Identification of ‘Haryejosaeng’ mandarin by multiplex SNP genotyping

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Abstract

Background

Most of the satsuma mandarin (*Citrus unshiu* Marc.) cultivars grown on Jeju Island farms, Korea, are difficult to improve through hybridization because of polyembryony and male sterility. Therefore, their improvement has mostly been based on the selection of nucellar embryo and bud mutation. These cultivars are supplied to breeders and farms at the seedling stage, which renders their identification based only on morphological traits. In addition, because these seedlings originate from nucellar embryo and bud mutation selection, they are genetically very similar. Therefore, the present study was carried out to develop markers that can specifically and rapidly distinguish 'Haryejosaeng,' which is generally supplied to breeders, from other satsuma mandarin cultivars that are planted on farms.

Results

Polymerase chain reaction (PCR) was performed to distinguish 'Haryejosaeng' from other 8 cultivars ('Haryejosaeng'- breeder's stock, 'Miyagawa wase,' 'Okitsu wase,' 'Yura wase,' 'Miyamoto wase,' 'Ueno wase,' 'Yonezawa wase,' and 'Nichinan 1 gou') using 6 single nucleotide polymorphism (SNP) markers specific for 'Haryejosaeng' and one SNP primer pair, which was used as the negative control. Using a multiplex PCR, SNP markers P1 (HL-SNP-SCAF_2-23997586-F and HL-SNP-SCAF_2-23997586-R), P2 (HL-SNP-SCAF_2-36059523-F and HL-SNP-SCAF_2-36059523-R), and P5 (HL-SNP-SCAF_9-30793978-F and HL-SNP-SCAF_9-30793978-R) simultaneously yielded 165, 150, and 526 bp amplicons, respectively, for Haryejosaeng only. The SNP markers were further validated by high-resolution melting analysis. The multiplex PCR based on P1/P5 and P2/P5 was also used to identify 'Haryejosaeng' on a farm growing 17 different cultivars of satsuma mandarin.

Conclusions

We developed specific molecular markers for accurate identification of 'Haryejosaeng,' which can be performed by multiplex PCR to save time and cost associated with the supply of 'Haryejosaeng' to farms.

Background

The most common satsuma mandarin (*Citrus unshiu* Marc.) cultivars on Jeju Island are 'Miyagawa wase' and 'Okitsu wase' [1]. Nucellar seedlings (plants developed from nucellar embryos) of the high-quality cultivar 'Haryejosaeng' [2], selected by crossing 'Tachima wase' with *C. × natsudaidai* 'Hayata' in the Citrus Research Institute, Jeju, Korea, are currently distributed to farmhouses. The fruits of this cultivar are bright orange-yellow and mature in early November; they weigh approximately 80–90 g and have a more compressed-oblate globose shape than 'Miyagawa wase' fruits (the main cultivar grown in Jeju). Furthermore, their rind has the characteristic thickness of mandarin fruits that allows easy peeling. The tree vigor of 'Haryejosaeng' is stronger, total soluble solids in its fruits are approximately 1 Brix higher, and acid content is approximately 0.1% lower than those of 'Miyagawa wase' [2].

It is generally difficult to improve satsuma mandarin cultivars through hybridization because of polyembryony and male sterility [3]. Accordingly, most of these cultivars have been improved based on nucellar embryos [one of the main features of seed reproduction in citrus cultivars; nucellar embryos are developed from nucellar cell tissues surrounding the embryo sac in citrus [4]] and selection of bud mutants with low citric acid and high sugar content (i.e., bud mutation selection). As such, these plants have the same genetic traits as the maternal line [5, 6], and variants are difficult to identify based on morphological traits only. For instance, 'Okitsu wase' is an improved cultivar originating from the selection of nucellar seedlings of 'Miyagawa wase' [7], whereas 'Morita Unshiu' was selected from a bud mutation of 'Miyagawa wase' and 'Nichinan 1 gou' was selected from a bud mutation of 'Okitsu wase' [2, 8, 9]. As per the guidelines of the International Union for the Protection of New Varieties of Plants (UPOV) and Korea Seed and Variety Service (KSVS), it is necessary to develop technologies that can specifically distinguish 'Haryejosaeng' from other mandarin cultivars being cultivated domestically and abroad [10] to protect the intellectual property rights of breeders. Molecular markers based on DNA sequences and amplified fragment-length polymorphisms (AFLPs), including restriction fragment-length polymorphisms (RFLPs), cleaved amplified polymorphic sequences (CAPS), random amplified polymorphic DNA (RAPD), expressed sequence tag (EST)-simple sequence repeats (SSRs), and genomic-SSRs, have been used for this purpose [11-17]. However, these methods have limitations; they are complex and time-consuming, and *the reproducibility of some of these molecular markers* is poor [18].

Single nucleotide polymorphism (SNP) markers are based on frequent variations in nucleotides at a specific position in the genome [19-21], and they have been widely used in genetic studies in plants and animals [20, 22-27]. Molecular markers can be categorized as dominant (such as RAPD and AFLPs) or co-dominant (such as SSRs, CAPS, and RFLPs), depending on the method used to search for these markers. Furthermore, depending on the method of analysis, SNPs may show dominance or co-dominance [28-31]. Different techniques have been used for searching SNPs, including microarray-, DNA chip-, and polymerase chain reaction (PCR)-based SNP search methods, such as high-resolution melting (HRM) analysis [18, 32, 33], single-strand conformation polymorphism (SSCP) [34, 35], CAPS [36, 37], and temperature gradient gel electrophoresis (TGGE) [38]. Compared to the other markers, PCR-based SNP markers can discriminate cultivars rapidly and at a low cost [20, 39-42]. These SNP markers have been utilized for selecting genes associated with useful traits in tomato varieties [18], disease-resistance genes in rice [43], and root rot-related genes in lettuce [18, 31, 44]. In citrus, SNP markers have been used for interspecific classification and genotype sequencing analysis [32, 45]. However, there are no reports on the use of these markers in cultivars that are difficult to distinguish morphologically and genetically, such as those of *C. unshiu* mandarin.

In this context, the present study was conducted to identify SNP markers that can discriminate between 'Haryejosaeng' and other cultivars, and to rapidly verify those markers using the HRM analysis. In addition, we developed a method based on multiplex PCR for rapid, cost-effective, accurate, and reproducible selection of 'Haryejosaeng.'

Results

Selection of SNP markers

After trimming the raw data obtained from sequencing, the genome coverage for each of the cultivars was contrasted with the standard genome of *C. × clementina* 'Clemenules' (approximately 367 Mbp); it was 20.70 times for 'Haryejosaeng,' 17.92 times for 'Miyagawa wase,' 18.14 times for 'Miyamoto wase,' 16.14 times for 'Okitsu wase' and 17.66 times for 'Nichinan 1 gou' (Table 1). The mapping ratio of the reads to the *C. × clementina* 'Clemenules' genome ranged from 90.45% to 91.44% (Table 1). Among the 49,111 SNPs from the interspecific integrated SNP matrix based on the consensus sequence obtained by mapping the reads of 'Haryejosaeng' and 'Miyagawa wase' to the genome sequence of *C. × clementina* 'Clemenules,' 3,639 SNPs were selected for designing the primers (data not shown). In addition, 77 candidate SNPs were selected by comparing the sequences of 'Haryejosaeng' with those of 'Miyagawa wase,' 'Miyamoto wase,' 'Okitsu wase,' and 'Nichinan 1 gou' using Integrative Genomics Viewer (IGV) software (Additional file 1: Table S1). Forty-one candidate markers with GC content suitable for primer design were selected. Based on the results mentioned above, 6 positive markers and one negative marker were selected using a PCR assay of 'Haryejosaeng' and control cultivars (Table 1 and Additional file 2: Table S2).

Selection of specific SNP markers for 'Haryejosaeng'

To specifically select 'Haryejosaeng,' PCRs were carried out for the SNP markers selected from among the 41 candidate markers (Table 2 and Additional file 2: Table S2) using samples from 8 *C. unshiu* mandarin cultivars (the breeder's stock of 'Haryejosaeng,' 'Miyagawa wase,' 'Okitsu wase,' 'Yura wase,' 'Miyamoto wase,' 'Ueno wase,' 'Yonezawa wase,' and 'Nichinan 1 gou'). The PCR products that were specifically amplified in 'Haryejosaeng' were obtained using only the SNP primer pairs P1 (Fig. 1a), P2 (Fig. 1b), and P5 (Fig. 1e), whereas, no amplification products were obtained with primer pairs P3 (Fig. 1c), P4 (Fig. 1d), and P6 (Fig. 1f); therefore, these 3 SNP primer combinations (P1, P2, and P5) were suitable markers for the identification of 'Haryejosaeng.' In addition, the primer set P7 amplified DNA fragments of the same size in all cultivars (Fig. 1g), suggesting that it could be used as a negative control.

Identification of 'Haryejosaeng' cultivars through HRM

Multiplex PCR using the SNP markers

A multiplex PCR was employed to obtain amplified products in a single PCR using the selected discrimination markers (P1/P5 and P2/P5) for 'Haryejosaeng.' The P1 and P5 primer pairs simultaneously produced amplicons of approximately 160 and 550 bp, respectively, in the multiplex PCR. *In contrast, none of the reactions performed on other cultivars yielded amplicons* (Fig. 3a). In addition, the exact size of the amplified product and the non-specific amplicons confirmed that the size of the amplified PCR products was 165 and 526 bp for P1 and P5, respectively; no non-specific bands appeared in other cultivars (Fig. 3b). The P2 and P5 primer pairs simultaneously produced amplicons of

approximately 150 and 550 bp, respectively, in the multiplex PCR. In contrast, none of the reactions performed on other cultivars generated amplicons (Fig. 3c). In addition, the exact size of the amplified product and the non-specific amplicons confirmed that the size of the amplified PCR products was 150 and 526 bp for P2 and P5, respectively, and no non-specific bands appeared in other cultivars (Fig. 3d).

Verification of 'Haryejosaeng' in farmhouses

A multiplex PCR was performed using the SNP markers P1/P5 and P2/P5 to specifically identify 'Haryejosaeng' among the 17 *C. unshiu* cultivars grown on the farms. The 165 and 526 bp PCR products were obtained only for 'Haryejosaeng' when using the P1 and P5 (Fig. 4a) and P2 and P5 (Fig. 4b) SNP primer sets (Table 2).

Discussion

Most citrus cultivars reproduce by sexual hybridization, frequent somatic mutation, and nucellar polyembryony; the latter is a feature of citrus in which the nucellar tissue that surrounds the embryonic sac containing the zygotic embryo develops one or more embryos that have the same genetic constitution as the maternal tissue cells [32, 51-53]. Satsuma mandarin cultivars have been particularly difficult to improve through hybridization because of polyembryony and male sterility [3, 54]. Accordingly, satsuma mandarin cultivars are bred by asexual methods, such as nucellar embryos [4, 53] and bud mutation [3, 6], and therefore have the same genetic and morphological traits as the mother plant [4]. 'Haryejosaeng' was developed from nucellar seedlings [4] of satsuma mandarin ('Tachima wase'), and it shows higher total soluble solids, and lower acidity than the most common satsuma mandarin ('Miyagawa wase') cultivated on farms [2] and has longer juvenile stages [55]. It is, therefore, difficult to morphologically distinguish the cultivars supplied to farms at the seedling stage [8].

Recently, the genotypes of satsuma mandarin were analyzed using SSR markers, but they could not be distinguished [56], suggesting both morphological and genetic similarities among the different cultivars. Although only 3 of the 7 sets of SNP markers used in the present study could differentiate 'Haryejosaeng' from other satsuma mandarin cultivars, this SNP-based method can be utilized to identify molecular markers capable of distinguishing very close varieties by relying on the differences among nucleotide sequences [18, 32]. Therefore, we identified SNP markers that were heterozygous in 'Haryejosaeng' and homozygous in 4 control cultivars, as well as SNP markers that were homozygous in 'Haryejosaeng' and heterozygous in 4 control cultivars (Additional file 1: Table S1). Using these markers, we could specifically distinguish 'Haryejosaeng' from other cultivars.

We identified 77 candidate SNP markers and selected 6 PCR-positive and one PCR-negative SNP markers among them. The efficacy of these markers in discriminating between cultivars was evaluated (Fig. 1a and e). Because DNA amplification and sequencing are not required for confirming the results of PCR-based HRM analysis, this methodology can be used to verify the efficiency of SNP markers and detect SNP mutations [41, 57, 58]. It is a simple, fast, and inexpensive method for detecting polymorphisms [41, 42, 59]. However, because of the high cost of the equipment required for the HRM analysis, PCR-based

SNP marker analysis is considered more efficient than PCR-based HRM analysis [40, 42]. Among the 6 positive SNP markers (P1–P6), we were able to select 3 (P1, P2, and P5) that could distinguish 'Haryejosaeng' from other cultivars at the early seedling stage. Sequence data of these 3 selected SNP markers are listed in Additional file 1: Table S1 (scaffold_2:23997586; scaffold_2:36059523; scaffold_9:30793978). We also carried out PCR using the 3 selected SNP primers (P1, P2, and P5) for 6 other satsuma mandarin cultivars ('Iwasaki wase,' 'Yamsitabeni wase,' 'Nankan 20 gou', 'Morita unshiu,' 'Ooura wase,' and 'Hiroshimakaken 7 gou') in addition to the 8 main satsuma mandarin cultivars. No amplified product was obtained in these 6 cultivars (data not shown), further confirming the specificity of the 3 selected SNP primers for 'Haryejosaeng.' The differences in amplicon size obtained by these 3 markers (P1, P2, and P5) was approximately 350–400 bp, which allowed us to perform a multiplex PCR. Thus, compared with the methods using a single primer pair [60–63], our method offers reduced time and cost of analysis. Overall, the application of the results obtained in the present study will reduce the time and cost involved in the identification of 'Haryejosaeng' mandarin at the seedling stage, a cultivar that is commercially supplied to farmers.

Conclusion

A method that can distinguish between *C. unshiu* mandarin cultivars based on their genetic characteristics has not yet been developed. It is, therefore, important to develop markers to identify breeds suitable for cultivation. The multiplex PCR method using the P1, P2 and P5 SNP markers selected in the present study is expected to reduce the time and cost associated with the supply of 'Haryejosaeng' to farms.

Methods

Plant materials and genomic DNA isolation

To identify the SNP markers and perform the HRM analysis suitable for selection of 'Haryejosaeng,' samples were collected from 7 main satsuma mandarin (*C. unshiu*) cultivars ('Miyagawa wase,' 'Okitsu wase,' 'Yura wase,' 'Miyamoto wase,' 'Ueno wase,' 'Yonezawa wase,' and 'Nichinan 1 gou') and from the original tree of 'Haryejosaeng' growing at the Citrus Research Institute (National Institute of Horticultural & Herbal Science, Jeju, Korea) (Table 3). For rapid and accurate selection of 'Haryejosaeng' at the seedling stage by multiplex PCR using the selected SNP markers, samples were collected from the following 17 cultivars: 'Taguchi wase,' 'Yura,' 'Hiroshimakaken 7 gou', and 'Higo' from the citrus nursery at the orchard of the Citrus Cooperative Society on Jeju Island; summer leaf of an outdoor cultivar 'Miyagawa wase'; 'Haryejosaeng-1' from a Daejeong farm; 'Haryejosaeng' from a Jeoji-ri farm; 'Haryejosaeng-2' and 'Imprecision wase-1' from agricultural cooperatives; 'Imprecision wase-2-1' and 'Imprecision wase-2-2' from a farm; 5 seedling cultivars, namely 'Haryejosaeng-S1', 'Miyagawa wase,' 'Haryejosaeng-S2', 'Nichinan 1 gou', and 'Yura wase'; breeder's stock of 'Haryejosaeng-BS,' which was improved by selection of nucellar seedlings (i.e., seedlings physiologically different from the mother plant, 'Tachima wase,' and reproduced by apomixis; 'Tachima wase' was produced by crossing the satsuma

mandarin ‘Tachima wase’ with pollens of *C. natsudaidai* ‘Hayata’; the total soluble solids to acidity rate is higher in the improved cultivar than in the common farm cultivar ‘Miyagawa wase’ and in the mother plant) (Table 3). Total genomic DNA was extracted from the samples by automatic nuclear extraction (MX 16; Promega, Madison, WI, USA) and stored at -20 °C until use.

Primer design for the SNP/HRM analysis

For designing the primers, we first performed standard genome sequencing of 4 control cultivars (‘Miyagawa wase,’ ‘Miyamoto wase,’ ‘Okitsu wase,’ and ‘Nichinan 1 gou’) on the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) using the genome of *Citrus* × *clementina* ‘Clemenules’ as reference. The sequence information (approximately 6 Gb) was obtained according to a previously established method [46]. A paired-end DNA library was constructed for genome sequencing using the TruSeq DNA Library Prep Kit (Illumina). The generated raw reads were trimmed using DynamicTrim and LengthSort in the SolexaQA package (v.1.13) [47]. The clean reads were mapped to the standard genome of *C.* × *clementina* ‘Clemenules’ [46], and the consensus sequence was obtained using the Burrows-Wheeler Aligner (BWA) program [48].

The SNPs were identified in the consensus polymorphic SNP locus through the comparative genomic analysis of ‘Haryejosaeng’ and ‘Miyagawa wase’ (Additional file 1: Table S1) using the IGV software (http://software.broadinstitute.org/Software/igv/). These SNPs were then compared with those in the genomes of ‘Miyamoto wase,’ ‘Okitsu wase,’ and ‘Nichinan 1 gou’ (Additional file 1: Table S1).

Using the selected SNPs, SNP-specific primers were synthesized for the identification of ‘Haryejosaeng.’ The SNPs were located at the 3'-end of each sequence, and, for specificity, an artificial SNP was introduced in the third base at the 3'-end. In addition, for the re-verification of the SNP marker, primer sets for the HRM analysis were designed on both sides of the SNP locus based on the results of genome sequencing (Table 4).

Cultivar selection using the PCR-based SNP markers

For the selection of ‘Haryejosaeng,’ multiple PCRs were carried out using the different primer sets (6 pairs) and a negative control primer set (Table 4). A multiplex PCR was performed by utilizing a combination of 2 selected SNP primers. All the PCRs were performed under the same conditions.

The PCR mixture contained 15–20 ng total genomic DNA, 0.25 μM of each primer, AccuPower® Multiplex PCR PreMix (250 μM dNTPs, 1.5 mM $MgCl_2$, 1.0 unit Taq DNA polymerase, 10 mM Tris–HCl [pH 9.0], 40 mM KCl; Bioneer Corp., Daejeon, Korea), and the volume was adjusted to 20 μL with nuclease-free water [49]. The PCRs were performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C (50 °C for P7 primer) for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were electrophoresed on a 1.2%–1.5% agarose gel at 100 V for 30 min or were reconfirmed using a QiAxcel Advanced System electrophoresis device (Qiagen, Hilden, Germany).

For the selection of ‘Haryejosaeng,’ multiple PCRs were carried out using the different primer sets (6 pairs) and a negative control primer (one set; Table 4). A multiplex PCR was performed using a combination of 2 selected SNP primers. All the multiplex PCRs were performed under the same conditions.

The multiplex PCR mixture contained 15–20 ng total genomic DNA, 0.25 μM each primer, and AccuPower® Multiplex PCR PreMix [250 μM dNTPs, 1.5 mM $MgCl_2$, 1.0 unit Taq DNA polymerase, 10 mM Tris–HCl (pH 9.0), 40 mM KCl; Bioneer Corp.]; the final volume was adjusted to 20 μL with nuclease-free water [49]. The PCRs were performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The multiplex PCR products were electrophoresed on a 1.2% agarose gel at 100 V for 30 min or were reconfirmed using a QiAxcel Advanced System electrophoresis device (Qiagen).

HRM analysis

The HRM analysis was performed according to the modified method described by Park et al. [50] on a Roche LightCycler® 480 II (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The reaction mixture included 15–20 ng total genomic DNA and 0.025 μM forward and reverse primers (Table 4), 1.8 μL of 25 mM $MgCl_2$, and 7.5 μL LightCycler 480 High-Resolution Melting Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), and the volume was adjusted to 15 μL with sterile water. The PCR conditions included pre-denaturation at 95 °C for 5 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. In the subsequent step, the mixture was subjected to 95 °C for 1 min and then to 40 °C for 1 min; this was followed by an increase in temperature from 65 °C to 95 °C at a rate of 0.1 °C/s, and the volume was adjusted to 20 μL using nuclease-free water.

Abbreviations

AFLP, amplified fragment-length polymorphism; CAPS, cleaved amplified polymorphic sequence; EST, expressed sequence tag; HRM, high-resolution melting; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment-length polymorphism; SNP, single nucleotide polymorphism; SSCP, single-strand conformation polymorphism; SSR, simple sequence repeat; TGGE, temperature gradient gel electrophoresis

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JSB and KHB performed database analysis for determining the SNP sequence. PSM, KMJ, and KSB helped in carrying out the experiments. YGR helped in preparing figures and tables. JSB wrote the manuscript with input from all other authors. YSH, JSB, and KHB reviewed the manuscript. All authors have read and approved the final manuscript.

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Supplementary Information

Additional file 1: Table S1. List of candidate single nucleotide polymorphisms (SNPs) identified based on the comparative analysis of 'Haryejosaeng' and control cultivars (*C.* × *clementina* 'Clemenules,' 'Haryejosaeng,' 'Miyagawa wase,' 'Miyamoto wase,' 'Okitsu wase,' and 'Nichinan 1 gou').

Additional file 2: Table S2. Candidate single nucleotide polymorphisms (SNPs) obtained from the comparative analysis of 'Haryejosaeng,' 'Miyagawa wase,' 'Miyamoto wase,' 'Okitsu wase,' and 'Nichinan 1 gou'.

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Tables

Table 1. Data obtained from the trimmed raw data and reads mapped onto the reference genome.

Sample	Number of reads	Average length (bp)	Total length (bp)	Trimmed/Raw[z]	Genome coverage (times)	Reads-mapping region[y]
Haryejosaeng	42 048 398	93.22	3 919 554 799	81.08%	20.70	275 590 654 (91.44%)
	42 048 398	87.46	3 677 734 261	76.08%		
Miyagawa wase	30 183 645	91.28	2 755 080 380	83.26%	17.92	273 017 614 (90.59%)
	30 183 645	87.63	2 644 989 353	79.93%		
Miyamoto wase	30 636 351	91.04	2 789 074 644	82.86%	18.14	272 946 847 (90.56%)
	30 636 351	87.39	2 677 445 575	79.55%		
Okitsu wase	27 143 763	91.40	2 480 973 959	83.46%	16.14	272 602 269 (90.45%)
	27 143 763	87.78	2 382 690 617	80.16%		
Nichinan 1 gou	29 701 139	91.42	2 715 191 322	83.37%	17.66	273 264 127 (90.67%)
	29 701 139	87.79	2 607 461 190	80.06%		

[z] (total length of trimmed reads/total length of raw reads) × 100

[y] Region except for base = N (unmapped region) within the consensus sequence (reads-mapping region/total length of the reference genome) × 100.

Table 2 Amplification of *Citrus unshiu* mandarin cultivar samples collected from farms by multiplex PCR using 2 single nucleotide polymorphism primer pairs (P1 and P5, P2, and P5).

No.	Cultivar abbreviation[Z]	Primers			Multiplex PCR amplification results		
		P1	P2	P5	P1	P2	P5
1	HRBS	P1	P2	P5	C[y]	C[y]	C
2	GCSL	P1	P2	P5	NC[x]	NC[x]	NC
3	HR-S1	P1	P2	P5	C	C	C
4	GCS	P1	P2	P5	NC	NC	NC
5	TGCN	P1	P2	P5	NC	NC	NC
6	HiroCN	P1	P2	P5	NC	NC	NC
7	YRSCN	P1	P2	P5	NC	NC	NC
8	HR-S2	P1	P2	P5	C	C	C
9	INS	P1	P2	P5	NC	NC	NC
10	YRS	P1	P2	P5	NC	NC	NC
11	IMPS2-1	P1	P2	P5	NC	NC	NC
12	IMPS2-2	P1	P2	P5	NC	NC	NC
13	HRD-1	P1	P2	P5	C	C	C
14	IMPS-1	P1	P2	P5	NC	NC	NC
15	HRJ	P1	P2	P5	C	C	C
16	HR-2	P1	P2	P5	C	C	C
17	HICN	P1	P2	P5	NC	NC	NC

[Z] See Table 1 for cultivar abbreviations. [y] C: PCR amplicon confirmed. [x]NC: PCR amplicon not confirmed.

Table 3. Satsuma mandarin (*Citrus unshiu* Marc.) cultivars used in the present study.

No	Abbreviation	Source	Cultivar name	Origin	Collection region	Analysis
1	HROT	Original tree	‘Haryejosaeng’	Nucellar seedling of ‘Tachima wase’	Citrus Research Institute	HRM, SNP
2	GCOT	Original tree	‘Miyagawa wase’	Limb sport of a Zairai tree in Fukuoka Prefecture	Citrus Research Institute	HRM, SNP
3	HJOT	Original tree	‘Okitsu wase’	Nucellar seedling of ‘Miyagawa wase’	Citrus Research Institute	HRM, SNP
4	YROT	Original tree	‘Yura wase’	Bud mutation of ‘Miyagawa Wase’	Citrus Research Institute	HRM, SNP
5	GBOT	Original tree	‘Miyamoto wase’	Bud mutation of ‘Miyagawa wase’	Citrus Research Institute	HRM, SNP
6	SYOT	Original tree	‘Ueno wase’	Bud mutation of ‘Miyagawa wase’	Citrus Research Institute	HRM, SNP
7	MTOT	Original tree	‘Yonezawa wase’	Bud mutation of ‘Owari’	Citrus Research Institute	HRM, SNP
8	INOT	Original tree	‘Nichinan 1 gou’	Bud mutation of ‘Okitsu wase’	Citrus Research Institute	HRM, SNP
9	TGCN	Citrus nursery	‘Taguchi wase’	Bud sport of ‘Okitsu wase’	Orchard of Citrus Cooperative Society	Multiplex PCR
10	HiroCN	Citrus nursery	‘Hiroshimakaken 7 gou’	Nucellar seedling of ‘Imada wase’	Orchard of Citrus Cooperative Society	Multiplex PCR
11	YRSCN	Citrus nursery	‘Yura’ nucellar seedling	Nucellar seedling of ‘Yura wase’	Orchard of Citrus Cooperative Society	Multiplex PCR
12	HICN	Citrus nursery	‘Higo’	Nucellar seedling of ‘Miyagawa wase’	Orchard of Citrus Cooperative Society	Multiplex PCR
13	GCSL	Summer leaf	‘Miyagawa wase’	Seedlings from scion of original ‘Miyagawa wase’ tree	Outdoor cultivar	Multiplex PCR
14	HRD-1	Seedlings	‘Haryejosaeng-1’	Seedlings from scion of original ‘Haryejosaeng’ tree	Daejeong farmhouse	Multiplex PCR
15	HRJ	Seedlings	‘Haryejosaeng’	Seedlings from scion of original ‘Haryejosaeng’ tree	Jeoji-ri farmhouse	Multiplex PCR
16	HR-2	Seedling	Haryejosaeng-2’	Seedlings from scion of original ‘Haryejosaeng’ tree	Agricultural cooperatives	Multiplex PCR
17	IMPS-1	Seedlings	‘Imprecision wase-1’	Unknown	Agricultural cooperatives	Multiplex PCR
18	IMPS2-1	Seedling	‘Imprecision	Unknown	Farmhouse	Multiplex

			wase-2-1’			PCR
19	IMPS2-2	Seedling	‘Imprecision wase-2-2’	Unknown	Farmhouse	Multiplex PCR
20	HR-S1	Seedling	‘Haryejosaeng-S1’	Seedlings from scion of original ‘Haryejosaeng’ tree	Citrus Research Institute	Multiplex PCR
21	INS	Seedling	‘Nichinan 1 gou’	Seedlings from scion of original ‘Nichinan 1 gou’ tree	Citrus Research Institute	Multiplex PCR
22	YRS	Seedling	‘Yura wase’	Seedlings from scion of original ‘Yura wase’ tree	Citrus Research Institute	Multiplex PCR
23	GCS	Seedling	‘Miyagawa wase’	Seedlings from scion of original ‘Miyagawa wase’ tree	Citrus Research Institute	Multiplex PCR
24	HR-S2	Seedling	‘Haryejosaeng-S2’	Seedlings from scion of original ‘Haryejosaeng’ tree	Citrus Research Institute	Multiplex PCR
25	HRBS	Seedling	‘Haryejosaeng-BS’	Seedlings from scion of original ‘Haryejosaeng’ tree	Breeder stock	Multiplex PCR

Table 4. Single nucleotide polymorphism/high-resolution melting (SNP/HRM) primer pairs used in the present study.

Primer set	Primer name	Primer sequence (5′→3′)	Amplicon size (bp)
P1	HL-SNP-SCAF_2-23997586-F	CAAAAGAGCTC**A**AATAA**G**GC	165
	HL-SNP-SCAF_2-23997586-R	TCCCTTTCTTGGCATGATCT	
	P1-HRM-F	CCTTGCTTGCCAAAAGAGCT	117
	P1-HRM-R	ACTGAACTTTCCTAGCAATCTAGC	
P2	HL-SNP-SCAF_2-36059523-F	TCTAGGCTGAGATGCAG**C**GC	150
	HL-SNP-SCAF_2-36059523-R	CAGAAAATTCAACTGACGGTGA	
	P2-HRM-F	TGGGCTTCTATGACTAACCACA	93
	P2-HRM-R	GCCTTGGCTGTTCTCTCTGA	
P3	HL-SNP-SCAF_2-176744-F	TGGCACTATTGCACCTG**C**TA	283
	HL-SNP-SCAF_2-176744-R	CAAAGAAACCAGGGACGAAG	
	P3-HRM-F	GGAGTCATCCCTTCTAGTCTAGGA	82
	P3-HRM-R	GGATAGCCCCACTGAGGTCA	
P4	HL-SNP-SCAF_9-24101032-F	CCATGTTATGAGTTCTGCA**G**TT	246
	HL-SNP-SCAF_9-24101032-R	AAATTGGGGTTCGAGACCTT	
	P4-HRM-F	TGCTATCACTCCAGGCCAGA	143
	P4-HRM-R	TGTGGTGAACTTGACGTGAA	
P5	HL-SNP-SCAF_9-30793978-F	ATTGAAATGCTGCCAAAGGT	526
	HL-SNP-SCAF_9-30793978-R	CTCCATACAAGAGAGTG**C**CG	
	P5-HRM-F	CCCCAATATCATCCTTTCTCAGT	150
	P5-HRM-R	AGCTGAAACCTATGCACTCCA	
P6	HL-SNP-SCAF_5-18939485-F	TGGATGGAAAGCTTATC**G**TT	340
	HL-SNP-SCAF_5-18939485-R	AACTTAACGGCAAGGGAGGT	
	P6-HRM-F	AGTCTTGGGAATCAGACTCT	130
	P6-HRM-R	GGCTCACATCTTGCTCTGGT	
P7	HL-SNP-SCAF_5-41248465-F	CACAGTGACGGTAATTGGT**T**AA	388
	HL-SNP-SCAF_5-41248465-R	CTGCCTTCGTTCTTATTTAC**C**CT	
	P7-HRM-F	AGTGCAGGCAGAAAGTGATGA	127
	P7-HRM-R	ACCTTTTTAATGATCTTCACTGGA	

The boldface nucleotides are SNPs among *Citrus unshiu* mandarin cultivars, and those that are underlined are artificial SNPs.

Figures

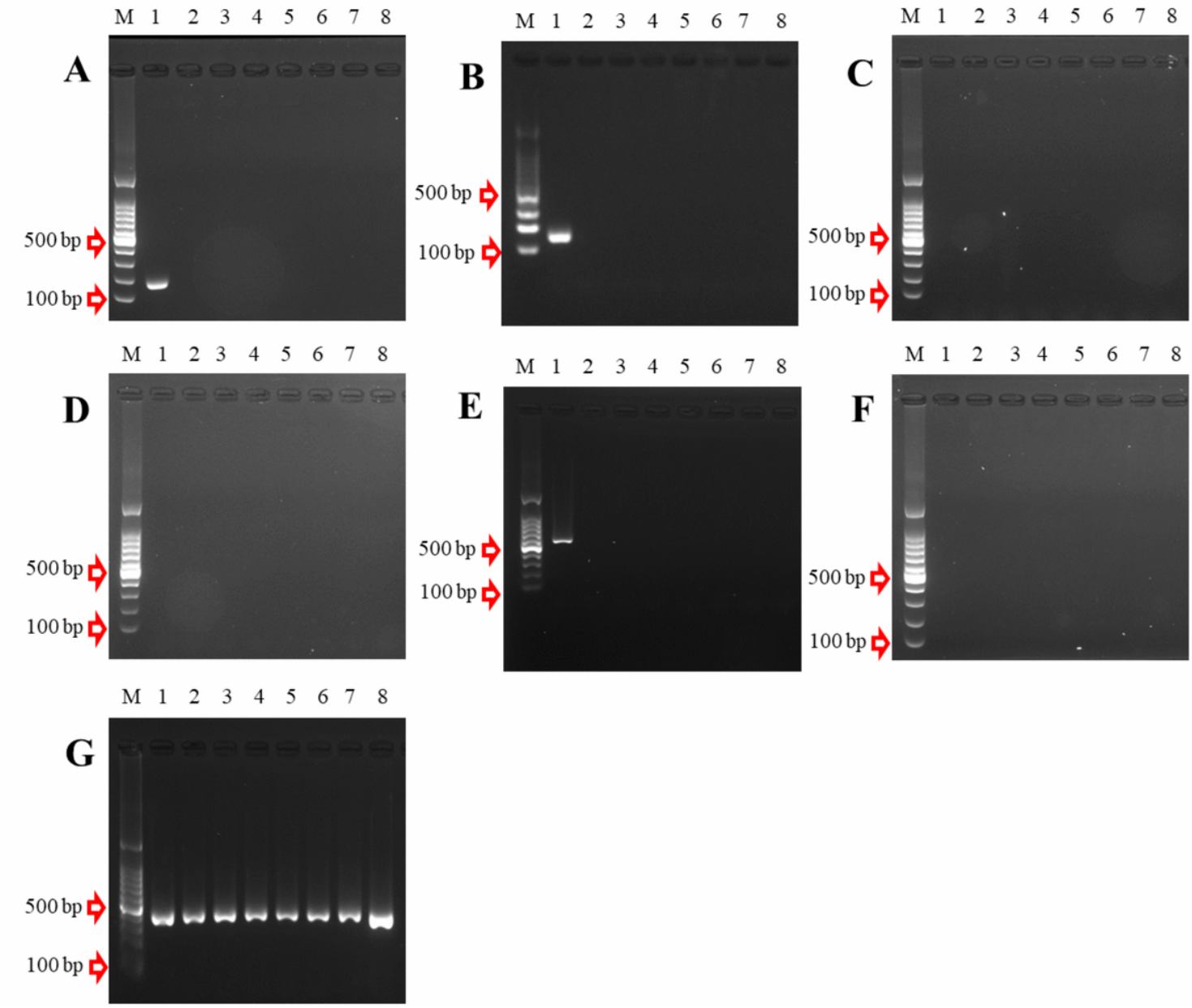

Figure 1

Amplification of ‘Haryejosaeng’-specific fragments using single nucleotide polymorphism primer pairs. The primer pairs are P1 (a), P2 (b), P3 (c), P4 (d), P5 (e), P6 (f), and P7 (negative control; g). M: Molecular weight marker (100 bp DNA ladder; Takara Bio Inc., Shiga, Japan); lane 1: ‘Haryejosaeng’ breeder’s stock; lane 2: ‘Miyagawa wase’; lane 3: ‘Okitsu wase’; lane 4: ‘Yura wase’; lane 5: ‘Miyamoto wase’; lane 6: ‘Ueno wase’; lane 7: ‘Yonezawa wase’; lane 8: ‘Nichinan 1 gou’.

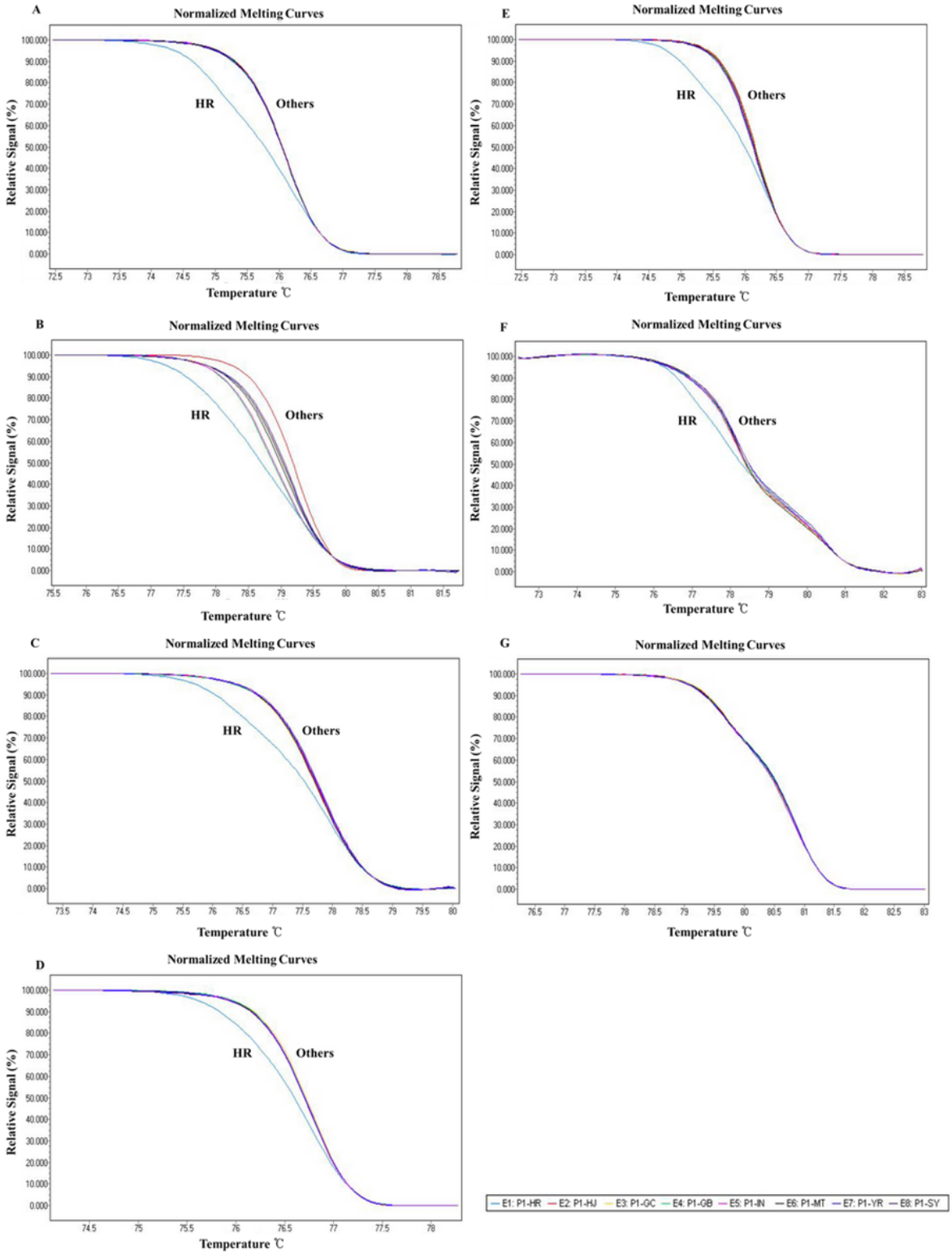

Figure 2

High-resolution melting analysis of Citrus unshiu mandarin cultivars using 7 single nucleotide polymorphism primer sets. The primer pairs are P1 (a), P2 (b), P3 (c), P4 (d), P5 (e), P6 (f), and P7 (g). HR, ‘Haryejosaeng;’ HJ, ‘Okitsu wase;’ GC, ‘Miyagawa wase;’ GB, ‘Miyamoto wase;’ IN, ‘Nichinan 1 gou’; MT, ‘Yonezawa wase’; YR, ‘Yura wase’; SY, ‘Ueno wase.’

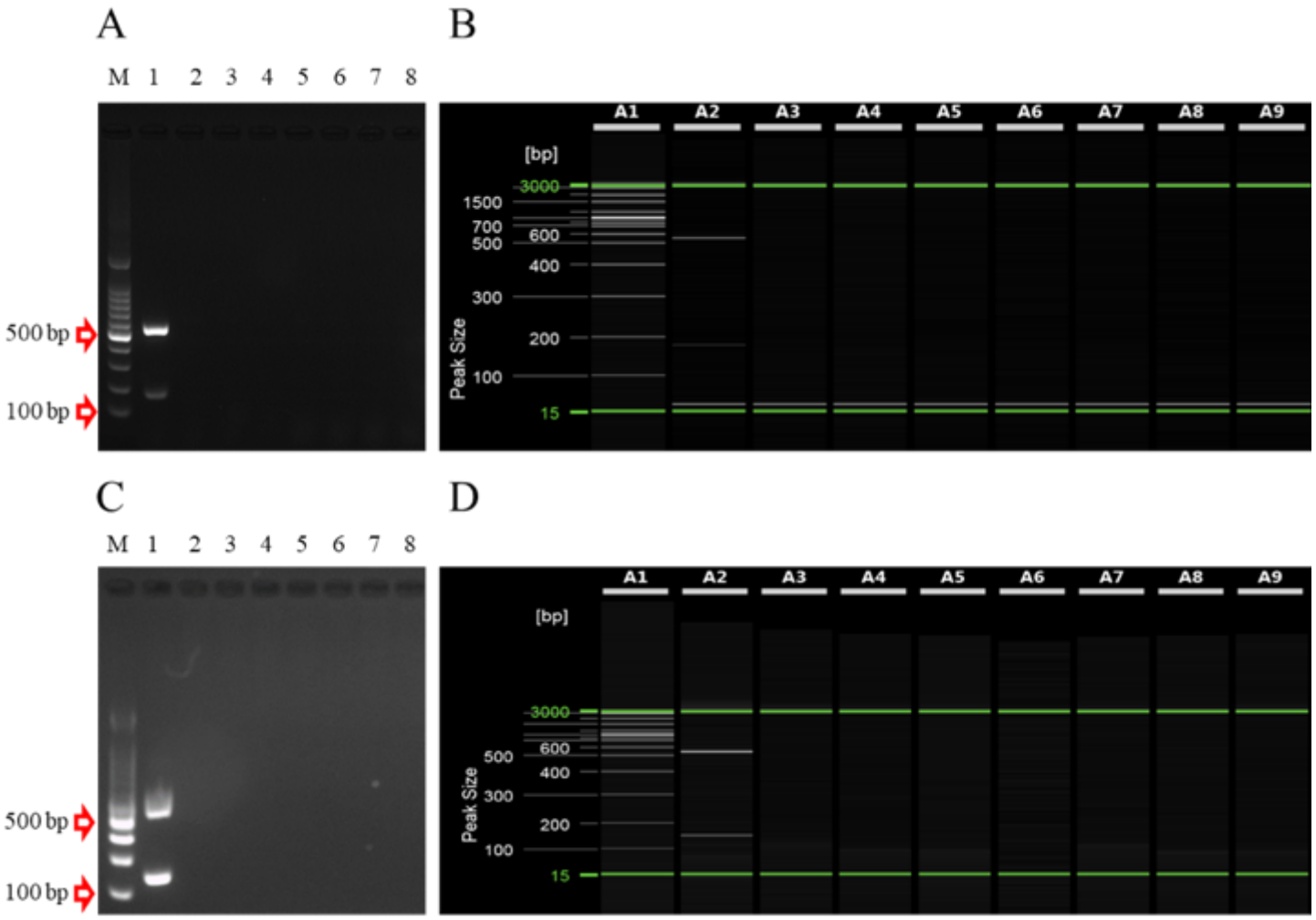

Figure 3

Amplicons obtained in the multiplex PCR of Citrus unshiu mandarin cultivars with 2 pairs of single nucleotide polymorphism primers (P1/P5 and P2/P5). (a, b) PCR analysis using P1 and P5 primer pairs; (c, d) PCR analysis using P2 and P5 primer pairs; (a and c) agarose gel electrophoresis; M: Molecular weight marker (100 bp DNA ladder; Takara); lane 1: 'Haryejosaeng' original tree; lane 2: 'Miyagawa wase'; lane 3: 'Okitsu wase'; lane 4: 'Yura wase'; lane 5: 'Miyamoto wase'; lane 6: 'Ueno wase'; lane 7: 'Yonezawa wase'; lane 8: 'Nichinan 1 gou'. (b and d) Polyacrylamide gel electrophoresis using the QiAxcel Advanced System (Qiagen); A1: molecular marker (20 bp and 100 bp DNA ladder, Qiagen); A2: 'Haryejosaeng' breeder's stock; A3: 'Miyagawa wase'; A4: 'Okitsu wase'; A5: Yura wase'; A6: 'Miyamoto wase'; A7: 'Ueno wase'; A8: 'Yonezawa wase'; A9: 'Nichinan 1 gou'.

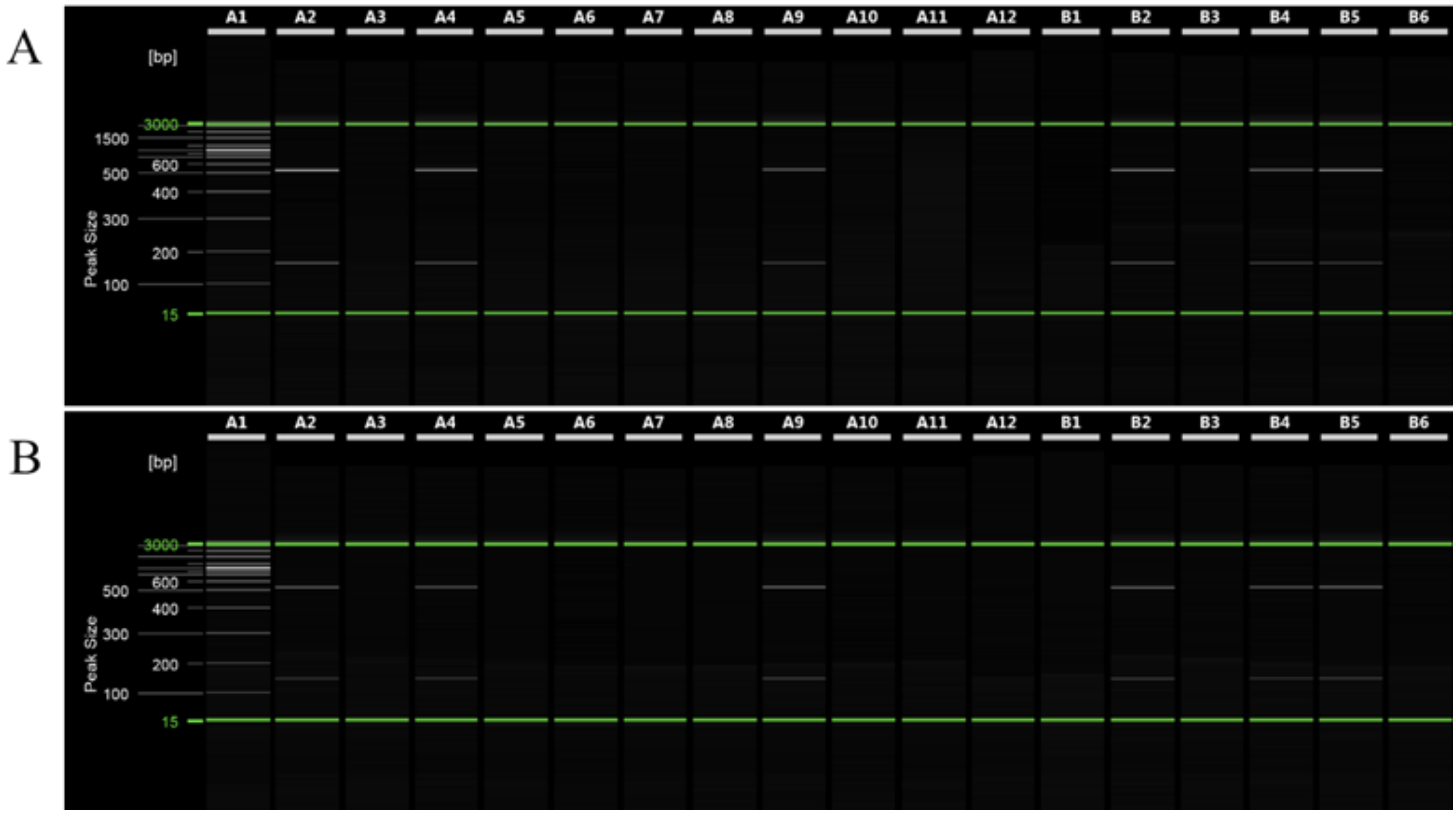

Figure 4

Amplicons obtained using multiplex PCR of Citrus unshiu mandarin cultivars from a farm with 2 pairs of single nucleotide polymorphism primer sets (P1/P5 and P2/P5). (a) PCR analysis using P1 and P5 primer pairs; (b) PCR analysis using P2 and P5 primer pairs. Polyacrylamide gel electrophoresis using the QiAxcel Advanced System (Qiagen); A1: molecular marker (20 bp and 100 bp DNA ladder, Qiagen); A2: HRBS; A3: GCSL A4: HR-S1; A5: GCS; A6: TGCN; A7: HiroCN; A8: YRSCN; A9: HR-S2; A10: INS; A11: YRS; A12: IMPS2-1; B1: IMPS2-2; B2: HRD-1; B3: IMPS-1; B4: HRJ; B5: HR-2; B6: HICN (See Table 1 for cultivar abbreviations).

Supplementary Files

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