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Research Paper

TaqMan-MGB SNP genotyping assay to identify 48 citrus cultivars distributed in the Japanese market

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A citrus cultivar identification system using CAPS marker has been developed on nursery trees, but this needs to be extended to include various product types, such as imported fruits and processed products. Here, we developed a new cultivar identification system using TaqMan-MGB SNP genotyping assay. Eight probe and primer sets were designed to amplify PCR fragments <100 bp to enable the genotyping of fresh and processed fruits in which predicted that insufficient quantities of DNA and residual impurities in the DNA extracts. The TaqMan-MGB SNP genotyping assay was stable and reproducible, and were confirmed to apply various sample sources, including leaves, fresh fruit, juice, canned fruit, and dry fruit. They could provide at least a single differentiating SNP to discriminate any paired combination among 48 citrus cultivars. Minimal marker subsets to identify the target cultivar were listed for each of 18 registered cultivars with valid patent. The allelic SNP genotypes of 48 citrus cultivars, which cover more than 98% of all citrus fruit shipment produced in Japan, is valuable for the referencing information in the DNA-based identification for fresh and processed fruits. This identification system will help protect registered cultivars and facilitate food fraud inspections.

Key Words: breeders' rights, food fraud, protection, processed fruit, fruit.

Introduction

Plant variety registration is an important system for protecting valuable plant varieties from imitation and unauthorized use, both in Japan and overseas. Once a plant variety is registered, it is protected for a period of 25 years from the date of registration. For perennial plants, such as fruit trees and lumber trees, the period of protection is 30 years. An amendment to the law in 2005 extended breeders' rights to include not only propagation materials, such as seeds and seedlings, but also the harvested materials and products manufactured from the cultivar. For the adequate enforcement of the breeder's right, it is necessary to prove that the breeder's right has been violated, but it takes an extraordinary amount of time to demonstrate that the agricultural products are identical to the registered cultivar, based on phenotypic features. Therefore, a reliable cultivar identification system using DNA diagnosis is required for economically important plants and their manufactured products, to protect breeders' rights from unauthorized use. On one hand, a reliable cultivar identification system using DNA diagnosis is also required for food safety inspections to detect food fraud and food adulteration. The authentication of plant ingredients is very important to protect product reliability, consumer safety, food processors and retailers from the infringement. Recently, protection of breeders' rights for fruit tree cultivars bred in Japan has become a problem in connection with the agricultural product export strategy promoted by the Japanese government. As representative cases, the sweet cherry cultivar, 'Benishuho' and the grape cultivar, 'Shine Muscat' have been illegally taken out overseas and were cultivated overseas (Ishikawa 2018, Tahira 2008). These issues would connect to the subsequent reverse import of pirated fruits into Japan and the loss of overseas markets, resulting in the potential damage to Japanese farmers. Therefore, cultivar identification systems using various type of DNA markers have been developed in fruit trees to protect the breeders' rights of the registered cultivars with valid patent in Japan such as Asian pear, grape and so on (Kimura *et al.* 2002, Yamamoto *et al.* 2019).

Citrus plants are one of the major cultivated fruits in Japan and various high-quality cultivars have been developed such as 'Shiranuhi', 'Setoka', 'Mihaya', 'Asumi' and so on. The citrus industry has benefited from those cultivars developed by conventional breeding methods. Because of global demand for new high-quality cultivar, Japanese new

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cultivars have been highly interested in world-wide. To protect the breeders' rights of those new cultivars, cultivar identification system using cleaved amplified polymorphic site (CAPS) markers have been developed (Fujii *et al.* 2019, Ninomiya *et al.* 2015, Nonaka *et al.* 2017) and then National Agriculture and Food Research Organization Institute of the Center for Seeds and Seedlings (NCSS) published the manual of citrus cultivar identification based on these reports on their web site (https://www.naro.affrc.go.jp/publicity_report/publication/pamphlet/tech-pamph/130601.html). The CAPS markers included in the manual are guaranteed to be stable and reproducible for citrus cultivar identification for the effective protection of breeders' rights during the legal procedures. This manual helps to deter the infringement of domestic regulations on nursery trees, such as the illegal diversion of the scion. While, assuming that breeder's rights were infringed from overseas, imported fruits and processed products would be targeted for inspection. DNA extraction from processed foods is generally difficult and PCR amplification is unstable by insufficient quantities of DNA and residual impurities in the DNA extracts (Lo and Shaw 2018). Therefore, some of the CAPS markers in the manual would not be applied to the processed products owing to long PCR amplicons and alternative PCR-based technologies using small-sized DNA markers are required for the authentication of processed foods. Single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) are frequently used for cultivar identification in fresh and processed fruits (Caramante *et al.* 2010, Consolandi *et al.* 2008, Corrado *et al.* 2011, Yamamoto *et al.* 2006). While, SNPs have several advantages over SSRs in food traceability. SNP detection uses smaller PCR amplicons than SSR detection and SNPs are genetically more stable than multiallelic or multilocus SSRs (Jones *et al.* 2007).

In this study, we developed TaqMan-MGB SNP genotyping assay that allow the stable and reproducible identification of citrus cultivars. We report SNP genotyping information for 48 citrus cultivars, representing more than 98% of all citrus fruit shipment produced in Japan. The PCR fragment of TaqMan-MGB SNP genotyping assay was designed to be <100 bp. The assays can, theoretically, be applied to both fresh and processed fruits, provided that the DNA sample is not degraded. The publishing of allelic SNP genotypes for major citrus cultivars distributed in the Japanese market is indispensable for the DNA-based identification of illegal fruits. The cultivar identification system reported here will reinforce the protection of Japanese citrus cultivar brands and also will contribute to help maintain food safety.

Materials and Methods

Plant material and DNA preparation

A total of 48 citrus cultivars (**Table 1**) preserved at the Division of Citrus Research in National Agriculture and Food Research Organization Institute of Fruit and Tea Tree Science (NIFTS) were used in this study. Taxonomic classification was performed according to Tanaka's system (Tanaka 1969). Sample accession numbers (JP No.) and registration numbers (Registration No.) in **Table 1** were based on the Genebank (http://www.gene.affrc.go.jp/databases-plant_search.php) at the Genetic Resources Center, National Agriculture and Food Research Organization (NARO) and Plant Variety protection office (http://www.hinshu2.maff.go.jp/en/en_top.html) at Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF), in order to understand which strain was used for SNP genotyping. Genomic DNA was isolated from fully expanded leaves using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Regarding for conventional varieties such as satsuma mandarin (*Citrus unshiu* Marc.), grapefruit (*C. paradisi* Macf.), sweet orange (*C. sinensis* (L.) Osbeck), lemon (*C. limon* (L.) Burm. f.), iyo (*C. iyo* hort. ex Tanaka), natsudaidai (*C. natsudaidai* Hayata), hassaku (*C. hassaku* hort. ex Tanaka), ponkan (*C. reticulata* Blanco), yuzu (*C. junos* hort. ex Tanaka) and sudachi (*C. sudachi* hort. ex Tanaka), mutant cultivars with some advantageous traits have developed from the same species among them during long-term cultivation periods. The representative cultivar wit JP No. was selected among those conventional varieties, of which all CAPS marker genotypes were confirmed to be identical (Fujii *et al.* 2019, Niimi *et al.* 2020).

The fresh fruit of the 'Shiranuhi' was sampled from the tree preserved in NIFTS. Juice, canned fruit, and dry fruit of the 'Shiranuhi' cultivar were purchased. Juice (straight juice) was made by squeezing whole fresh fruit and sterilizing at 95°C for 5 s. Canned fruit was made by preparing juice sac by 0.3% of sodium hydroxide treatment to remove pulp segment and adhering albedo, sealing juice sac soaking in the syrup including around 18% sugar in a can, and sterilizing 80°C for 15 m. Dry fruit was made by drying the sliced fruit under 50°C for 10 h in food dry machine. The juice sacs without the segment membrane were collected in fresh and canned fruits. The pellets were collected from juice by the centrifuge at 6,000 g for 10 min (CR21N, Hitachi, Tokyo, Japan). Whole sliced fruit was utilized in dry fruit. Those samples were powdered by liquid nitrogen and genomic DNA was isolated by the CATB method (Doyle and Doyle 1987) with the twice prewash steps using the wash buffer (0.1 M Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 1% polyvinylpyrrolidone (PVP), 10% polyethylene glycol (PEG) #6,000 and 2% β-mercaptoethanol). The extracted DNA was purified using DNeasy mini spin column under the DNA clean protocol (DNeasy plant mini kit, Qiagen). Genomic DNA was also isolated from the leaves of 'Shiranuhi' by the same protocol as a control for the data comparison.

Design of TaqMan minor groove binder probes and primers

From a citrus 384-SNP array previously developed using

Table 1. Citrus culitvars used for TaqMan-MGB SNP analysis

No.	JP No.[a]	Registration No.[b]	Cultivar name (conventional variety)	Parentage or scientific name[c]
1	117351		'Miyagawa-wase' (Satsuma mandarin)	*Citrus unshiu* Marc.
2	168864		'Duncan' grapefruit (Grapefruit)	*C. paradisi* Macf.
3	172154		'Trovita' orange (Sweet orange)	*C. sinensis* (L.) Osbeck
4	117289		'Lisbon' lemon (Lemon)	*C. limon* (L.) Burm. f.
5	117159		'Shiranuhi' [Dekopon[d]]	'Kiyomi' × 'Nakano 3 gou' ponkan
6	117373		Iyo (Iyo)	*C. iyo* hort. ex Tanaka
7	117297		'Kawanonatsudaidai' (Natsudaidai)	*C. natsudaidai* Hayata
8	117286		Hassaku (Hassaku)	*C. hassaku* hort. ex Tanaka
9	171505	413	'Ohta ponkan' (Ponkan)	*C. reticulata* Blanco
10		24081	'Rinoka'	'Lisbon' lemon × Hyuganatsu
11	251815	23722	'Mihaya'	'Tsunonozomi' × No. 1408
12	245233	23723	'Asumi'	Okitsu 46 gou × 'Harumi'
13		32235 (Application No.)	'Asuki'	Okitsu 46 gou × 'Harumi'
14		13542	'Reikou'	Unknown × 'Murcott'
15		17970	'Tsunokagayaki'	KyOw No. 14 × 'Encore'
16		17969	'Seinannohikari'	EnOw No. 21 × 'Youkou'
17		20788	'Tsunonozomi'	'Kiyomi' × 'Encore'
18	237599	20679	'Haruhi'	Okitsu 46 gou × 'Awa-orange'
19	115521		'Kiyomi'	'Miyagawa-wase' × 'Trovita' orange
20	118842	9398	'Setoka'	KyEn No. 4 × 'Murcott'
21	117468	7506	'Harumi'	'Kiyomi' × ponkan F-2432
22		12069	'Harehime'	E-647 × 'Miyagawa-wase'
23		15548	'Kanpei'	'Nishinokaori' × Ponkan
24		12981	'Ehime Kashi No. 28 gou' [Benimadonna[e]]	'Nankou' × 'Amakusa'
25	117380		Yuzu (Yuzu)	*C. junos* hort. ex Tanaka
26			Tosabuntan (Buntan)	*C. grandis* Osbeck
27	117412		Kawachibankan	*C. sp*
28			Hyuganatsu (Hyuganatsu)	*C. tamurana* hort. ex Tanaka
29	117383		Sudachi (Sudachi)	*C. sudachi* hort. ex Tanaka
30			Ogimi Kugani (Shiikuwasha)	*C. depressa* Hayata
31			'Kara'	'Owari-unshiu' × 'King' mandarin
32	113507		Tankan-T132 (Tankan)	*C. tankan* Hayata
33			'Seminole'	'Dunkan' grapefruit × 'Dancy' tangerin
34	117381		Kabosu (Kabosu)	*C. sphaerocarpa* hort. ex Tanaka
35		4596	'Amakusa'	KyOw no. 14 × 'Page'
36	172148		Ougonkan	*C. sp*
37	171490		Kishu mikan	*C. kinokuni* hort. ex Tanaka
38		298	'Sweet spring'	'Ueda-unshiu' × Hassaku
39	117315		Sanboukan	*C. sulcata* hort. ex. Ik. Takahashi
40			'Encore'	'King' mandarin × 'Willowleaf'
41		14543	'Tamami'	'Kiyomi' × 'Willking'
42		8557	'Nishinokaori'	'Kiyomi' × 'Trovita' orange
43		638	'Saga madanrin'	'Konishi-wase' × 'Fairchild'
44			'Murcott'	Unknown
45		1857	'Nankou'	'Miho-wase' × Clementine
46		7507	'Amaka'	'Kiyomi' × 'Encore'
47		12070	'Kankitsuchukanbohon nou 6 gou'	'King' mandarin × Mukaku kishu
48	117365		Kabusu (Kabusu)	*C. aurantium* L.

[a] Discription by Genebank (http://www.gene.affrc.go.jp/databases-plant_search.php). [b] Discription by Plant Variety protection office at MAFF, Japan (http://www.hinshu2.maff.go.jp/en/en_top.html). [c] Tanaka's system was used for scientific name. [d] The registered trademark of the Federation of Kumamoto Prefectural Fruit Agriculture Cooperatives. [e] The registered trademark of Ehime Prefectural Headquarters, National Federation of Agricultural Cooperative Associations.

the GoldenGate system (Illumina, San Diego, CA, USA; Fujii *et al.* 2013a), the genomic sequences flanking to SNP were referred to design TaqMan minor groove binder (MGB) probe and primer sets. TaqMan-MGB probe and primer sets were designed using Primer Express ver. 3.0 (Applied Biosystems, Foster city, CA, USA). The size of the amplified products was designed to be <100 bp. 5-carboxyl-fluorescein (FAM) and hexachloro-fluorescein (HEX) were used for labeling the 5′-end of the oligonucleotides. Since these two dyes had different emission wavelengths (FAM: 518 nm, HEX: 556 nm), they could be discriminated in the same tube. A total of 8 TaqMan-MGB probe and primer sets were developed for the identification of 48 citrus cultivars.

TaqMan-MGB SNP genotyping assay

TaqMan-MGB SNP genotyping reagents were prepared as follows: 7.5 µL of TaqMan Genotyping Master Mix (Thermo Fisher, Waltham, MA, USA), 10 µM each primer, 5 µM FAM- and HEX-labeled TaqMan-MGB probes, 10 ng of purified DNA, and RNase-free water to a final volume of 15 µL. Cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; and 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. All reactions were performed in a QuantoStudio™ 3 real-time PCR system (Thermo Fisher). Regarding for the fresh and processed fruit samples, the PCR cycle (45 cycles) and DNA template amount (1 ng) were modified owing to poor amount of DNA extracts from those samples. Data analysis was performed using QuantStudio™ Design & Analysis software v 1.4.1 (Thermo Fisher) to calculate the threshold cycle (Ct) and delta Rn values, which indicated a fluorescent signal significantly above the background fluorescence. The fluorescence of the reporter dyes was normalized to the fluorescence of the passive dye in each well. The normalized intensities of the 2 reporter dyes in each sample were plotted on an allelic discrimination plot and were algorithmically clustered. Genotype calls were assigned according to the sample position on the allelic discrimination plot.

Validation of TaqMan-MGB assays and minimal marker subset for cultivar identification

To evaluate the reliability of 8 TaqMan-MGB SNP assays, their inheritance fashion with co-dominancy was evaluated using the subsets of cultivars with parent-offspring relationship by MARCO software (Fujii *et al.* 2010). MARCO evaluated a parent-offspring relationship based on the transmission of one allele at each SNP locus from the parents to the offspring. The minimal marker subset required to discriminate 48 cultivars and the registered cultivars with valid patents, was calculated using MinimalMarker software (Fujii *et al.* 2013b). The values of observed homozygosity (Ho), expected heterozygosity (He), and polymorphism information content (PIC) were calculated by MarkerToolKit v 1.0 (Fujii *et al.* 2008).

Results

Design of TaqMan-MGB probes and primers and SNP genotyping

The 384 previously reported SNPs (Fujii *et al.* 2013a) were reviewed to identify those that met the following criteria: (a) high allele frequency; (b) co-dominant allelic genotype; (c) a PCR amplicon <100 bp, suitable for genotyping fresh and processed fruits; and (d) genomic region of SNP loci should be spread to different chromosome to enable genome-wide SNP assay. Most of the preliminarily selected SNP candidates failed to generate TaqMan-MGB probe and primer set designs because the design criteria used in the TaqMan-MGB SNP genotyping system are more stringent than those used in the GoldenGate system. Extra SNPs located around the target SNP also interfered to the probe and primer design when a wide range of citrus cultivars were analyzed *in silico*. In addition, some of the designed probe and primer sets were unable to be analyzed due to low signal or unclear allelic discrimination plot patterns. Finally, 8 TaqMan-MGB SNP assays were developed, and these could be applied to the genotyping 48 citrus cultivars at the following loci: SI312, SI001, SI322, SI149, SI306, SI201, SI210, and SI381 (**Table 2**). The PCR amplicon size of all assays ranged from 60 to 99 bp. The 8 SNPs were scattered across scaffolds 1, 2, 4, 6, 7, 8, and 9 in the clementine mandarin genome sequence (Wu *et al.* 2014). They were located within coding regions that are highly conserved among citrus and related species, rather than non-coding regions. Using these TaqMan-MGB assays, SNP genotyping of 48 citrus cultivars was performed using a QuantoStudio™ 3 real-time PCR system and allelic SNP genotypes for all 48 citrus cultivars were determined (**Table 3**). **Fig. 1** shows the allelic discrimination plot of the 8 SNPs among the 48 citrus cultivars. All TaqMan-MGB assays showed clear allelic discrimination plot patterns and genotyping calls could be assigned reliably. The He, Ho, and PIC values of each SNP were calculated to determine the genetic diversity of the 48 citrus cultivars and were listed in **Table 3**. He values ranged from 0.36 to 0.50, with an average of 0.45. Ho values ranged from 0.35 to 0.65, with an average of 0.50 and PIC values ranged from 0.30 to 0.38, with an average of 0.35. Thus, the 8 SNPs assayed here were moderately polymorphic and they provided genome-wide SNP detection on various scaffolds and clear allelic discrimination plot patterns among 48 citrus cultivars. The allelic genotypes at these 8 SNPs in 48 citrus cultivars provide valuable reference information in citrus cultivar identification system by DNA diagnosis.

Validation of 8 TaqMan-MGB SNP markers by parentage analysis

Among the 48 citrus cultivars examined, there were 9 different combinations with parent-offspring relationship as follows: ‘Shiranuhi’ (‘Kiyomi’ × ponkan), ‘Rinoka’

Table 2. Infromation of 8 TaqMan-MGB SNP markers applied in this study

SNP marker	Forward Primer	Reverse Primer	Probe (Fam-labelled)[a]	Probe (Hex-labelled)[a]
SI312	ACGTTCGCGAAAGTGTATTACG	ATTCCTTCTTCAATTGATCCTTGTTG	AAGCAGGTGATGACAG	AAGCAGCTGATGACAG
SI001	TGATGTTGACCATGATAAAGAGAGA	CTCCATCAAAACTAAATCCATTTG	CACAAGCCCTAGATC	CACAAGGCCTAGATC
SI322	TGGTGCGTACTTACTCTGTGATGTT	CAATTCTCCGGCACTCATCAT	TGATAATGAATTTTCAGCCC	TGATAATGATTTTTCAGCCC
SI149	TGAAGAAGCAACAACAACATCCA	CATATGTAGAGAGGGAGAGAGAGATTTG	AGAGACCATCCCAACAA	AGAGACCATCCCAGCAA
SI306	TTATGTGGGTGGAGATGAGTAGGA	GCACTCAAATCTTTCAGCTGATAGC	CCGCCAAAATATT	CGCCAAAGTATTT
SI201[c]	GCAGTTGAGATCAATGGTGCTT	TGATCGGCTGGTGATCCA	CTGATAGATACATTGACC	CTGATAGATACATCGACC
SI210	TGCTTTTGGAGATGGATCAGACT	TCAGCCACTTTCGCTTTCAGA	ACTTGCTCGAATCAC	CACTTGCTCGAGTCA
SI381[c]	CCCTTGTTTAGTTAAGTCCAATTTTCC	CATGACGTGCGCTTCAAGTATT	TAACAAGACCCTATTCTTA	ATAACAAGACCCTATCCT

Polymorphism	PCR product size (bp)	Position of clementine genome ver1.0[b]		
		Scaffold	Locus	Gene function
G/C	91	1	Ciclev10008225m	Serine/threonine-protein kinase
C/G	71	2	Ciclev10014319m	Glycosyl transferase
A/T	71	4	Ciclev10031443m	ATPase
A/G	94	4	Ciclev10032902m	30S ribosomal protein
A/G	80	6	Ciclev10011780m	F-box domain containing protein
T/C	60	7	Ciclev10024949m	1-Deoxy-d-xylulose-5-phosphate synthase
A/G	76	8	Ciclev10027999m	Polyadenylate-binding protein
T/C	99	9	Ciclev10006006m	Chlorophyll A-B binding protein

[a] SNP site is underlined in the nucleotide sequence of probe. [b] Refer Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) for clementine genome information. [c] Compermentary sequces are used to design TaqMan-MGB pobe and primer set.

(lemon × hyuganatsu [*C. tamurana* hort. ex Tanaka]), 'Kiyomi' (satsuma mandarin [*C. unshiu* Marc.] × sweet orange), 'Harumi' ('Kiyomi' × ponkan), 'Kanpei' ('Nishinokaori' × ponkan), 'Ehime Kashi 28 gou' ('Nankou' × 'Amakusa'), 'Sweet spring' (satsuma mandarin × hassaku), 'Nishinokaori' ('Kiyomi' × sweet orange), and 'Amaka' ('Kiyomi' × 'Encore'). The genotypes of the cultivars at the 8 SNP loci were analyzed using MARCO software (Fujii *et al.* 2010) to trace the lineage from parent to progeny. The alleles in the 'Shiranuhi', 'Rinoka', 'Kiyomi', 'Harumi', 'Kanpei', 'Ehime Kashi 28 gou', 'Sweet spring', 'Nishinokaori', and 'Amaka' cultivars were confirmed to be inherited from either of the parental alleles without any discrepancies (**Supplemental Table 1**). Therefore, these 8 SNPs were inheritable among cultivars with parent-offspring relationships, demonstrating that they could also be applied to parentage and cultivar identification.

Minimal marker set to identify the registered citrus cultivars with valid patent

The genotyping data for the 8 TaqMan-MGB SNP markers were analyzed by MinimalMarker software (Fujii *et al.* 2013b) to calculate a minimal marker set to discriminate the 48 citrus cultivars. There were 2 minimal marker subsets consisting of 7 TaqMan-MGB SNP markers which could discriminate any pair combination among 48 citrus cultivars by the difference of at least a single SNP (**Table 4**), as following; subset 1; SI312, SI001, SI322, SI149, SI306, SI210 and SI381, subset 2: SI312, SI001, SI322, SI149, SI306, SI201 and SI210. Allelic genotypes of those minimal marker subsets for 48 citrus cultivars were listed in **Supplemental Tables 2**, **3**.

Among the 48 citrus cultivars, 18 have a valid patent. Using genotyping data from the TaqMan-MGB SNP genotyping assay, a minimal marker subset was calculated to identify registered cultivars with valid patent (**Table 5**). 'Reikou', 'Haruhi', 'Rinoka', 'Mihaya' and 'Kankitsu-chukanbohon nou 6 gou' could be identified by at least 2 differentiating SNPs. 'Tsunokagayaki', 'Seinannohikari', 'Tsunonozomi', 'Setoka', 'Harehime', 'Asumi', 'Asuki', 'Kanpei', 'Ehime Kashi No. 28 gou' (Benimadonna), 'Tamami', 'Nishinokaori' and 'Amaka' could be identified by at least 3 differentiating SNPs. 'Harumi' could be identified by at least 4 differentiating SNPs. For example, 'Reikou' could be identified from the other 47 citrus cultivars by genotypes at the SI210 (AA) and SI312 (CC) loci, while 'Tsunokagayaki' could be identified by genotypes at the following 2 subsets: subset 1—SI001 (CG), SI210 (AA), and SI312 (CG) and subset 2—SI210 (AA), SI312 (CG), and SI381 (CT). Depending on the cultivar, total number of minimal marker subsets with different SNP marker combination varied from 1 ('Reikou', 'Rinoka', 'Mihaya', 'Kanpei' and 'Tamami') to 18 ('Asumi'). The information on the minimal marker subset to identify the patent cultivar will facilitate the inspection in cultivar identification.

Application of TaqMan-MGB SNP genotyping assay for the genotyping of fresh and processed fruits

To verify whether the TaqMan-MGB SNP genotyping assay developed here could be applied to the genotyping of fresh and processed fruits, genotyping assay was performed using DNA samples isolated from fresh fruit, juice, canned fruit, and dry fruit of the 'Shiranuhi' cultivar. DNA isolated from the leaf of 'Shiranuhi' was used as a control for comparison. The 8 TaqMan-MGB SNP markers were applied to analyze DNA samples from fresh and processed fruits using 45 cycles of PCR amplification owing to poor amount of DNA extracts from those samples. The allelic SNP genotypes detected in fresh and processed fruit samples were identical to those in the leaf sample in all assays

Table 3. Allelic genotypes of 8 TaqMan-MGB SNP markers for 48 citrus cultivars distributed in the Japanese market

No.	Cultivar name (conventional variety)	SNP marker	SI312	SI001	SI149	SI306	SI210	SI381	SI322	SI201
		He[a]	0.36	0.49	0.40	0.49	0.50	0.45	0.47	0.42
		Ho[b]	0.35	0.60	0.44	0.65	0.56	0.48	0.44	0.48
		PIC[c]	0.30	0.37	0.32	0.37	0.38	0.35	0.36	0.33
1	‘Miyagawa-wase’ (Satsuma mandarin)		GG	GG	GG	AA	AA	CC	AA	CT
2	‘Duncan’ grapefruit (Grapefruit)		GG	CC	AA	AG	AG	TT	AT	CT
3	‘Trovita’ orange (Sweet orange)		GG	CC	AG	AG	AG	CT	AT	CT
4	‘Lisbon’ lemon (Lemon)		CG	CG	AG	GG	GG	CT	TT	TT
5	‘Shiranuhi’ [Dekopon[d]]		CG	CC	AG	AG	AG	TT	AA	TT
6	Iyo (Iyo)		CG	CG	GG	AA	AG	CT	AT	CT
7	‘Kawanonatsudaidai’ (Natsuaidai)		GG	CG	AG	AG	AA	CT	TT	CT
8	Hassaku (Hassaku)		GG	CC	AG	AG	AA	CT	AT	CT
9	‘Ohta ponkan’ (Ponkan)		CG	CG	GG	AG	GG	TT	AA	TT
10	‘Rinoka’		CG	CG	AG	GG	AG	TT	AT	CT
11	‘Mihaya’		GG	CC	GG	AA	AG	CT	AT	CT
12	‘Asumi’		GG	CC	GG	GG	GG	CT	AA	CT
13	‘Asuki’		GG	CC	GG	AG	GG	CT	AA	CT
14	‘Reikou’		CC	CG	GG	AG	AA	TT	AA	TT
15	‘Tsunokagayaki’		CG	CG	GG	AG	AA	CT	AA	TT
16	‘Seinannohikari’		GG	CG	GG	AA	GG	CT	AA	CT
17	‘Tsunonozomi’		CG	CC	GG	AG	AA	TT	AA	TT
18	‘Haruhi’		GG	CG	AG	AA	AG	CT	AT	CC
19	‘Kiyomi’		GG	CG	AG	AG	AA	CT	AA	TT
20	‘Setoka’		GG	CC	GG	AG	AA	TT	AA	TT
21	‘Harumi’		GG	CG	GG	AG	AG	TT	AA	TT
22	‘Harehime’		GG	CG	GG	AA	AA	CT	AT	CT
23	‘Kanpei’		CG	CG	AG	AG	GG	CT	AT	TT
24	‘Ehime Kashi No. 28 gou’ [Benimadonna[e]]		GG	CC	AG	AG	AG	TT	TT	CT
25	Yuzu (Yuzu)		GG	GG	GG	AG	GG	TT	AT	TT
26	Tosabuntan (Buntan)		GG	CC	AG	GG	AG	TT	TT	CC
27	Kawachibankan		GG	CG	AG	AG	AG	TT	TT	CC
28	Hyuganatsu (Hyuganatsu)		GG	CG	AA	AG	AG	TT	AT	CT
29	Sudachi (Sudachi)		GG	CG	AA	AG	GG	TT	AT	TT
30	Ogimi Kugani (Shiikuwasha)		CG	GG	AG	AA	GG	CT	AT	TT
31	‘Kara’		CG	CG	GG	AA	AG	CT	AA	CT
32	Tankan T132 (Tankan)		CG	CG	GG	AG	AG	TT	AA	TT
33	‘Seminole’		GG	CG	AG	AG	AG	TT	AA	CT
34	Kabosu (Kabosu)		GG	CG	GG	AG	GG	TT	TT	TT
35	‘Amakusa’		CG	CC	AG	AG	AG	CT	AT	CT
36	Ougonkan		GG	CG	AG	AA	GG	TT	TT	CT
37	Kishu mikan		CG	GG	GG	AA	AG	CC	AT	TT
38	‘Sweet spring’		GG	CG	AG	AG	AA	CC	AT	CT
39	Sanboukan		GG	CG	AG	AG	AG	CT	TT	CT
40	‘Encore’		CC	CG	GG	GG	AG	TT	AA	TT
41	‘Tamami’		CG	GG	GG	AG	AG	CT	AA	TT
42	‘Nishinokaori’		GG	CG	AG	AA	AG	CC	AT	TT
43	‘Saga madanrin’		GG	CG	GG	AA	AG	CT	AT	CT
44	‘Murcott’		CG	CC	GG	AG	AG	TT	AA	TT
45	‘Nankou’		GG	CG	GG	AG	AG	CT	AT	CT
46	‘Amaka’		CG	GG	AG	AG	AG	CT	AA	TT
47	‘Kankitsuchukanbohon nou 6 gou’		CC	CG	GG	AG	AG	CT	AT	TT
48	Kabusu (Kabusu)		CG	CG	AG	AG	AG	CC	AT	CT

[a] Expected Heterozygosity, [b] Observed Heterozygosity, [c] Polymorphic Information Contents, [d] The registered trademark of the Federation of Kumamoto Prefectural Fruit Agriculture Cooperatives. [e] The registered trademark of Ehime Prefectural Headquarters, National Federation of Agricultural Cooperative Associations.

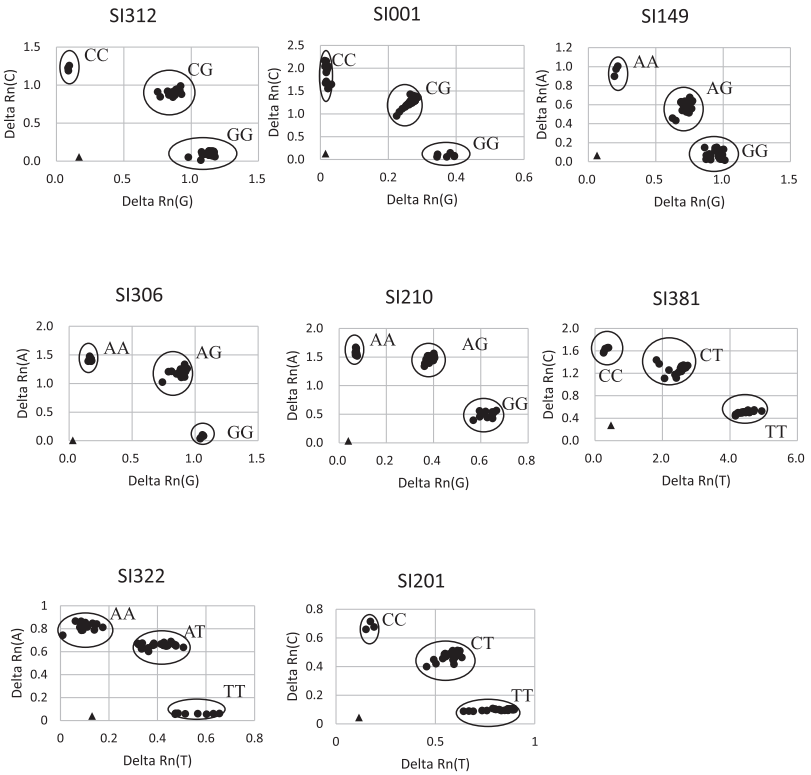

Fig. 1. Allelic discrimination plots of 48 citrus cultivars distributed in the Japanese market by TaqMan-MGB SNP genotyping analysis. Genomic DNA isolated from leaves was used as the PCR template. FAM fluorescent signal values are plotted on the x-axis and HEX fluorescent signal values are plotted on the y-axis. Triangle dot indicates the no-template control (NTC).

Table 4. Two minimal marker subsets to disriminate the 48 citrus cultivars by at least a single differentiating SNP

Minimal marker subset No.	TaqMan-MGB SNP marker (Scaffold number[a])						
Subset 1	SI312 (1)	SI001 (2)	SI322 (4)	SI149 (4)	SI306 (6)	SI210 (8)	SI381 (9)
Subset 2	SI312 (1)	SI001 (2)	SI322 (4)	SI149 (4)	SI306 (6)	SI201 (7)	SI210 (8)

[a] Refer Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) for clementine genome information.

(**Fig. 2**). Although the amount of template DNA was identical among all sample types, the Ct values varied in the following order: leaf < fresh fruit < juice < dry fruit < canned fruit (**Fig. 3**). This order was consistent across all assays. The delta Rn values tend to correspond to the degree of DNA degradation, with canned fruit and dry fruit samples showing decreased values compared with fresh fruit and juice samples.

Discussion

The infringement of breeders' rights for Japanese superior citrus cultivars with valid patent is getting concerned along the agricultural product export strategy advanced by the Japanese government. The scions of the patent cultivars were unwillingly outflowed overseas and the reverse import of pirated fruits and processed products into Japan is faced on the real problem. To obstruct this problem, a rapid and stable DNA diagnosis system, which is applicable to fresh and processed fruits, have been required in addition to previously developed system for nursery trees. In this experiment, we developed a new alternate basis of Japanese citrus cultivar identification system using TaqMan-MGB SNP genotyping assay and confirmed the applicability to fresh and processed fruits. The developed 8 TaqMan-MGB SNP markers were validated by MARCO software to confirm the inheritability of the target SNPs among the cultivars with parent-offspring relationship and it was demonstrated that they could also be applied to parentage and cultivar identification. The mean PIC value of the 8 TaqMan-MGB SNP markers was 0.35 among the examined 48 citrus cultivars. The mean PIC value for citrus species, including related species of trifoliate orange (*P. trifoliata*), papeda (*C. honghensis*), kumquat (*Fortunella* spp), was 0.45, ranging from 0.40 to 0.48 in the genome-wide SSR analysis (Liu *et al.* 2013). Meanwhile, the mean PIC value of SNPs in open reading frames (ORFs) among 18 representative *Citrus* species has been reported to be 0.34, ranging from 0.32 to 0.35 (Distefano *et al.* 2013), which is similar to our result. The result indicated that the 8

Table 5. Minimal marker subset to identify the registered cultivar with valid patent among 48 citrus cultivars in **Table 1**

Cultivar	Expired date of patent	Number of minimal SNP marker for identification	Example of minimal marker subset				Total number of minimal marker subsets comprising different SNP marker combination
'Reikou'	December in 2035	2	SI210	SI312			1
'Tsunokagayaki'	March in 2039	3	SI001	SI210	SI312		2
'Seinannohikari'	March in 2039	3	SI149	S210	SI306		2
'Tsunonozomi'	May in 2041	3	SI001	SI210	SI312		2
'Haruhi'	March in 2041	2	SI201	SI306			3
'Setoka'	October in 2026	3	SI001	SI201	SI312		2
'Harumi'	Nobember in 2024	4	SI149	SI210	SI312	SI381	6
'Harehime'	June in 2029	3	SI001	SI210	SI306		4
'Rinoka'	March in 2045	2	SI306	SI322			1
'Mihaya'	September in 2044	2	SI001	SI306			1
'Asumi'	September in 2044	3	SI149	SI306	SI312		18
'Asuki'	N/A	3	SI001	SI210	SI306		2
'Kanpei'	August in 2037	3	SI149	SI210	SI306		1
'Ehime Kashi No. 28 gou' [Benimadonna[a]]	March in 2035	3	SI001	SI306	SI322		2
'Tamami'	December in 2036	3	SI001	SI149	SI381		1
'Nishinokaori'	December in 2025	3	SI201	SI306	SI312		10
'Amaka'	November in 2024	3	SI001	SI149	SI306		3
'Kankitsuchukanbohon nou 6 gou'	June in 2029	2	SI312	SI381			2

[a] The registered trademark of Ehime Prefectural Headquarters, National Federation of Agricultural Cooperative Associations.

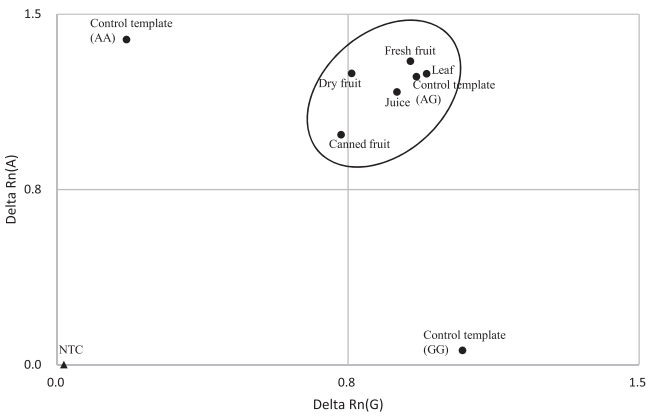

Fig. 2. Allelic discrimination plots of various DNA samples isolated from leaves, fresh fruit, juice, canned fruit, and dry fruit of the 'Shiranuhi' in SI306 marker. FAM fluorescent signal values are plotted on the x-axis and HEX fluorescent signal values are plotted on the y-axis. Triangle dot indicates the no-template control (NTC). The leaf DNA samples of Iyo, 'Duncan' grapefruit and 'Lisbon' lemon are used as the reference genotypes of AA, AG and GG, respectively.

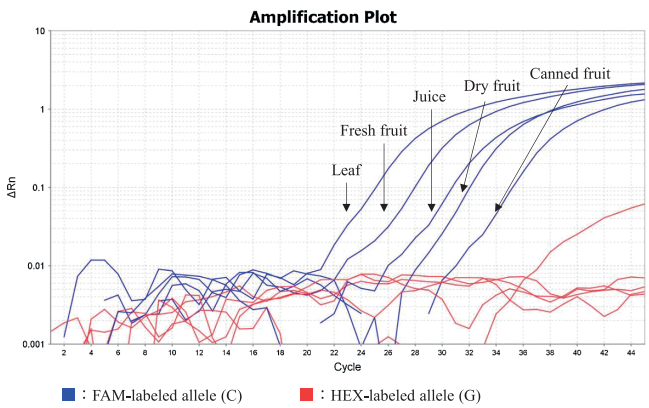

Fig. 3. Amplification plot pattern of various DNA samples isolated from leaves, fresh fruit, juice, canned fruit, and dry fruit of 'Shiranuhi' in SI001 marker. Blue line indicates FAM fluorescent signal and red line indicates HEX fluorescent signal. The allelic genotypes of the examined 'Shiranuhi' samples in SI001 are assigned to CC. The Ct value increases in the following order: leaf < fresh fruit < juice < dry fruit < canned fruit.

TaqMan-MGB SNP markers possessed the moderate polymorphism for the cultivar identification in citrus. The average size of their PCR amplicons was 80.3 bp ranging from 60 to 99 bp, and they were confirmed to detect SNPs on DNA samples isolated from fresh fruit, juice, canned fruit and dry fruit in 'Shiranuhi'. In addition, fresh fruits, juices, canned fruits and dry fruits of plural other cultivars, which were made by the same procedure with the processed fruits of 'Shiranuhi', were applied to TaqMan-MGB SNP genotyping assay. It was also confirmed that SNP was stably detected in those samples (**Supplemental Fig. 1**) and the same allelic genotype for 8 TaqMan-MGB SNP markers was obtained with their leaf sample. Considering that small PCR amplicons of approximately 150 bp were utilized as DNA markers in the analysis of degraded DNA samples from canned tuna, bonito, and pear (Ram *et al.* 1996, Yamamoto *et al.* 2006), the TaqMan-MGB SNP assay developed here is theoretically applicable to processed

fruits. It is well known that DNA degradation occurs during food manufacturing processes, due to heat and physical and chemical factors (Quirasco *et al.* 2004, Vijayakumar *et al.* 2009). Mano *et al.* (2017) compared the amplification curves in real-time PCR analysis between undamaged DNA samples and DNA samples degraded by a time-course of heat treatment. They reported that the Ct values of degraded DNA samples increased in parallel with the degree of DNA degradation. Similar result was obtained in our experiment that more PCR cycles are required to reach exponential PCR amplification in processed samples than fresh samples. Therefore, the modification of PCR cycle (45 cycles) ensured the stable and reliable assignment of genotypes for processed fruit DNA samples. In TaqMan-MGB SNP genotyping assay, the target specific PCR product is detected by the third oligo of TaqMan-MGB probe. Non-specific products such as primer dimer, which might be amplified simultaneously in long PCR cycles, would not reflect the detected fluorescence value. The manufacture's protocol recommends increasing the number of PCR cycles up to 50 cycles for the samples with small delta Rn value. Thus, it was demonstrated that TaqMan-MGB SNP genotyping assay can be used to successfully genotype both fresh and processed fruit samples. Scarano and Rao (2014) evaluated various types of DNA markers for food product authentication in plants, meats and fishes. They concluded that SNPs are useful in food traceability and SNPs can be adapted to highly fragmented DNA because the detection of their polymorphism is based on the amplification of very small fragments. In fact, SNP genotyping was successful in the analysis of degraded DNA samples of salmon and olive oil (Agrimonti *et al.* 2011, Johnston *et al.* 2013). Among the examined processed fruits, SNP genotyping was failed in jam and purified juice without pulp, from which sufficient level of DNA was not available due to long-term heating and lack of tissues with nucleus (data not shown). We considered that the application scope of TaqMan-MGB SNP genotyping assay would depend on the quality of DNA extracts of the processed fruits. Even in the same category of processed fruit that SNP detection was successful in this experiment, it is plausible that SNP detection may fail when the processing condition is different and only poor quality of DNA was available. Therefore, the experiment condition should be customized for each procced product such as DNA extraction method, PCR cycle, template amount of reference sample and so on.

In conclusion, we developed a SNP genotyping system using TaqMan-MGB SNP genotyping assay for the reliable and reproducible identification of citrus cultivars. The genotype information of 48 citrus cultivars reported here, covers more than 98% of all citrus fruit shipments produced in Japan and is an indispensable reference source in citrus cultivar identification and food safety. The developed TaqMan-MGB SNP genotyping assay was confirmed to be applicable for both fresh and representative processed fruit samples. It could provide stable DNA polymorphism detection in fresh and processed fruits in which lower poor quality of DNA is expectedly available and makes up for a shortcoming of the previously developed system of CAPS marker. Recently, the development of various SNP detection systems, such as multiplex systems and array systems, has allowed the rapid and efficient genotyping of large numbers of samples using methods that are amenable to high-throughput automation. The cultivar identification system reported here is expected to play an important role in reinforcing the protection of registered cultivar breeders' rights and in inspections related to food fraud and food safety.

Author Contribution Statement

Dr. T. Endo designed the TaqMan-MGB SNP marker and conducted SNP genotyping assays. Dr. H. Fujii validated the 8 TaqMan-MGB SNP markers using MARCO software and calculated the minimal marker subset to identify the registered cultivars by MinimalMarker software. Dr. T. Yoshioka managed the examined citrus cultivars and collected the samples from original trees. Dr. M. Omura searched the candidate SNPs applicable to the SNP genotyping of fresh and processed fruits out of the past developed DNA markers. Dr. T. Shimada isolated DNA from the examined citrus cultivars and is a corresponding author to organize the manuscript.

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