATT)6	55	181-205	
47	BM-CiSSR-264	*TGTAAAACGACGGCCAGT*AGGGGTGCTGAGCATAAAAT	ATACCCCGTCGTGGAATTAG	(TAA)9	55	415-442	
48	BM-CiSSR-266	*TGTAAAACGACGGCCAGT*CGTAGCCAAAACTCCCAAAT	CCGAAGATGGAGGGAACTAA	(GAA)8	55	293-342	
49	BM-CiSSR-268	*TGTAAAACGACGGCCAGT*TCTGTGGCTCACTTCACTCC	GAAGACGACAGATGCTGGAA	(TCAC)7	55	355-365	
50	BM-CiSSR-270	*TGTAAAACGACGGCCAGT*TGCTGTAAGTGCAGTGCAAA	GGGACGAGCATCTTCCTTTA	(TATG)5	55	342-347	
51	BM-CiSSR-271	*TGTAAAACGACGGCCAGT*CCCCCAAAATGCTGAGTAGT	AAAGGGAGAGAGTTGGCTGA	(TATG)13	55	382-422	
52	BM-CiSSR-272	*TGTAAAACGACGGCCAGT*ATAGGTCCCCACAATGGAAA	GGGCATAAATGAATTGGGTC	(GAA)8	55	405-419	
53	BM-CiSSR-273	*TGTAAAACGACGGCCAGT*GCATCATACGTTCAAGCCAC	TCTTGTGCTCTCCTGTGACC	(CAT)12	55	298-336	

[z]The italicized letters in the forward primer indicate the M13 primer sequence.

[y]Ta indicates annealing temperature.

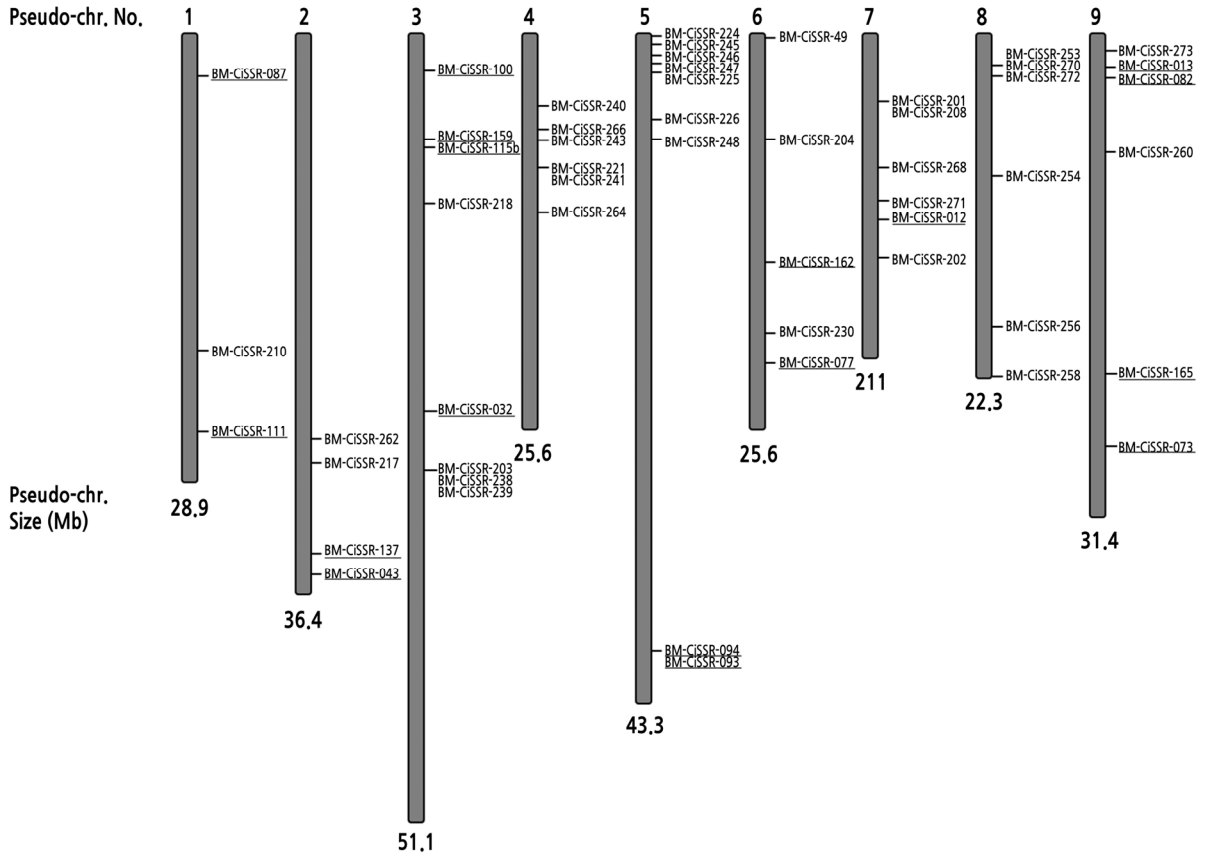

Fig. 1. Physical map of 53 microsatellite loci on the nine pseudochromosomes of *C. clementina* cv. Clemenules. The underlined markers are 17 polymorphic microsatellites developed previously (Woo et al., 2019). Pseudo-chr. = pseudochromosome.

Identification of the Citrus Varieties Bred in Korea using Polymorphic Microsatellite Markers

Up to now, 34 citrus varieties were developed from bud sport mutants, nucellar mutants, and traditional genetic hybridizations by breeders at governmental institutes and breeding companies as well as private breeders in Korea (Table 2). Fifty-three microsatellite markers were applied to an available 32 Korean citrus varieties to determine their genotypes. Genetic characteristics of microsatellite loci based on the genotype analysis of 32 accessions are summarized in Table 3. A total of 245 alleles, ranging from 2 (BMCi-SSR084) to 11 (BMCi-SSR012) per locus, were observed among the 32 citrus accessions, with an average of five alleles per locus. Major allele frequency (M_{AF}) varied from 0.22 (BMCi-SSR012) to 0.98 (BM-CiSSR-245). The average genetic diversity (GD) value was 0.53, ranging from 0.03 (BM-CiSSR-245) to 0.87 (BMCi-SSR012), and average polymorphism information content (PIC) value was 0.47, from 0.14 (BM-CiSSR-224) to 0.85 (BMCi-SSR012). The average observed heterozygosity (HO) was 0.47, with the lowest value in BM-CiSSR-245 and BM-CiSSR-270 (0.03) and the highest value in BM-CiSSR-262 (1.00).

A total of 245 alleles derived from the 53 polymorphic microsatellite loci were used to evaluate genetic relationships among the 32 varieties. A UPGMA dendrogram was constructed based on the genetic similarity matrices among the accessions. Fig. 2 illustrates the results of the cluster analysis based on microsatellite data. The resulting dendrogram revealed that the accessions used in the analysis could be distinctly classified into lemon and non-lemon groups. The

Table 2. Citrus varieties developed in Korea

Cultivar name	Abbr.	Breeding details	Breeders
'Haryejosaeng'	HRJ	Nucellar mutant from *C. unshiu* 'Tachima Wase'	CRI, NIHHS[z]
'Gaonhyang'	n.a.*	Bud sport mutant from *Citrus* hybrid 'Ehime Kashi 28 gou'	Private breeder
'Gaeulhyang'	GEH	*Citrus* hybrid 'Ehime Kashi 28 gou' × *Citrus* hybrid 'Kanpei'	ARES, JSSGP[y]
'Noeulhyang'	NEH	CRS0215 (*Citrus* hybrid 'Kiyomi' × Orange) × Osceola	CRI, NIHHS
'Redsanta'	RSN	*Citrus* hybrid 'Ehime Kashi 28 gou' × *C. reticulata* 'Ponkan'	HBI[x]
'R.O.Y.G'	ARD	Nucellar mutant from *Citrus* hybrid 'Ecriec 65'	HBI
'Tarachogeuk'	TRG	Bud sport mutant from *C. unshiu* 'Nichinan 1 gou'	HBI
'Moobong'	MUB	*Citrus* hybrid 'Shiranuhi' × *C. hassaku*	CRI, NIHHS
'Minihyang'	MNH	*C. kinokuni* × *C. reticulata* 'Ponkan'	CRI, NIHHS
'Sarahyang'	SRH	Nucellar mutant from *Citrus* hybrid 'Setoka'	CRI, NIHHS
'Samdajosaeng'	SMJ	Nucellar mutant from *C. unshiu* 'Tachima Wase'	CRI, NIHHS
'Sandojosaeng'	SNJ	Bud sport mutant from *C. unshiu* 'Sasaki unshiu'	ARES, JSSGP
'Saebyeolbong'	SBB	Nucellar mutant from *Citrus* hybrid 'Shiranuhi'	CRI, NIHHS
'Sunking'	SNK	*Citrus* hybrid 'Nankou' × *C. reticulata* 'Encore'	CRI, NIHHS
Seolbongmi'	SBM	*Citrus* hybrid 'Kiyomi' × *Citrus* hybrid 'Seminole'	CRI, NIHHS
'Sinyegam'	SYG	*Citrus* hybrid 'Kiyomi' × *C. reticulata* 'Wilking'	CRI, NIHHS
'Suneat'	SNE	Bud sport mutant from *Citrus* hybrid 'Shiranuhi'	Private breeder
'Winterprince'	WTP	*Citrus* hybrid 'Harehime' × *C. reticulata* 'Ootaponkan'	CRI, NIHHS
'Injajosaeng'	n.a.*	Bud sport mutant from *C. unshiu* 'Takabayashi Wase'	Private breeder
'Jeramon'	JRM	Nucellar mutant from *C. limon* 'Frost Lisbon' × *C. limon* 'Mayor'	CRI, NIHHS
'Tamnaneunbong'	TNB	Nucellar mutant from *Citrus* hybrid 'Shiranuhi'	CRI, NIHHS
'Tamnajoseang'	TNJ	Nucellar mutant from *C. unshiu* 'Shigeta unshiu'	CRI, NIHHS
'Tamdo No. 1'	TMD1	*Citrus* hybrid 'Kiyomi' × *C. platymmama*	CRI, NIHHS
'Tamdo No. 3'	TMD3	*Citrus* hybrid 'Kiyomi' × *Citrus* hybrid 'Seminole'	CRI, NIHHS
'Tamdori'	TDR	*Citrus* hybrid 'Kiyomi' × *C. reticulata* 'Fortune'	CRI, NIHHS
'Tambit No. 1'	TMB1	*Citrus* hybrid 'Nishinokaori' × *C. reticulata* 'Ponkan'	CRI, NIHHS
'Poonggwang navel'	PGN	Nucellar mutant from *C. sinensis* 'Washington navel'	CRI, NIHHS
'Hayangjosaeng'	HYJ	Nucellar mutant from *C. unshiu* 'Ueno Wase'	CRI, NIHHS
'Minimon'	MNM	Seedlings (*C. limon* 'Mayor')	CRI, NIHHS
'Hallamon'	HRM	Seedlings (*C. limon* 'Mayor')	CRI, NIHHS
'Jerajin No. 1'	JRJ1	*Citrus* hybrid 'Ehime Kashi 28 gou' × *C. reticulata* 'Ponkan'	JN[w]
'Jerajin No. 2'	JRJ2	*Citrus* hybrid 'Ehime Kashi 28 gou' × *C. reticulata* 'Ponkan'	JN
'Jerajin No. 3'	JRJ3	Open pollination (*C. reticulata* 'Lee' as a female)	JN
'Jerajin No. 4'	JRJ4	Open pollination (*C. reticulata* 'Lee' as a female)	JN

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[x]Hannong Bio Industry Corp., Korea.

[w]Jenong Co., Ltd., Korea.

*Not available.

Table 3. Characteristics of 53 microsatellite loci based on the genotype analysis of 32 citrus varieties developed in Korea

Locus name	SS	N_{OBS}	Availability	N_G	M_{AF}	N_A	GD	H_O	PIC
BMCi-SSR012	32	32	1.00	13	0.22	11	0.87	0.34	0.85
BMCi-SSR013	32	30	0.94	6	0.52	3	0.59	0.23	0.50
BMCi-SSR032	32	32	1.00	7	0.72	7	0.46	0.44	0.43
BMCi-SSR043	32	31	0.97	5	0.69	4	0.46	0.06	0.40
BMCi-SSR073	32	32	1.00	8	0.58	6	0.59	0.66	0.54
BMCi-SSR077	32	31	0.97	4	0.81	4	0.33	0.35	0.31
BMCi-SSR082	32	32	1.00	5	0.69	4	0.46	0.41	0.39
BMCi-SSR084	32	32	1.00	3	0.63	2	0.47	0.25	0.36
BMCi-SSR093	32	32	1.00	2	0.77	2	0.36	0.47	0.29
BMCi-SSR094	32	31	0.97	7	0.65	4	0.51	0.58	0.45
BMCi-SSR100	32	32	1.00	7	0.45	4	0.64	0.59	0.57
BMCi-SSR111	32	32	1.00	5	0.59	4	0.51	0.31	0.41
BMCi-SSR115b	32	30	0.94	10	0.40	5	0.71	0.47	0.66
BMCi-SSR137	32	32	1.00	10	0.41	8	0.67	0.41	0.62
BMCi-SSR159	32	31	0.97	11	0.31	8	0.77	0.87	0.73
BMCi-SSR162	32	31	0.97	13	0.24	7	0.81	0.81	0.78
BMCi-SSR165	32	30	0.94	7	0.48	6	0.59	0.27	0.51
BM-CiSSR-201	32	32	1.00	6	0.77	5	0.39	0.38	0.36
BM-CiSSR-202	32	32	1.00	6	0.84	6	0.28	0.22	0.26
BM-CiSSR-203	32	32	1.00	8	0.66	5	0.54	0.50	0.51
BM-CiSSR-204	32	31	0.97	6	0.68	6	0.50	0.23	0.46
BM-CiSSR-208	32	32	1.00	5	0.83	3	0.30	0.22	0.28
BM-CiSSR-210	32	32	1.00	3	0.61	3	0.51	0.78	0.42
BM-CiSSR-217	32	32	1.00	5	0.81	4	0.33	0.31	0.31
BM-CiSSR-218	32	32	1.00	5	0.56	3	0.53	0.75	0.43
BM-CiSSR-221	32	32	1.00	6	0.53	4	0.55	0.50	0.45
BM-CiSSR-224	32	32	1.00	4	0.92	3	0.15	0.09	0.14
BM-CiSSR-225	32	32	1.00	8	0.58	6	0.60	0.47	0.55
BM-CiSSR-226	32	31	0.97	9	0.45	5	0.68	0.84	0.63
BM-CiSSR-230	32	29	0.91	14	0.31	7	0.79	0.93	0.76
BM-CiSSR-238	32	32	1.00	5	0.72	5	0.45	0.25	0.42
BM-CiSSR-239	32	32	1.00	6	0.61	4	0.52	0.44	0.44
BM-CiSSR-240	32	32	1.00	3	0.56	2	0.49	0.69	0.37
BM-CiSSR-241	32	32	1.00	6	0.72	4	0.43	0.44	0.38
BM-CiSSR-243	32	32	1.00	5	0.52	3	0.55	0.38	0.45
BM-CiSSR-245	32	32	1.00	2	0.98	2	0.03	0.03	0.03
BM-CiSSR-246	32	32	1.00	6	0.56	5	0.54	0.50	0.45
BM-CiSSR-247	32	32	1.00	4	0.64	3	0.52	0.53	0.46
BM-CiSSR-248	32	32	1.00	7	0.34	5	0.71	0.94	0.65
BM-CiSSR-249	32	32	1.00	7	0.50	5	0.61	0.56	0.54
BM-CiSSR-253	32	32	1.00	6	0.67	4	0.49	0.53	0.43
BM-CiSSR-254	32	32	1.00	5	0.50	3	0.57	0.44	0.47

Table 3. Characteristics of 53 microsatellite loci based on the genotype analysis of 32 citrus varieties developed in Korea (Continued)

Locus name	SS	N_{OBS}	Availability	N_G	M_{AF}	N_A	GD	H_O	PIC
BM-CiSSR-256	32	32	1.00	11	0.42	8	0.69	0.38	0.65
BM-CiSSR-258	32	32	1.00	8	0.53	5	0.65	0.84	0.61
BM-CiSSR-260	32	31	0.97	3	0.71	2	0.41	0.52	0.33
BM-CiSSR-262	32	32	1.00	2	0.50	3	0.52	1.00	0.40
BM-CiSSR-264	32	32	1.00	9	0.59	6	0.57	0.50	0.51
BM-CiSSR-266	32	31	0.97	4	0.50	3	0.52	0.45	0.40
BM-CiSSR-268	32	31	0.97	6	0.55	4	0.59	0.48	0.53
BM-CiSSR-270	32	32	1.00	3	0.52	3	0.51	0.03	0.40
BM-CiSSR-271	32	30	0.94	8	0.60	6	0.55	0.27	0.48
BM-CiSSR-272	32	32	1.00	11	0.39	6	0.74	0.72	0.70
BM-CiSSR-273	32	31	0.97	9	0.34	5	0.73	0.42	0.68
Mean	32	32	0.99	6	0.58	5	0.53	0.47	0.47

SS, sample size; N_{OBS}, number of observations; availability is defined as 1-OBS/n, where OBS is the number of observations and n is the number of individuals sampled; N_G, genotype number; M_{AF}, major allele frequency; N_A, number of alleles; GD, genetic diversity. GD, often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different; observed heterozygosity (H_O) is simple proportion of heterozygous individuals in the population.; PIC, polymorphism information content.

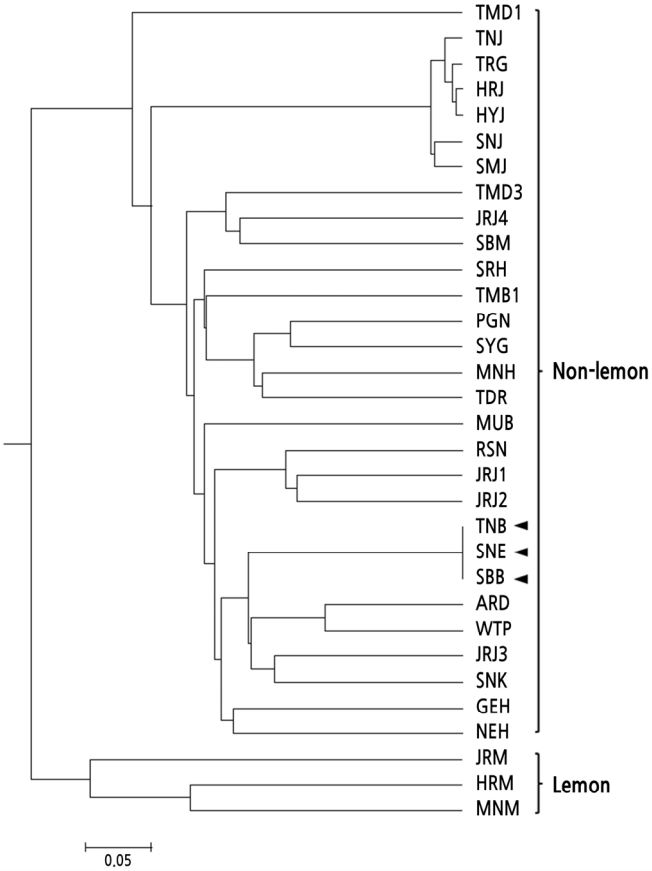

Fig. 2. UPGMA dendrogram based on genetic distances among 32 citrus varieties developed in Korea using 53 polymorphic microsatellite markers. Arrowheads indicate somatic mutant varieties originated from the *Citrus* hybrid 'Shiranuhi'.

microsatellite markers clearly discriminated most of the citrus varieties developed in Korea, except for 'Tamnaneunbong' (TNB), 'Saebyeolbong' (SBB), and 'Suneat' (SNE), which originated from nucellar offspring (TNB and SBB) and bud sport mutation (SNE) of *Citrus* hybrid 'Shiranuhi' (Table 2). Nucellar embryos develop from somatic embryos derived from maternal nucellar tissue surrounding the sexual embryo sac. This process is a kind of apomixis and is widely found in many citrus varieties. The offspring derived from nucellar embryony in citrus possesses the same genetic constitution as the female parent (Kepiro and Roose, 2007; Zhang et al., 2018). Our results indicate that somatic mutants, including nucellar offspring, originating from an identical parental plant have identical or very similar genetic constitutions to each other.

In conclusion, we developed 53 polymorphic microsatellite markers covering nine pseudochromosomes of citrus and applied them to the identification of citrus varieties bred in Korea. The microsatellite markers clearly discriminated most of the citrus varieties, whereas somatic mutant varieties that were derived by nucellar embryony or bud sport mutation from a parental plant showed identical genotypes on all loci investigated due to identical or very similar genetic constitution. The microsatellite markers developed in this study will be a useful molecular tool to protect breeders' rights and guarantee the quality of nursery plants in the citrus industry.

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