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Evaluation of polyembryony for genetic resources and efficacy of simple sequence repeat markers for the identification of nucellar and zygotic embryo-derived individuals in citrus

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Abstract

Many citrus cultivars have the polyembryony trait that develops many nucellar embryos alongside a single zygotic embryo in an individual seed by sporophytic apomixis. This unique botanical trait hinders citrus breeding by genetic hybridization and affects breeding efficiency and cost. Techniques to efficiently identify nucellar and zygotic individuals in citrus are still very limited. For a systematic and targeted citrus breeding program, we collected 101 citrus genetic resources and determined their embryo types, which revealed 22 monoembryo, 54 polyembryo, and 25 mixed types. We also developed 17 simple sequence repeat (SSR) markers showing polymorphism among the genetic resources from the public resources and our own comparative genome analysis. Seventeen SSR markers detected a total of 181 alleles, ranging from 5 to 16 alleles per locus. The average polymorphism information content value was 0.67, ranging from 0.43 to 0.84. Genetic cluster analysis based on similarity matrices of alleles revealed that several genetic resources of the genus *Citrus* were fragmented and/or scattered throughout the entire dendrogram, not forming unique groups, due to frequent natural or intended genetic crossings. Application of these polymorphic SSR markers to F_1 individuals derived from several genetic crosses using polyembryonic citrus cultivars as a female parent revealed that the polyembryony trait decreased the breeding efficiency due to the poor occurrence rate of zygotic individuals. Therefore, our results suggest that identification of nucellar and zygotic embryo-derived F_1 individuals using SSR markers as a genotyping technology may be a powerful tool for establishing a systematic molecular breeding program in citrus.

Keywords: Citrus, Genetic resource, Molecular breeding, Polyembryony, SSR marker, Zygotic embryo

Introduction

Citrus is a major fruit crop that generates the largest amount of fruit production in the world and is grown throughout the tropical and subtropical regions worldwide. Citrus fruit is an important nutritional source for human health due to its high content of vitamin C and other health-promoting compounds, including unique flavonoids such as tangeretin and hesperidin [1, 2]. Despite the tremendous extent and value of citrus production, most of the citrus cultivars have arisen from natural hybridizations and bud sport mutations, not as a consequence of systematic and targeted breeding programs [3]. Citrus is considered the most difficult crop for developing new cultivars through conventional breeding approaches due to its large plant size, long juvenility, high level of heterozygosity, and various unique reproductive

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biological characteristics, including apomictic nuclear embryony.

In most flowering plants, an individual seed usually develops a single zygotic embryo in the embryo sac. However, in many citrus cultivars, many nucellar embryos develop in an individual seed alongside a zygotic embryo by sporophytic apomixis, resulting in the development of polyembryos in a single seed. The nucellar embryos are initiated directly from the maternal, nucellar tissue surrounding the embryo sac containing a developing zygotic embryo and give rise to seedlings with the same genotype as the female parent [4, 5]. Apomictic polyembryony is a heritable trait found in many citrus varieties and greatly impedes progress in scion breeding via genetic crosses in citrus. Nucellar embryony is also essential in citrus rootstocks because it allows rootstock breeders to propagate F_1 hybrid trees on highly heterozygous but genetically uniform seedling populations [3, 5]. Systematic citrus breeding programs require reproducible, objective, and labor-saving methods to easily identify nucellar and zygotic individuals.

Several methods have been developed and used for identifying nucellar and zygotic plants in citrus, including vegetative morphology characterization [6], infrared spectroscopy [7], chromatography [8], isozyme pattern analysis [9], random amplified polymorphic DNA (RAPD) analysis [10], and inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) [11]. However, these methods are unreliable due to problems with reproducibility, accuracy and efficiency of the selection and labor intensiveness of these techniques. Simple sequence repeats (SSRs) are highly informative DNA markers due to their high degree of polymorphism and co-dominant mode of inheritance and genome-wide distribution. SSR markers have been successfully used for the identification of zygotic seedlings or nucellar rootstock candidates for rootstock breeding programs [12–14]. However, these previous studies used seedling populations derived from a very limited number of genetic crosses. The versatility of the previously reported SSR markers has also not been determined.

To establish systematic and targeted citrus breeding programs, we collected 101 citrus genetic resources. In this paper, we determined the polyembryony of the germplasm collection. We isolated 17 SSR markers showing polymorphism among several citrus cultivars from previous reports [15, 16] and the comparative genome analysis between *Citrus clementina* 'Fina Sodea' and *C. platymamma* Hort. et Tanaka and between *C. natsudaidai* and *C. natsudaidai* 'Whanggumhagyul'. Using 17 polymorphic SSR markers that we selected from the polymorphism test, we determined allele sizes from the fragment analysis for all citrus germplasms that we collected.

Based on the genotype information, we applied the polymorphic SSR markers for the identification of zygotic or nucellar individuals derived from several genetic crosses using polyembryonic cultivars as female parents.

Materials and methods

Plant materials and genomic DNA isolation
Leaf tissues were harvested from the citrus genetic resources that were collected by Agricultural Research and Extension Services, Jeju Special Self-Governing Province, Republic of Korea. The harvested leaf tissues were rinsed with tap water and then were stored at − 80 °C until use. Genomic DNA was purified using the Biomedic® Plant gDNA Extraction Kit (Biomedic Co., Ltd., Korea; www.ibiomedic.co.kr). The DNA quantity and quality for genotyping were determined using the DeNovix DS-11+ Spectrophotometer (DeNovix, Wilmington, DE, USA).

Polyembryony test of citrus genetic resources
Seed number was measured by counting the number of total seeds in five ripened fruits. After counting the seed number, total number of seeds from each cultivar were mixed together and five seeds were randomly selected to determine embryo type. An individual seed was cut in half and observed under a stereoscopic microscope for the determination of embryo type: monoembryony, polyembryony or mixed type. For cultivars showing mixed embryo type, an additional five fruits were further investigated for the final confirmation of embryo type.

Whole genome sequencing
The paired-end DNA libraries for whole genome sequencing were constructed using TruSeq® DNA Library Prep Kits (Illumina, San Diego, CA, USA) for four citrus cultivars: *C. clementina* 'Fina Sodea', *C. platymamma* Hort. et Tanaka, *C. natsudaidai*, and *C. natsudaidai* 'Whanggumhagyul'. Approximately six gigabytes of sequence information per each sample were obtained from whole genome sequencing by the Illumina HiSeq 2500 platform. The produced raw reads were trimmed using the DynamicTrim and LengthSort softwares of the SolexaQA package (v.1.13) [17]. Then left clean reads were mapped to the reference genome of *C. clementina* 'Clemenules' [18] to obtain consensus sequences using Burrows-Wheeler Aligner (BWA) program (0.6.1-r104) [19].

SSR identification, primer design, and in silico analysis of SSR polymorphism
MIcroSAtelitte (MISA) was used to identify SSRs from the consensus sequences of each sample (http://pgrc.

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ipk-gatersleben.de/misa/). To identify the presence of SSRs, only 2–10 nucleotide motifs were considered, and the minimum repeat unit was defined as 6 for di-, 5 for tri-, 4 for tetra-, 3 for penta-, and 2 for hexa-, hepta-, octa-, nona- and deca-nucleotides [20]. The characteristics of SSRs were obtained by statistical analysis from the MISA files. The information from MISA was used for designing primers flanking the SSR motif. The Primer3 software (v2.3.5) was used to design primers flanking the SSR loci [21]. The following parameters were used: primer length 18–24 base pairs (bp), with 20 bp as an optimum; primer GC % = 20–80%, with an optimum value of 50%; primer Tm 55–65 °C, with 60 °C as an optimum, and product size ranging from 150 to 500 bp. In silico polymorphism analysis of SSR markers was performed using the virtual PCR approach, in which pairs of primer sequences from the reference were mapped onto the consensus sequence of each sample [22]. We also obtained SSR marker information from the previous reports [15, 16].

Polymorphism test of SSR marker candidates

Putative polymorphic SSR markers were selected from the routine PCR using genomic DNAs from 11 cultivars belonging to six citrus groups, such as lemon, mandarin, sour orange, citron, orange, and tangor. PCR was conducted using the ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 μL, containing 20 ng DNA, 5 μL 2x HS™ Taq mix (Dongsheng Biotech, China), and 0.2 μL of each 10 pmol forward and reverse primers. The conditions for PCR amplification were as follows: 5 min for initial denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55–58 °C, and 1 min at 72 °C, concluding with 1 cycle of 10 min at 72 °C. PCR products were separated on a 2.5% (w/v) agarose gel to check PCR amplification and polymorphism among the tested cultivars.

Genotyping using M13-tailed PCR

The M13-tailed PCR method was used for the genotyping analysis using the selected polymorphic SSR markers [23]. PCR was conducted using the ABI 2720 thermal cycler (Applied Biosystems) in a total volume of 10 μL, containing 20 ng DNA, 5 μL 2x HS™ Taq mix (Dongsheng Biotech), 0.2 μL 10 pmol M13-tailed forward primer, 1 μL 10 pmol reverse primer, and 1 μL 10 pmol 6-FAM labeled M13 primer (5′-6-FAM-TGTAAAACGACGGCCAGT-3′). The conditions for PCR amplification followed the protocol described previously by Schuelke [23]. Fragment analysis of the PCR products was described previously [24]. Allele sizes were analyzed using the GeneMapper software (ver. 4.0; Applied Biosystems).

Data analysis

Genetic parameters such as major allele frequency, number of alleles, genetic diversity, observed heterozygosity, and polymorphic information content were measured by calculating the shared allele frequencies using the PowerMarker software (v. 3.25) [25]. The unweighted pair group method with arithmetic mean (UPGMA) tree was implemented using the MEGA software (v. 5.0) [26], which is embedded in the PowerMarker, with the UPGMA algorithm.

Results and discussion

Polyembryony in citrus genetic resources

We collected 101 accessions of citrus genetic resources for systematic and targeted breeding programs. In citrus, the polyembryony trait is a barrier for the conventional citrus breeding by cross hybridization. Prior to the use of the collected germplasms for our citrus breeding program, we first performed polyembryony tests of mature seeds to investigate which germplasms had a polyembryo phenotype caused by nucellar embryony. Monoembryo and polyembryo phenotypes were observed in 22 and 54 genetic resources, respectively. The other 25 accessions showed a mixed type, with a mixture of seeds containing a single embryo or multiple embryos. These results of the current citrus germplasm revealed that the polyembryony trait is widely distributed in various citrus groups (Table 1). According to previous reports, the frequency of nucellar seedlings in major citrus groups ranged from 0 ('Kishiu' mandarin and 11 pumello cultivars) to 100% ('Dancy' and 'Kara' mandarins), depending on the citrus group [5, 27]. Except for the *C. grandis* 'Dangyooja' and the *Citrus* hybrid 'Haruka' that were classified into a mixed type, all of the pumellos investigated in this study were monoembryonic. In contrast, all accessions belonging to the papeda, its hybrid, and sweet orange groups showed the polyembryony phenotype. Not every seed produced by a tree with nucellar embryony trait has multiple mature embryos. Normal sexual reproduction can also occur in genotypes with the nucellar embryony trait. A genotype with this trait can produce several different types of seeds, that is, seeds with one mature nucellar embryo, seeds with multiple mature nucellar embryos, and seeds with one mature zygotic and one or more mature nucellar embryos [5, 28]. The 25 accessions showing a mixed type could be therefore classified into the polyembryony group.

Development of polymorphic SSR markers and genotyping of citrus genetic resources

We performed whole genome sequencing for four citrus cultivars: *C. clementina* 'Fina Sodea', *C. platymamma*,

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Table 1 Polyembryony test of citrus germplasm collection used in this study. M, P, and Mx indicate monoembryony, polyembryony, and mixed type, respectively

Citrus group	Species/Cultivar name	Abbr.	Embryo type	Citrus group	Species/Cultivar name	Abbr.	Embryo type
Kumquat	*Fortunella crassifolia*	YG	P	Lemon	*C. limon*	LM	P
	Fortunella spp. 'Puchimaru'	PM	M		*C. limon* 'Allen-Newman Eureka'	LM-AN	Mx
Pumello	*C. grandis* 'Dangyooja'	DYJ	Mx		*C. limon* 'Frost Nucellar Eureka'	LM-FN	Mx
	C. hassaku	PS	M		*C. limon* 'Lisbon'	RM	Mx
	C. hassaku 'Beni Hassaku'	HP	M	Lime	*C. latifolia*	Li–Cl	M
	C. maxima	MD	M	Sour orange	*C. aurantium* 'Jigak'	JI	P
	C. maxima 'Banbeiyu'	MB	M		*C. obovoidea*	KG	P
	C. maxima 'Mato Buntan'	MM	M		*C. sulcata*	SBK	Mx
	C. pseudogulgul	CH	M		*C. taiwanica*	JD	Mx
	C. pseudogulgul 'Sadoogam'	SD	M	Tangelo	*C. natsudaidai*	HK	Mx
	C. tamurana	IH	M		*C. natsudaidai* 'Kawanonatsudaidai'	GHK	MX
	Citrus spp. 'Suyou'	SU	M		*C. natsudaidai* 'Shinamanatsu'	SGH	Mx
	Citrus hybrid 'Haruka'	HRK	Mx		*C. natsudaidai* 'Tachibana Orange'	LHO	Mx
Grapefruit	*C. paradisi*	GF	P		*C. natsudaidai* 'Whanggumhagyul'	HH	Mx
	C. paradisi 'Golden Special'	GS	Mx		*Citrus* hybrid 'Minneola'	MN	P
	C. paradisi 'P. Star Ruby'	SR	P		*Citrus* hybrid 'Nova'	NV	P
Sweet orange	*C. sinensis* 'Fukuichi Navel'	BIN	P		*Citrus* hybrid 'Orlando'	OL	P
	C. sinensis 'Hamlin'	HR	P		*Citrus* hybrid 'Seminole'	SN	P
	C. sinensis 'Morita Navel'	SJN	P		*Citrus* hybrid 'Sweetspring'	SS	P
	C. sinensis 'Sanguinelli'	SG	P		*Citrus* spp. 'Sunholt'	SHT	Mx
	C. sinensis 'Seike Navel'	CGN	P	Tangor	*C. iyo* 'Miyauchiyokan'	GN	M
	C. sinensis 'Shirayanagi Navel'	BU	P		*C. iyo* 'Ootaniiyokan'	DG	Mx
	C. sinensis 'Suzuki Navel'	YMN	P		*Citrus* hybrid '56-423'	423	Mx
	C. sinensis 'Tarocco'	TR	P		*Citrus* hybrid 'Akemi'	JK	Mx
	C. sinensis 'Valencia Late'	VL	P		*Citrus* hybrid 'Amakusa'	CC	Mx
	C. sinensis 'Yoshida Navel'	GV	P		*Citrus* hybrid 'Ariake'	TG-YM	P
Mandarin	*C. clementina*	CL-02	M		*Citrus* hybrid 'Ehime Kashi No. 28'	AH28	M
	C. clementina 'Fina Sodea'	CL-01	M		*Citrus* hybrid 'Harehime'	HM	M
	C. erythrosa 'Dingjeongkyul'	DJK	Mx		*Citrus* hybrid 'Harumi'	HRM	P
	C. kinokuni	KJ	M		*Citrus* hybrid 'Hinoyutaka'	BP	P
	C. leiocarpa 'Binkyul'	BN	Mx		*Citrus* hybrid 'Kiyomi'	CK	M
	C. reticulata	BG	P		*Citrus* hybrid 'Okitsu No. 46'	HJ46	Mx
	C. reticulata (Seedless line)	BG-seedless	P		*Citrus* hybrid 'Okitsu No. 47'	HJ47	M
	C. reticulata 'Daisy'	DSY	Mx		*Citrus* hybrid 'S-1129'	S-1129	M
	C. reticulata 'Dancy'	DS	P		*Citrus* hybrid 'Satonokaori'	STNK	Mx
	C. reticulata 'Kousyun'	HC	P		*Citrus* hybrid 'Seihou'	TG-CB	P
	C. reticulata 'Nakano no.3'	JY3	P		*Citrus* hybrid 'Setoka'	SE	P
	C. reticulata 'Page'	PG	P		*Citrus* hybrid 'Shiranuhi'	BJ-01	P
	C. reticulata 'Yosida Ponkan'	GJ	P		*Citrus* hybrid 'Tamami'	TM	M
	C. sunki 'Jinkyul'	JG	P		*Citrus* hybrid 'Tsunokaori'	JJH	P
	C. tachibana 'Hongkyul'	HG	Mx		*Citrus* hybrid 'Wonkyoah-dan-baiseong No.1'	WK1	M

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Table 1 (continued)

Citrus group	Species/Cultivar name	Abbr.	Embryo type	Citrus group	Species/Cultivar name	Abbr.	Embryo type
	C. tangerina 'Pyunkyul'	PK	Mx	Papeda	*C. sphaerocarpa* 'Kabosu'	CB	P
	C. unshiu 'Aoshima 4 Gou'	M-CS	P		*C. sphaerocarpa*	DB	P
	C. unshiu 'Chungchon'	M-CC	P		*C. sudachi*	SDC	P
	C. unshiu 'Hyangmoongam'	M-HM	P		*Citrus* spp. 'Sankyul'	SK	P
	C. unshiu 'Imamura Unshiu'	M-GC	P	Papeda hybrid	*C. junos* 'Yooja'	YJ	P
	C. unshiu 'Katayama Unshiu'	M-PS	P		*C. junos* 'Tadanishiki'	DJ	P
	C. unshiu 'Kinokuni Unshiu'	M-KK	P		*C. junos* 'Sibori'	CT-MD	P
	Citrus spp. 'Inchangkyul'	IC	P		*C. junos* 'Yamane'	YS	P
	Citrus hybrid 'Hayaka'	JH	P		*C. junos* 'Jaerae'	YR	P
	Citrus hybrid 'Mihocore'	MH	P				

C. natsudaidai, and *C. natsudaidai* 'Whanggumhagyul' using the *C. clementina* 'Clemenules' genome as a reference [25], and obtained genome sequence information covering 17.68-, 16.42-, 19.18-, and 16.3-fold of the citrus reference genome (Additional file 1: Table S1). The comparative genome analysis resulted in in silico mining of 521 putative polymorphic SSRs between *C. clementina* 'Fina Sodea' and *C. platymamma* (Additional file 2: Table S2) and 169 SSRs between *C. natsudaidai* and *C. natsudaidai* 'Whanggumhagyul' (Additional file 3: Table S3).

As a first step to select polymorphic SSR markers for identifying nucellar and zygotic embryos or plants in citrus, we selected 41 EST (expressed sequence tag)-derived SSRs [15] and 77 BAC (bacterial artificial chromosome) end-derived SSRs [16]. To obtain more polymorphic SSR markers, we further selected 40 putative polymorphic SSRs mined in silico from the comparative genome analysis between *C. clementina* 'Fina Sodea' and *C. platymamma* along with 10 SSRs from the comparative analysis between *C. natsudaidai* and *C. natsudaidai* 'Whanggumhagyul'. Based on agarose gel electrophoresis and fragment analyses using these SSR markers, we screened polymorphic SSR markers among 11 cultivars belonging to several citrus groups: *C. limon* 'Lisbon' for lemon; *C. clementina*, *C. unshiu* 'Miyagawa Wase', *C. unshiu* 'Okitsu Wase', and *C. unshiu* 'Nichinan 1 gou' for mandarin; *C. natsudaidai* for tangelo, *C. sinensis* 'Sanguinelli' for sweet orange; *C. sphaerocarpa* and *C. junos* for papeda and its hybrid; and *Citrus* hybrid 'Shiranuhi' and *Citrus* hybrid 'Kiyomi' for tangor. 'Okitsu Wase' was obtained from nucellar embryo-derived seedlings of the crossing 'Miyagawa Wase' × *Poncirus trifoliata*. 'Miyagawa Wase' and 'Nichinan 1 gou' were originated from bud sport mutation of *C. unshiu* and 'Okitsu Wase', respectively [29]. No polymorphic SSR loci were identified between

nucellar embryo- or somatic mutation-derived cultivar and its corresponding parent (data not shown). From the screening, we finally selected 17 polymorphic SSR markers with high specificity and high amplification efficiency (Table 2).

As a next step, we determined the genotypes of 17 polymorphic SSR loci among 101 citrus genetic resources showing the monoembryony, polyembryony and mixed embryo phenotypes (Additional file 4: Table S4). Table 3 summarizes the characteristics of 17 SSR loci based on the fragment analysis of the 101 accessions belonging to 12 citrus groups. Several markers such as BM-CiSSR-013, -43, -137, -162, -165 failed to achieve PCR amplification from several accessions, possibly due to sequence variation, resulting in null alleles in the accessions (Additional file 4: Table S4). A total of 181 alleles, ranging from 5 (BM-CiSSR-087, and -93) to 16 alleles (BM-CiSSR-137 and -162), were observed among the 101 citrus accessions with a mean value of 10.6 alleles per locus. Major allele frequency (M_{AF}) varied from 0.22 (BM-CiSSR-159 and -162) to 0.73 (BM-CiSSR-043). The average values of genetic diversity (GD, expected heterozygosity) and polymorphism information content (PIC) were 0.70 and 0.67, ranging from 0.46 (BM-CiSSR-043) to 0.85 (BM-CiSSR-159) and from 0.43 (BM-CiSSR-087) to 0.84 (BM-CiSSR-159), respectively. The average observed heterozygosity (H_O) was 0.56 with the lowest average in BM-CiSSR-043 (0.14) and the highest average in BM-CiSSR-159 (0.86) (Table 3).

A total of 181 alleles from 17 polymorphic SSRs were used to evaluate genetic diversity and relationships among the 101 citrus accessions. A UPGMA tree was constructed based on the genetic similarity matrices among the accessions. Figure 1 illustrates results from cluster analysis based on the SSR data. The resulting tree revealed that the citrus genetic resources could be largely classified into 2 clusters. Cluster I contained

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Table 2 Information on 17 polymorphic SSR markers selected finally from the screening

Marker	Previous name	Primer sequence (5′ → 3′)	Repeat motif	Ta (°C)[a]	Size range (bp)
BM-CiSSR-012	CiBE0914[b]	GGGCTCAGTTCTTCTCTACTC GCATTAGGCTTCTCTCATACC	(TTA)15	58	283–308
BM-CiSSR-013	CiBE1098	GGTGGCATACATACATACATACA GCAACATCTGGAACTACTCA	(TA)6	58	127–175
BM-CiSSR-032	CiBE2380	GCCTGAGTTTCTTTGTTATG CATTCCATCGTCTCCTATTGT	(TATG)4	58	137–188
BM-CiSSR-043	CiBE3458	ATTAGTGCGGGTAAGATGAA AAGGATTTGGTGTAGGAAGTAA	(AAAAT)3	58	261–332
BM-CiSSR-073	CiBE6092	CGGACAAGGAGATGAAGATAG TTCTAACAGCACCAAGCAG	(GA)16	58	319–345
BM-CiSSR-077	CiBE6256	TGTATTTATTTCTGACTACGACC ATGCGTTTGGTGTGTGTT	(AT)12	58	182–207
BM-CiSSR-082	Luro0016[c]	ACCTGAGCCCTTTTTGGTTT GCCAGATCAAGGCTCAAATC	(TC)13	58	145–158
BM-CiSSR-087	Luro0032	CAGATCCTATTGCAGAGGCA GCCCATTTGTATTGCCATTT	(CAG)6	58	183–192
BM-CiSSR-093	Luro0115	CCCCCTCTTCTTTCACACAA GGTGAGCAGCCATCTTCTTC	(TA)6	58	145–166
BM-CiSSR-094	Luro0116	GAATTGGGAGGACGAACTGA CGAGCCCTAGACAGAGATGG	(AGA)7	58	265–277
BM-CiSSR-100	Luro0164	GTTTTCAGCTGGATTCGAGG CACGTGTCCTCCTGGAACTT	(GCC)5	58	192–206
BM-CiSSR-111	Luro0430	CCGATACAGCACAAAGCAAA TGGAAAGAGAGAAGCCAAGC	(AAT)7N15(AGC)7	58	134–147
BM-CiSSR-115b	Luro0817	CGGTGTGTATTGGGTACACG GCTTTTTCGAAAGCGTCAAG	(TA)17	58	231–255
BM-CiSSR-137	In this study	GCAACGTGTACTGACGCTTG GCTCGTATCTGAAGCTCGCC	(TAT)7	53	292–330
BM-CiSSR-159	In this study	ATGACCTCAAACGGTGAGCA CTTCCACATCCGAACCGACA	(GAGG)5	53	378–404
BM-CiSSR-162	In this study	GCTAGGGTTCCAGACTTCCAG GATTTGGCCGATCGAAAGCC	(AAT)10(CAT)6	53	180–210
BM-CiSSR-165	In this study	AGCAACTTAAGGTCCTTCACGA TTCTCTGCTCTGCTGTGCAT	(AAT)6	53	393–448

[a] Ta means annealing temperature. [b]Information of SSR markers starting with CiBE was obtained from Ollitrault et al. [16]. [c]SSR markers starting with Luro were obtained from Luro et al. [15]

2 accessions of the kumquat group (*F. crassifolia* and *Fortunella* spp. 'Puchimaru'), which belongs to the genus *Fortunella*. Cluster II contained the remaining 99 accessions belonging to the genus *Citrus*. However, except for the papeda and its hybrid, limon, lemon, and sweet orange groups, several groups were fragmented and/or scattered throughout the entire UPGMA tree and did not form unique groups. For example, accessions of the mandarin group were largely fragmented into 3 subclades. This fragmentation and/or scattering patterns are possibly attributable to frequent natural or intended genetic crossings. *C. sinensis* 'Hamlin'

and *C. sinensis* 'Tarocco' of the sweet orange group showed completely identical genotypes for 17 SSR loci. These isogenic genotypes were also found between *C. sinensis* 'Suzuki Navel' and 'Valencia Late', between *C. reticulata* 'Dancy' and 'Yosida Ponkan', and between *C. reticulata* (seedless line) and 'Nakano no.3.' To discriminate two accessions with isogenic genotypes, more polymorphic SSR loci would be needed. Barkley et al. [30] assessed genetic diversity and population structure in a citrus germplasm collection utilizing 24 SSR markers. The kumquat group also consisted of an independent cluster in the previous report [30], as shown in our

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Table 3 Characteristics of 17 polymorphic SSR markers developed in this study

Markers	SS	N_{OBS}	Availability	M_{AF}	N_A	GD	H_O	PIC
BM-CiSSR-012	101	101	1.00	0.42	7	0.75	0.62	0.72
BM-CiSSR-013	101	98	0.97	0.35	10	0.77	0.29	0.73
BM-CiSSR-32	101	101	1.00	0.49	14	0.72	0.67	0.70
BM-CiSSR-043	101	92	0.91	0.73	12	0.46	0.14	0.45
BM-CiSSR-073	101	101	1.00	0.37	13	0.77	0.84	0.75
BM-CiSSR-077	101	101	1.00	0.55	14	0.67	0.62	0.65
BM-CiSSR-082	101	101	1.00	0.48	9	0.71	0.40	0.68
BM-CiSSR-087	101	101	1.00	0.62	5	0.51	0.50	0.43
BM-CiSSR-093	101	101	1.00	0.63	5	0.53	0.62	0.47
BM-CiSSR-094	101	101	1.00	0.41	8	0.67	0.60	0.62
BM-CiSSR-100	101	101	1.00	0.42	7	0.68	0.85	0.62
BM-CiSSR-111	101	101	1.00	0.47	8	0.64	0.43	0.58
BM-CiSSR-115b	101	101	1.00	0.47	11	0.74	0.48	0.71
BM-CiSSR-137	101	100	0.99	0.34	16	0.80	0.84	0.78
BM-CiSSR-159	101	101	1.00	0.22	14	0.85	0.86	0.84
BM-CiSSR-162	101	99	0.98	0.22	16	0.84	0.58	0.82
BM-CiSSR-165	101	99	0.98	0.31	12	0.80	0.18	0.78
Mean	101	100	0.99	0.44	10.6	0.70	0.56	0.67

SS sample size, N_{OBS} number of observations, M_{AF} major allele frequency, N_A number of alleles, *GD* genetic diversity, which is often referred to as expected heterozygosity, H_O observed heterozygosity, *PIC* polymorphism information content
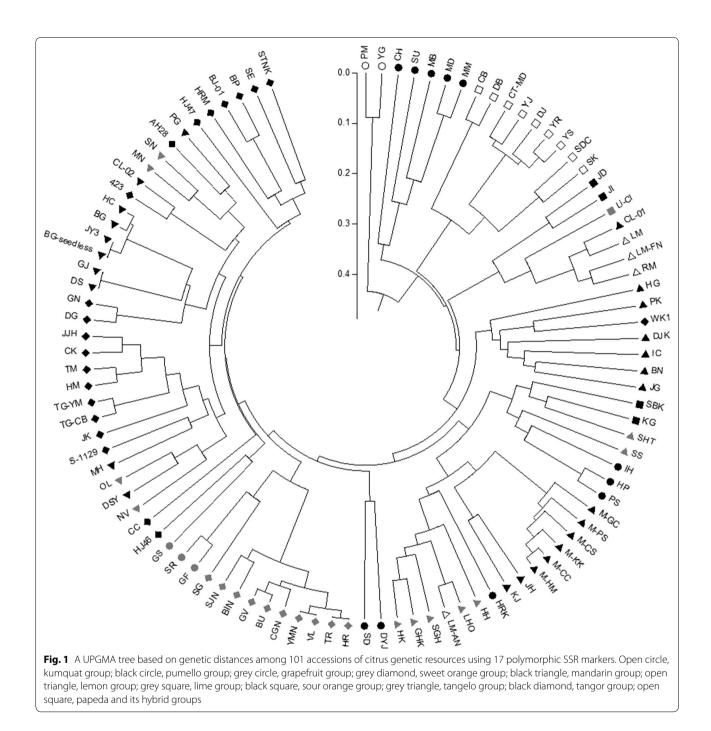
UPGMA tree (Fig. 1). The PIC value obtained from 17 SSR markers in this study was higher than 0.625 of the previous report [30]. Biswas et al. [20] mined a total of 50,846 SSRs from genome wide in silico analysis of the sweet orange and identified 534 SSRs showing polymorphism among only 16 citrus accessions covering 7 citrus species. Two-thirds of the marker were transferable to 16 citrus relatives [20]. In contrast, 17 markers developed in this study were transferable to all 101 accessions belonging to 28 species (Additional file 4: Table S4), indicating the universality and versatility of our SSR markers.

Application of SSR markers to genetic crosses

While polyembryony is a unique characteristic in citrus, it serves as an obstacle of citrus breeding by traditional genetic hybridization. To better understand the underlying molecular mechanism of the polyembryony phenomenon in citrus, genes associated with the polyembryony have been characterized [31]. Several markers associated with polyembryony trait in citrus, including QTL (quantitative trait loci), SCAR (sequence characterized amplified regions), and AFLP (amplified fragment length polymorphism), have been reported [32–34]. To increase breeding efficiency and to save breeding cost by identifying nucellar and zygotic embryo-derived plants, molecular markers including SSR, ISSR, and SCAR also have been developed [11–13, 33]. Polyembryony test for the

germplasm collection revealed that the polyembryony trait is widely distributed in various citrus groups, except for pumello (Table 1).

We investigated whether SSR markers showing polymorphism between female and male parents could effectively identify zygotic embryo-derived individuals from F_1 plant population, which was derived from the genetic crossing combinations using polyembryonic cultivars as a female partner. We crossed polyembryonic cultivars, such as *Citrus* hybrid 'Setoka', *Citrus* hybrid 'Harumi', *C. reticulata* 'Nova', and *C. reticulata* 'Page' as a female parent, to either *C. maxima* 'Banbeiyu' or *C. maxima* as a male parent. As a control, we crossed monoembryonic cultivars, such as *Citrus* hybrid 'Kiyomi', *Citrus* hybrid 'Ehime Kashi No. 28', and *Citrus* hybrid 'Harehime' as a female parent, to either *Citrus* hybrid 'Seminole' or *Citrus* hybrid 'Orlando' as a male parent (Table 4). SSR markers and their allele sizes showing polymorphism between female and male parents are listed in Table 4 and Additional file 4: Table S4. We performed genotyping analysis for identifying between nucellar and zygotic embryo-derived individuals for eight different genetic crosses using the selected SSR markers. Among the five genetic crosses using polyembryonic cultivars as a female parent, we found one zygotic individual among 98 plants investigated from the 'Setoka' × 'Banbeiyu' cross and three zygotic individuals among 48 plants
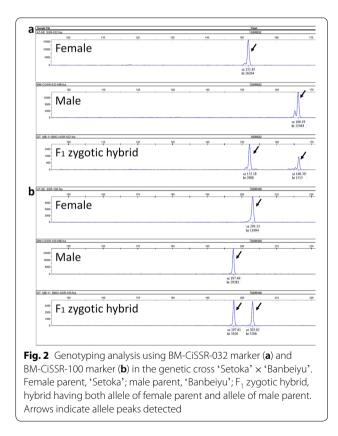
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Fig. 1 A UPGMA tree based on genetic distances among 101 accessions of citrus genetic resources using 17 polymorphic SSR markers. Open circle, kumquat group; black circle, pumello group; grey circle, grapefruit group; grey diamond, sweet orange group; black triangle, mandarin group; open triangle, lemon group; grey square, lime group; black square, sour orange group; grey triangle, tangelo group; black diamond, tangor group; open square, papeda and its hybrid groups

from the 'Page' × 'Banbeiyu' cross (Table 4, Figs. 2 and 3). However, no zygotic individuals were found from the 'Harumi' × 'Banbeiyu', 'Nova' × 'Banbeiyu', and 'Setoka' × *C. maxima* crosses (Table 4). In contrast, all F$_1$ individuals from three genetic crosses using monoembryonic cultivars as a female parent (that is, 'Kiyomi' × 'Seminole', 'Ehime Kashi No. 28' × 'Orlando', and 'Harehime' × 'Orlando' crosses)

were zygotic plants that have both alleles of the female parent and alleles of the male parent (Table 4).

Three plants from the genetic cross 'Page' × 'Banbeiyu' were identified as zygotic individuals. Genotype analysis using BM-CiSSR-115b resulted in either the allele of the male parent having low peak intensity for two individuals (No. 37 and 45) or the allele of the male parent without the allele of
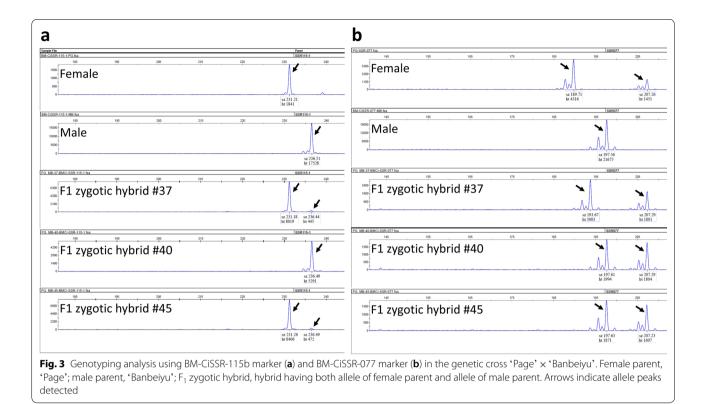
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Table 4 Frequency test of genetic hybrids from various genetic crosses using polymorphic SSR markers

Genetic crosses	Embryo type of female plant	Used SSR markers	No. of tested plant	No. of genetic hybrid
Citrus hybrid 'Setoka' × *C. maxima* 'Banbeiyu'	P	BM-CiSSR-032 BM-CiSSR-100	98	1
Citrus hybrid 'Harumi' × *C. maxima* 'Banbeiyu'	P	BM-CiSSR-115b	38	0
C. reticulata 'Nova' × *C. maxima* 'Banbeiyu'	P	BM-CiSSR-115b	98	0
C. reticulata 'Page' × *C. maxima* 'Banbeiyu'	P	BM-CiSSR-115b BM-CiSSR-077	48	3
Citrus hybrid 'Setoka' × *C. maxima*	P	BM-CiSSR-032	54	0
Citrus hybrid 'Kiyomi' × *Citrus* hybrid 'Seminole'	M	BM-CiSSR-013	5	5
Citrus hybrid 'Ehime Kashi No. 28' × *Citrus* hybrid 'Orlando'	M	BM-CiSSR-012 BM-CiSSR-087	5	5
Citrus hybrid 'Harehime' × *Citrus* hybrid 'Orlando'	M	BM-CiSSR-012 BM-CiSSR-013 BM-CiSSR-087	6	5

P polyembryony, *M* monoembryony

Fig. 2 Genotyping analysis using BM-CiSSR-032 marker (**a**) and BM-CiSSR-100 marker (**b**) in the genetic cross 'Setoka' × 'Banbeiyu'. Female parent, 'Setoka'; male parent, 'Banbeiyu'; F₁ zygotic hybrid, hybrid having both allele of female parent and allele of male parent. Arrows indicate allele peaks detected

the female parent for one individual (No. 40) (Fig. 3a). To further determine if these three individuals are zygotic plants, we performed genotype analysis using the BM-CiSSR-077 marker. The results identified two individuals (No. 40 and 45) with both alleles of the female parent and allele of the male parent, indicating that the plants are zygotic. In contrast, one of the two

alleles detected from the No. 37 plant was identical to that of the female parent, whereas the other allele was a new allele that was not present in either female or male parent (Fig. 3b). In the No. 40 plant, the allele of only the male parent was detected from the genotype analysis using BM-CiSSR-115b (Fig. 3a), whereas alleles of both female and male parents were detected from the genotyping analysis using BM-CiSSR-077 (Fig. 3b). Genotyping analysis on the female parent 'Page' for the Bm-CiSSR115b locus detected a single allele (231 bp) for the genetic locus (Additional file 4: Table S4), indicating that the locus is possibly homozygous. However, the Bm-CiSSR115b locus in the female parent 'Page' was essentially in the heterozygous state containing a null allele on either chromosome. Therefore, the F₁ plant derived from fertilization with a female gamete having the null allele contained an allele only from the male parent for the Bm-CiSSR115b locus. A new allele that was not present in either the female or male parent was detected from the genotyping analysis in the No. 37 plant for the BM-CiSSR-077 locus. The BM-CiSSR-077 marker is composed of a 2 bp repeat (AT) motif (Table 2). The new allele detected in the F1 plant was 4 bp longer or shorter than the allele of either female or male parent (Fig. 3B). SSR mutation rates are known to be very high (10^{-2} to 10^{-6} events/genetic locus/generation) compared with the point mutation rates at coding gene loci [35]. The instability of SSR is largely manifested as changes in the numbers of the repeat motif. Two major mechanisms of SSR mutation (increase or decrease in repeat units) involve DNA slippage during DNA replication and recombination between DNA strands [35, 36]. Therefore, the occurrence of a new allele for BM-CiSSR-077 in the No. 37 plant is possibly

Fig. 3 Genotyping analysis using BM-CiSSR-115b marker (**a**) and BM-CiSSR-077 marker (**b**) in the genetic cross 'Page' × 'Banbeiyu'. Female parent, 'Page'; male parent, 'Banbeiyu'; F₁ zygotic hybrid, hybrid having both allele of female parent and allele of male parent. Arrows indicate allele peaks detected

caused by one of these two mechanisms during the production of female and/or male gametes.

The genotyping analysis using polymorphic SSR markers on F_1 plants that were derived from the genetic crosses using various polyembryonic cultivars as a female parent revealed that polyembryony not only decreases the breeding efficiency, but also enormously increases the breeding cost. Therefore, our results demonstrate that the identification of zygotic embryo-derived individuals using the polymorphic SSR markers may be a powerful tool for establishing a systematic molecular breeding program in citrus.

In conclusion, polyembryony, which is caused by sporophytic apomixis and is a unique botanical trait in citrus, hinders its breeding by genetic hybridization and affects breeding efficiency and cost. In this study, we determined embryo types of 101 citrus genetic resources that we collected for targeted and systematic breeding program. We also developed 17 polymorphic SSR markers to efficiently identify nucellar and zygotic individuals. Application of the polymorphic SSR markers to F_1 individuals derived from the genetic crosses using polyembryonic cultivars as an ovule donor revealed that the identification of zygotic embryo-derived individuals using the SSR markers would be a powerful genetic tool for establishing a systematic molecular breeding program in citrus. The powerful transferability and versatility

of the markers developed in this study suggest that the markers are valuable for diverse genetic studies including population structure analyses, cultivar identification, and identification of genetic hybrids in citrus and its relatives.

Additional files

Additional file 1. Genome sequence information obtained from *C. clementina* 'Fina Sodea', *C. platymamma*, *C. natsudaidai*, and *C. natsudaidai* 'Whanggumhagyul' after trimming of raw reads.

Additional file 2. Distribution pattern of putative SSRs showing polymorphism between *C. clementina* 'Fina Sodea' and *C. platymamma*.

Additional file 3. Distribution pattern of putative SSRs showing polymorphism between *C. natsudaidai* and *C. natsudaidai* 'Whanggumhagyul'.

Additional file 4. Allele size data of 101 citrus genetic resources determined by 17 polymorphic SSR markers.

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Authors' contributions

Competing interests

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