JARQ 56 (4), 329-340 (2022) https://www.jircas.go.jp

Development of Cultivar Identification System Using 12 InDel Markers for Widely Distributed Citrus Cultivars in Japan

Tomoko ENDO, Hiroshi FUJII and Takehiko SHIMADA*

Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization, Shimizu, Japan

Abstract

Citrus cultivar identification systems using cleaved amplified polymorphic site and single-nucleotide polymorphism markers have been developed for nursery trees and flesh fruits, but time and cost efficiency require improvement for the inspection process. Here, we developed a new cultivar identification system using the InDel marker. Twelve InDel markers, which revealed clear bi-allelic PCR fragment patterns, were selected from 185 InDel markers. Their primer sets were redesigned to generate less than 650 bp PCR fragments, and all were confirmed to apply to leaf and fresh fruit samples. It was confirmed that they were inherited in a codominant fashion among cultivars with parent–offspring relationships. At least two differentiating InDel polymorphisms to discriminate any paired combination among 33 citrus cultivars were provided, including 14 ancestral varieties and a reference genome cultivar of the clementine mandarin. Minimal marker subsets to identify the target cultivar are listed for each of the 14 registered cultivars with valid breeder's rights. The developed cultivar identification system features a simple experimental procedure with PCR and electrophoresis, saving time and cost during the inspection process. It could help protect registered cultivars from the illegal distribution of nursery trees and the reimportation of illegal fruits from abroad.

Discipline: Crop Science
Additional key words: breeder's rights, DNA diagnosis, fruit, nursery tree, breeder's rights protection

Introduction

Citrus plants are one of the major cultivated fruits in Japan, and various high-quality cultivars have been developed, such as "Shiranuhi," "Setoka," "Mihaya," and "Asumi." The Japanese citrus industry has benefited from cultivars developed using conventional breeding methods. Owing to the global demand for high-quality cultivars, new Japanese cultivars have generated interest worldwide. Recently, the unauthorized overseas outflow of nursery trees of citrus new cultivars has been frequently reported for "Asumi," "Mihaya," and "Kanpei." This connects to the subsequent reverse importation of pirated fruits into Japan and the loss of overseas markets, resulting in potential damage to Japanese farmers. Therefore, cultivar identification systems using various types of DNA markers have been developed to protect breeders' rights regarding the registered Japanese citrus cultivars with valid patents. Using cleaved amplified polymorphic site (CAPS) markers, a cultivar identification system was initially developed (Fujii et al. 2019). Next, the 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 website (https://www.naro.affrc.go.jp/publicity_report/publication/pamphlet/tech-pamph/130601.html). The CAPS markers included in the manual are guaranteed stable and reproducible for citrus cultivar identification to protect breeders' rights during legal procedures effectively. This manual helps to deter the infringement of domestic regulations regarding nursery trees, such as the illegal diversion of the scion. Additionally, an alternative cultivar identification system using TaqMan-MGB single-nucleotide polymorphism (SNP) genotyping assays has been developed to protect breeder's rights to the sale of their fruits and processed products from overseas (Endo et al. 2020). Some of the CAPS markers with long PCR amplicons in the manual

*Corresponding author: tshimada@affrc.go.jp
Received 20 October 2021; accepted 22 December 2021.

would not be applied to the processed products because PCR amplification is unstable with insufficient quantities of DNA and residual impurities in the DNA extracts. Unless the extracted DNA sample is incompatible because of excessive heat or chemical treatment degradation, the developed system is promised to apply to both fresh and processed fruits. These cultivar identification systems contribute to the protection of Japanese citrus cultivar brands, whereas a more convenient system must reduce time and cost in the inspection sites and apply DNA diagnostics for various purposes other than infringement.

Recent advances in next-generation sequencing technologies and bioinformatics have greatly accelerated genomics research on perennial crops. The draft genome sequences of citruses, such as pummelo (*Citrus grandis* Osbeck), satsuma mandarin (*Citrus unshiu* Marc), sweet orange (*Citrus sinensis* Osbeck), citron (*Citrus medica* L.), and Ichang papeda (*Citrus ichangensis* Swingle), have been assembled de novo (Wu et al. 2014, Wang et al. 2017, Kawahara et al. 2020). These sequence resources could facilitate the exploration of DNA polymorphisms required for cultivar identification. Insertion/deletion polymorphisms (InDels) are derived from the insertion of transposable elements, slippage in simple-sequence replication, or unequal crossover events (Britten et al. 2003). InDel markers display higher genetic diversity at the interspecific level than simple-sequence repeat markers (Garcia-Lor et al. 2012). Ollitrault et al. (2012) and Fang et al. (2018) identified several InDels by comparing the genome sequences of citrus species and developed PCR-based polymorphic InDel markers. Noda et al. (2020) also developed 119 InDel markers with three PCR fragments from the satsuma mandarin genome sequence and applied them to discriminate satsuma mandarin hybrid seedlings. For phylogenetic analysis and marker-assisted selection in citrus breeding and cultivar identification, these InDel markers are expected to be useful.

In this study, we developed a new cultivar identification system using InDel markers. The system provides a simple experimental procedure consisting of PCR and electrophoresis. The 12 InDel markers with clear biallelic PCR fragment patterns were selected out of 185 previously developed InDel markers (Fang et al. 2018, Kawahara et al. 2020). Here, we report the allelic genotyping information for 33 citrus cultivars, including 14 ancestral varieties and reference genome of clementine mandarin, representing more than 94% of all shipments of citrus fruits produced in Japan. The 12 InDel markers were confirmed to apply to DNA samples isolated from fresh fruits. In this study, the cultivar identification system reported is very useful and saves costs during the inspection of nursery trees and illegal fruits and could be utilized as a convenient DNA diagnosis for various purposes in addition to breeder's rights infringement.

Materials and methods

1. Plant material and DNA preparation

A total of 33 citrus cultivars and varieties preserved at the Division of Citrus Research at the National Agriculture and Food Research Organization Institute of Fruit and Tea Tree Science (NIFTS) were used in this study (Table 1). Taxonomic classification was performed according to the method described by Tanaka (1969). Sample accession numbers (JP No.) in Table 1 are based on GenBank (http://www.gene.affrc.go.jp/databases-plant_search.php) at the Genetic Resources Center, National Agriculture and Food Research Organization (NARO), to understand the origin of the strain for InDel genotyping. These plant materials included 24 widely distributed citrus cultivars in the Japanese market, 14 ancestral varieties for the Japanese breeding population, in which some cultivars overlap, and a reference genome cultivar of the clementine mandarin (Table 1).

Genomic DNA was isolated from fully expanded leaves using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Fresh fruits of the "Rinoka," "Mihaya," "Asumi," and "Asuki" were sampled from the trees preserved in NIFTS. Peels were excised from the fruits and powdered using liquid nitrogen, and genomic DNA was isolated using a DNeasy Plant Mini Kit (Qiagen).

2. Screening of InDel markers and redesigning of the primer set

Of the 185 InDel markers developed by Fang et al. (2018) and Kawahara et al. (2020), InDel markers were screened using 14 ancestral varieties by the following condition: 1) clear biallelic pattern comprising 2 PCR fragments and 2) more than 50 bp of length difference between 2 PCR fragments in fragment size. Using GENETYX ver. 15 (GENETYX, Tokyo, Japan), a primer set for the InDel marker with more than 650 bp of the PCR amplicon was redesigned to enable the inspection of fruit samples. The annotation information of the 12 selected InDel markers was investigated using the BLAST search function in the Mikan Genome Database (https://mikan.dna.affrc.go.jp/).

3. PCR and electrophoresis

The PCR reaction mixture was prepared in a 10 µL solution, which contained 5 ng of genomic DNA, 5 pmol of the forward and reverse primers, and 5 µL GoTaq®

Green Master Mix (Promega, Madison, WI, USA). PCR cycling conditions were as follows: 1 cycle denaturation at 95°C for 2 min, 32 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, an extension at 72°C for 1 min, and 1 cycle for the final extension at 72°C for 5 min. All reactions were performed using a ProFLEX PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The PCR products were electrophoresed in 2.0% agarose gel (Agarose standard 01, Solana; Rikaken, Nagoya, Japan) electrophoresis and visualized by ethidium bromide staining.

4. Validation of InDel markers and minimal marker subsets for cultivar identification

To evaluate the reliability of the 12 InDel markers, we selected several trios of cultivars that had a parent–child relationship from the genotyped cultivars and confirmed the inheritance pattern of marker genotypes using MARCO software (Fujii et al. 2010). MARCO evaluated the validity of a parent–offspring relationship by confirming that each parent contributed one marker allele to its offspring. The minimal marker subset required to discriminate between 24 cultivars and registered cultivars with valid breeder's rights was calculated using MinimalMarker software (Fujii et al. 2013). The values of observed homozygosity (Ho), expected heterozygosity (He), and the Polymorphic Information Content (PIC) were calculated using MarkerToolKit v 1.0 (Fujii et al. 2008).

Results

1. Screening InDel markers suitable for cultivar identification

Of the 185 InDel markers in previous reports (Fang et al. 2018, Kawahara et al. 2020), InDel markers with clear biallelic PCR fragment patterns were screened using 14 ancestral varieties (Table 1). Most InDel

Table 1. Citrus cultivars and ancestral varieties used in this study

No.	JP No.[a]	Cultivar name (native species)	Parentage or scientific name [b]	Selecting reason
1	117351	"Miyagawa-wase" (satsuma mandarin)	*Citrus unshiu* Marc.	Widely-distributed cultivar
2	168864	"Duncan" grapefruit (grapefruit)	*C. paradisi* Macf.	Widely-distributed cultivar & ancestral variety
3	172154	"Trovita" orange (sweet orange)	*C. sinensis* (L.) Osbeck	Widely-distributed cultivar & ancestral variety
4	117289	"Lisbon" lemon (lemon)	*C. limon* (L.) Burm. f.	Widely-distributed cultivar & ancestral variety
5	117159	"Shiranuhi" [Dekopon [c]]	"Kiyomi" × "Nakano 3 gou" ponkan	Widely-distributed cultivar
6	117373	Iyo (Iyo)	*C. iyo* hort. ex Tanaka	Widely-distributed cultivar & ancestral variety
7	117297	"Kawanonatsudaidai" (natsuaidai)	*C. natsudaidai* Hayata	Widely-distributed cultivar
8	117286	Hassaku (hassaku)	*C. hassaku* hort. ex Tanaka	Widely-distributed cultivar & ancestral variety
9	171505	"Ohta ponkan" (ponkan)	*C. reticulata* Blanco	Widely-distributed cultivar & ancestral variety
10		"Rinoka"	"Lisbon" lemon × "Hyuganatsu"	Widely-distributed cultivar
11	251815	"Mihaya"	"Tsunonozomi" × "No.1408"	Widely-distributed cultivar
12	245233	"Asumi"	"Okitsu 46 gou" × "Harumi"	Widely-distributed cultivar
13		"Asuki"	"Okitsu 46 gou" × "Harumi"	Widely-distributed cultivar
14		"Reikou"	Unknown × "Murcott"	Widely-distributed cultivar
15		"Tsunokagayaki"	"KyOw No.14" × "Encore"	Widely-distributed cultivar
16		"Seinannohikari"	"EnOw No.21" × "Youkou"	Widely-distributed cultivar
17		"Tsunonozomi"	"Kiyomi" × "Encore"	Widely-distributed cultivar
18	237599	"Haruhi"	"Okitsu 46 gou" × "Awa-orange"	Widely-distributed cultivar
19	115521	"Kiyomi"	"Miyagawa-wase" × "Trovita" orange	Widely-distributed cultivar
20	118842	"Setoka"	"KyEn No.4" × "Murcott"	Widely-distributed cultivar
21	117468	"Harumi"	"Kiyomi" × Ponkan "F-2432"	Widely-distributed cultivar
22		"Harehime"	"E-647" × "Miyagawa-wase"	Widely-distributed cultivar
23		"Kanpei"	"Nishinokaori" × Ponkan	Widely-distributed cultivar
24		"Ehime Kashi No. 28" [Benimadonna [d]]	"Nankou" × "Amakusa"	Widely-distributed cultivar
25		Dancy tangerine	*Citrus tangerina* hort. ex Tanaka	Ancestral variety
26		"Mukakukishu"	*C. kinokuni* hort. ex Tanaka	Ancestral variety
27		"Tanikawa" buntan	*C. grandis* Osbeck	Ancestral variety
28		Hyuganatsu	*C. tamurana* hort. ex Tanaka	Ancestral variety
29		Kunenbo	*C. nobilis* Lour. var. kunep Tanaka	Ancestral variety
30		Willowleaf mandarin	*C. deliciosa* Ten.	Ancestral variety
31		King Mandarin	*C. nobilis* Lour.	Ancestral variety
32		Murcot	Hybrid	Ancestral variety
33		Clementine mandarin	*C. clementina* hort. ex Tanaka	Reference of genome sequence

[a] Description by Genebank (http://www.gene.affrc.go.jp/databases-plant_search.php)

[b] Tanaka's system was used for scientific name (Tanaka 1969).

[c] The registered trademark of the Federation of Kumamoto Prefectural Fruit Agriculture Cooperatives

[d] The registered trademark of the National Federation of Agricultural Cooperative Associations

markers revealed multiple PCR fragment patterns with more than 2 PCR fragments among 14 ancestral varieties. These InDel markers were not considered suitable as DNA markers for cultivar identification because it would be difficult to judge whether the PCR fragments were derived from the same or different loci, and the validation test based on allelism would become extremely difficult. A total of 12 InDel markers were selected based on the criteria that 2 PCR fragments could be fractionated in 2% agarose gel electrophoresis.

The genetic loci in the clementine mandarin genome sequence (Wu et al. 2014) and PCR fragment size of the 12 InDel markers are summarized in Table 2. Five InDel markers were derived from scaffold 2, three InDel markers were derived from scaffold 1, and the remaining InDel markers were derived from scaffolds 5, 8, 6, and 9. Three InDel markers (CI-IND1, 2, and 9) were located in the coding region, whereas the remaining nine InDel markers were located in the noncoding region. A biallelic PCR fragment pattern was observed among 14 ancestral varieties; a large PCR fragment was assigned as the A allele, and a small PCR fragment was assigned as the B allele in each InDel marker. No other alleles were observed among 14 ancestral varieties.

The 12 selected InDel markers were applied to 24 widely distributed citrus cultivars in the Japanese market, which account for more than 94% of all citrus fruit shipments produced in Japan. A clear biallelic PCR fragment pattern was acquired from DNA isolated from the leaves for each InDel marker (Fig. 1). Allelic genotypes were scored based on the PCR fragment pattern and are summarized in Table 3. The He, Ho, and PIC values of each InDel marker were calculated to determine the genetic diversity of the 33 citrus cultivars, including 14 ancestral varieties and clementine mandarin genome sequence for referencing (Table 3). The He values ranged from 0.36 to 0.51, with an average of 0.44. The Ho values ranged from 0.27 to 0.66, with an average of 0.51, and PIC values ranged from 0.20 to 0.37, with an average of 0.34. The allelic genotypes at these 12 InDel markers in 33 citrus cultivars provided valuable reference information for identifying citrus cultivars by DNA diagnosis.

2. Validation of 12 InDel markers by parentage analysis

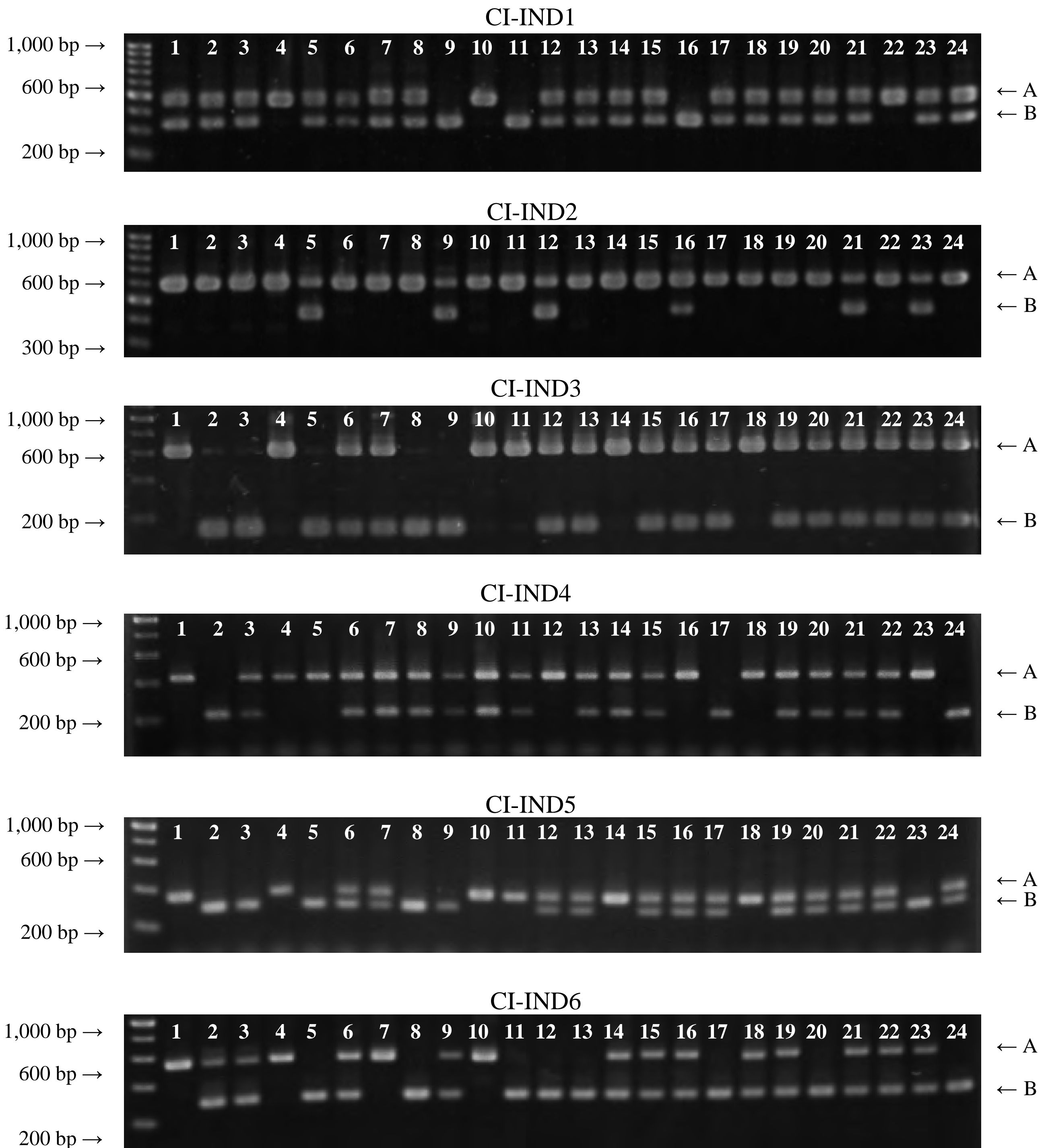
Among the 33 examined citrus cultivars including 14 ancestral varieties, there were six different combinations with parent–offspring relationships as follows: satsuma mandarin (Kishu mandarin [*Citrus kinokuni*] × kunenbo [*Citrus nobilis* var. *knep*]), "Shiranuhi" ("Kiyomi" × ponkan [*Citrus reticulata*]), "Rinoka" (lemon [*Citrus lemon*] × hyuganatsu [*Citrus tamurana* hort. ex Tanaka]), "Kiyomi" (satsuma mandarin × sweet orange), "Harumi" ("Kiyomi" × ponkan), and clementine mandarin [*Citrus clementina*] (Willow leaf mandarin [*Citrus deliciosa*] × sweet orange). The allelic genotypes of the cultivars of the 12 InDel markers were analyzed using MARCO software (Fujii et al. 2010) to trace the lineage from parent to progeny. The alleles in the satsuma mandarin, "Shiranuhi," "Rinoka," "Kiyomi," "Harumi," and clementine mandarin were confirmed to be inherited from

Table 2. Information of 12 InDel markers applied in this study

SNP marker	Forward Primer	Reverse Primer	PCR product size (bp)		Position of clementine genome ver1.0[a]		
			A allele	B allele	Scaffold	Locus	Gene function
CD-IND1	GATTTGGGTATGCGGCTTTCGG	TGAGAGGGTTCCATCTGAAAGCTG	448	259	1	Ciclev10008211m.g	Amino acid trasnporter
CI-IND2	TTTGTCTTCGGCCCCTCAGACAC	TCGCACAAGCTGCACCACATCA	600	431	5	Ciclev10002215m.g	ATP-dependent Clp protease
CI-IND3	TGGATTGAAGTCAGATTTCCGTCTATC	GTGCCACTTCAGCCTTGGTC	600	150	2	32464505::32465104	Non-coding
CI-IND4	GCCAAGCAATGCCGATATCA	ATTGGGTTGGGGAGGCAAAA	425	213	9	7702525::7702737	Non-coding
CI-IND5	TGTGGTTACTATCAAGGAGACC	GCTCAATTATTGTCAGCGC	341	280	2	32706742::32707082	Non-coding
CI-IND6	TTGAAAGAGGACTGAACGTAC	ACACTTCTAATTTCGGCTACTT	548	285	2	35520021::35520568	Non-coding
CI-IND7	AATACTTGATCCGTGGCGCACTACG	AGTTAACAACATCACAAGAGCAGTT	499	280	2	36073972::36074470	Non-coding
CI-IND8	ACTCTACCCAGCTAGTTGTT	TGGTTAAAATGCCAGATGAACT	642	286	2	33702664::33703305	Non-coding
CI-IND9	TGGTGAGGACTGAGGAGATTCT	ATGAAGTAGCCTGGACCACC	520	287	1	Ciclev10024995m.g	Serine/threonine protein kinase
CI-IND10	GCCCAGATCTCTCAGCCGTA	AGAAATTACGCAGGGCTCAGT	540	280	6	22729611:22730150	Non-coding
CI-IND11	CCAACCGACAGTCCATATGCT	GCAGGGCTCCATTGATCCTT	596	278	8	1015651::1015929	Non-coding
CI-IND12	GCTGCGGTTTTGTCTTTCCC	ACAATAGTGGCAGAGTAGTTTT	505	300	1	106049::106553	Non-coding

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

either of the parental alleles without any discrepancy. Thus, these 12 InDel markers were correctly inheritable among cultivars with parent–offspring relationships. These alleles were originally derived from 14 ancestral varieties and could be applied to parentage and cultivar identification in Japanese cultivars.



(Continued on next page)

(Continued)

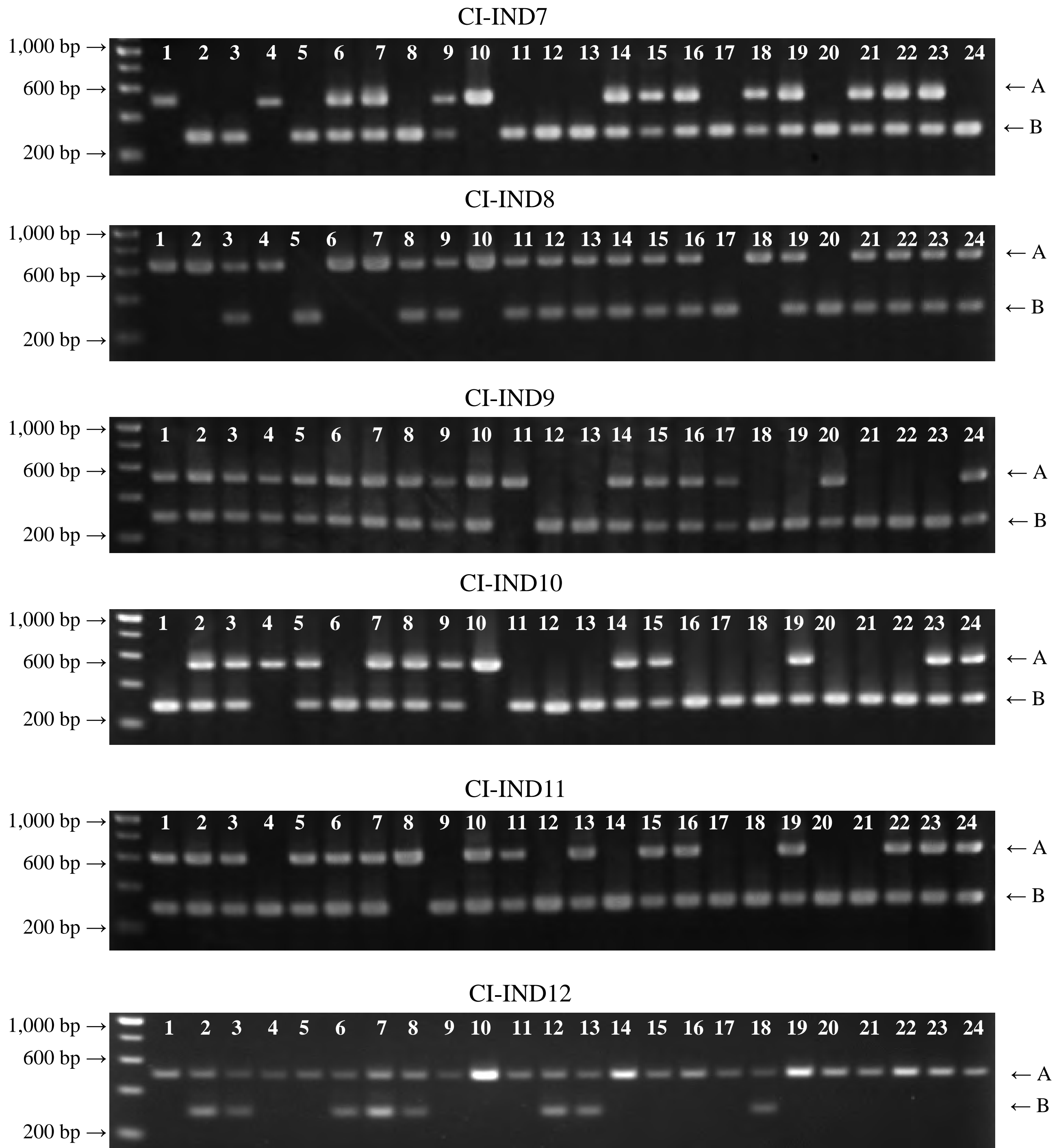


Fig. 1. PCR fragment pattern of 12 InDel markers for 24 citrus cultivars widely distributed in the Japanese market

Genomic DNA isolated from leaves was used as the PCR template. The number indicates plant material; the same number as in Table 1 is used. The letter on the right side indicates the allele in each InDel marker. A 100 bp DNA ladder is used as a size marker in CI-ND1 and CI-IND2, and a 200 bp DNA ladder is used in the remaining markers.

Table 3. Genotypes of 33 citrus cultivars and ancestral varieties by 12 InDel markers

No.	Cultivar name (native species)	InDel marker	CI-IND1	CI-IND2	CI-IND3	CI-IND4	CI-IND5	CI-IND6	CI-IND7	CI-IND8	CI-IND9	CI-IND10	CI-IND11	CI-IND12
		He [a]	0.50	0.24	0.50	0.49	0.50	0.50	0.48	0.48	0.49	0.44	0.45	0.24
		Ho [b]	0.67	0.27	0.61	0.61	0.49	0.49	0.49	0.58	0.64	0.49	0.58	0.27
		PIC [c]	0.37	0.21	0.37	0.37	0.37	0.37	0.36	0.37	0.37	0.35	0.35	0.21
1	"Miyagawa-wase" (satsuma mandarin)		AB	AA	AA	AA	AA	AA	AA	AA	AB	BB	AB	AA
2	"Duncan" grapefruit (grapefruit)		AB	AA	BB	BB	BB	AB	BB	AA	AB	AB	AB	AB
3	"Trovita" orange (sweet orange)		AB	AA	BB	AB	BB	AB	BB	AB	AB	AB	AB	AB
4	"Lisbon" lemon (lemon)		AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	BB	AA
5	"Shiranuhi" [Dekopon [d]]		AB	AB	BB	AA	BB	BB	BB	BB	AB	AB	AB	AA
6	Iyo (iyo)		AB	AA	AB	AB	AB	AB	AB	AA	AB	BB	AB	AB
7	"Kawanonatsudaidai" (natsuaidai)		AB	AA	AB	AB	AB	AA	AB	AA	AB	AB	AB	AB
8	Hassaku (hassaku)		AB	AA	BB	AB	BB	BB	BB	AB	AB	AB	AA	AB
9	"Ohta ponkan" (ponkan)		BB	AB	BB	AB	BB	AB	AB	AB	AB	AB	BB	AA
10	"Rinoka"		AA	AA	AA	AB	AA	AA	AA	AA	AB	AA	AB	AA
11	"Mihaya"		BB	AA	AA	AB	AA	BB	BB	AB	AA	BB	AB	AA
12	"Asumi"		AB	AB	AB	AA	AB	BB	BB	AB	BB	BB	BB	AB
13	"Asuki"		AB	AA	AB	AB	AB	BB	BB	AB	BB	BB	AB	AB
14	"Reikou"		AB	AA	AA	AB	AA	AB	AB	AB	AB	AB	BB	AA
15	"Tsunokagayaki"		AB	AA	AB	AB	AB	AB	AB	AB	AB	AB	AB	AA
16	"Seinannohikari"		BB	AB	AB	AA	AB	AB	AB	AB	AB	BB	AB	AA
17	"Tsunonozomi"		AB	AA	AB	BB	AB	BB	BB	BB	AB	BB	BB	AA
18	"Haruhi"		AB	AA	AA	AA	AA	AB	AB	AA	BB	BB	BB	AB
19	"Kiyomi"		AB	AA	AB	AB	AB	AB	AB	AB	BB	AB	AB	AA
20	"Setoka"		AB	AA	AB	AB	AB	BB	BB	BB	AB	BB	BB	AA
21	"Harumi"		AB	AB	AB	AB	AB	AB	AB	AB	BB	BB	BB	AA
22	"Harehime"		AA	AA	AB	AB	AB	AB	AB	AB	BB	BB	AB	AA
23	"Kanpei"		AB	AB	AB	AA	BB	AB	AB	AB	BB	AB	AB	AA
24	"Ehime Kashi No. 28" [Benimadonna [e]]		AB	AA	AB	BB	AB	BB	BB	AB	AB	AB	AB	AA
25	Dancy tangerine		BB	AB	AB	AA	BB	AB	AB	AB	AB	BB	BB	AA
26	"Mukakukishu"		AB	AA	AA	AA	AA	AA	AA	AA	AB	BB	BB	AA
27	"Tanigawa" buntan		AB	AA	AB	AB	AA	BB	BB	AB	BB	AA	AA	AB
28	Hyuganatsu		AA	AA	AB	AB	AA	AA	AB	AA	AB	AB	AB	AA
29	Kunenbo		BB	AA	AB	AB	AB	AB	AB	AB	AB	BB	AB	AA
30	Willowleaf mandarin		BB	AB	AA	AB	AA	AA	AA	AA	AA	AB	BB	AA
31	King mandarin		AB	AA	AB	AB	AB	AB	AB	AB	AA	AB	AB	AA
32	Murcot		BB	AA	AB	AB	AB	BB	BB	BB	AB	AB	BB	AA
33	Clementine mandarin		AB	AB	AB	BB	AB	AB	AB	AB	AA	AB	AB	AA

[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 the National Federation of Agricultural Cooperative Associations

3. Application of 12 InDel markers for the genotyping of fresh fruits

To verify whether the newly developed cultivar identification system using 12 InDel markers could be applied to the genotyping of fresh fruits, InDel marker analysis was conducted using DNA samples isolated from fresh fruits of the "Asumi," "Asuki," "Mihaya," and "Rinoka" under the same PCR reaction conditions as the leaf samples. Clear biallelic PCR fragment patterns were obtained from fresh fruit samples (Fig. 2). All allelic genotypes of the 12 InDel markers detected in fresh fruit samples of the four examined cultivars were identical to those in the leaf samples. Therefore, it was confirmed that the 12 selected InDel markers could be used to inspect fresh fruit samples.

4. Minimal marker set to identify the registered citrus cultivars with valid breeder's right

We analyzed the genotyping data for the 12 InDel markers in Table 3 using MinimalMarker software (Fujii et al. 2013) to calculate a minimal marker set to discriminate 33 citrus cultivars. There was a single minimal marker subset consisting of six InDel markers that could discriminate any pair combination among

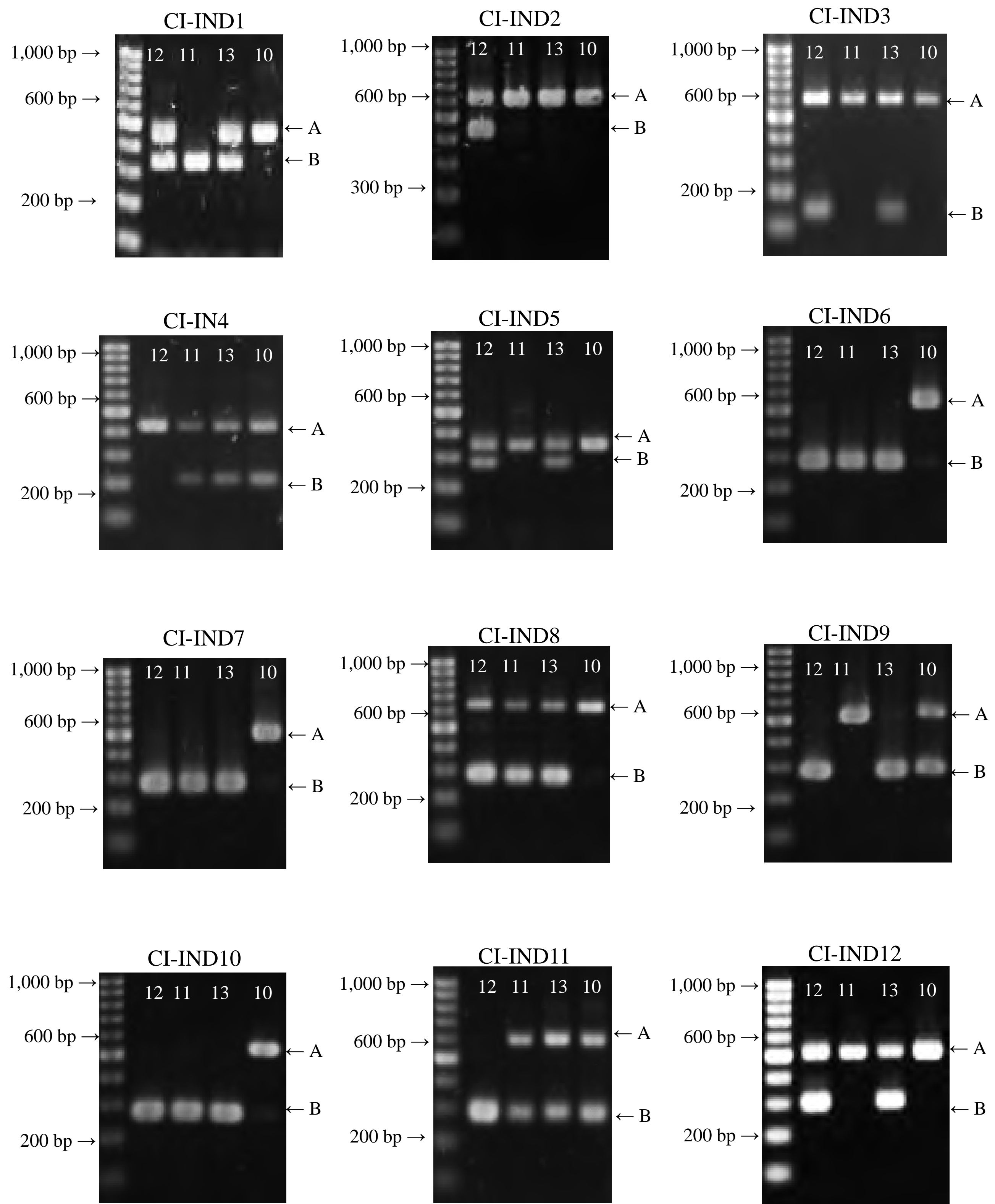


Fig. 2. PCR fragment pattern of 12 InDel markers amplified from genomic DNA isolated from fresh fruit samples of four representative citrus cultivars "Asumi," "Mihaya," "Asuki," and "Rinoka"

The number indicates plant material; the same number as in Table 1 is used. The letter on the right side indicates the allele in each InDel marker. A 100 bp DNA ladder is used as a size marker.

33 citrus cultivars by the difference of at least one InDel polymorphism (Table 4), as follows: CI-IND4 (scaffold 9), CI-IND5 (scaffold 2), CI-IND9 (scaffold 1), CI-IND10 (scaffold 6), CI-IND11 (scaffold 8), and CI-IND12 (scaffold 1). A total of 2 subsets consisting of 10 InDel marker combinations could discriminate any pair combination among the 33 citrus cultivars by two differentiating InDel polymorphisms, in the following: subset 1: CI-IND1 (scaffold 1), CI-IND2 (scaffold 5), CI-IND4 (scaffold 9), CI-IND5 (scaffold 2), CI-IND6 (scaffold 2), CI-IND7 (scaffold 2), CI-IND8 (scaffold 2), CI-IND9 (scaffold 1), CI-IND10 (scaffold 6), and CI-IND11 (scaffold 8) and subset 2: CI-IND1 (scaffold 1), CI-IND2 (scaffold 5), CI-IND3 (scaffold 2), CI-IND4 (scaffold 2), CI-IND5 (scaffold 2), CI-IND6 (scaffold 2), CI-IND8 (scaffold 2), CI-IND9 (scaffold 1), CI-IND10 (scaffold 6), and CI-IND11 (scaffold 8). Among the 33 citrus cultivars examined, 14 had valid breeders' rights. Using allelic genotype data of the 12 InDel markers, a minimal marker subset was calculated to identify registered cultivars with valid breeder's rights (Table 5). "Tsunonozomi," "Haruhi," "Harehime," "Rinoka," "Mihaya," "Asumi," and "Kanpei" could be identified by at least two differentiating InDel markers. "Reikou," "Seinannohikari," "Setoka," "Harumi," "Asuki," and "Ehime Kashi No. 28 gou" ("Benimadonna") could be identified by at least three differentiating InDel markers. At least four differentiating InDel markers could identify "Tsunokagayaki." Depending on the cultivar, the total number of minimal marker subsets with differentiating InDel marker combinations varied from 1 ("Rinoka," "Asumi," and "Kanpei") to 30 ("Reikou"). For example,

Table 4. The minimal marker set to disriminate the 33 citrus cultivars and ancestral varieties by at least a single differentiating InDel polymorophism

InDel marker (Scaffold number [a]) of minimal marker set :	CI-IND4 (9)	CI-IND5 (2)	CI-IND9 (1)	CI-IND10 (6)	CI-IND11 (8)	CI-IND12 (1)
Cultivar	Allelic genotype					
"Lisbon" lemon (lemon)	AA	AA	AB	AA	BB	AA
"Miyagawa-wase" (satsuma mandarin)	AA	AA	AB	BB	AB	AA
"Mukakukishu"	AA	AA	AB	BB	BB	AA
"Haruhi"	AA	AA	BB	BB	BB	AB
"Seinannohikari"	AA	AB	AB	BB	AB	AA
"Asumi"	AA	AB	BB	BB	BB	AB
"Shiranuhi" [Dekopon [b]]	AA	BB	AB	AB	AB	AA
Dancy tangerine	AA	BB	AB	BB	BB	AA
"Kanpei"	AA	BB	BB	AB	AB	AA
Willowleaf mandarin	AB	AA	AA	AB	BB	AA
"Mihaya"	AB	AA	AA	BB	AB	AA
"Rinoka"	AB	AA	AB	AA	AB	AA
Hyuganatsu	AB	AA	AB	AB	AB	AA
"Reikou"	AB	AA	AB	AB	BB	AA
"Tanigawa" buntan	AB	AA	BB	AA	AA	AB
King Mandarin	AB	AB	AA	AB	AB	AA
"Tsunokagayaki"	AB	AB	AB	AB	AB	AA
"Kawanonatsudaidai" (natsuaidai)	AB	AB	AB	AB	AB	AB
Murcot	AB	AB	AB	AB	BB	AA
Kunenbo	AB	AB	AB	BB	AB	AA
Iyo (iyo)	AB	AB	AB	BB	AB	AB
"Setoka"	AB	AB	AB	BB	BB	AA
"Kiyomi"	AB	AB	BB	AB	AB	AA
"Harehime"	AB	AB	BB	BB	AB	AA
"Asuki"	AB	AB	BB	BB	AB	AB
"Harumi"	AB	AB	BB	BB	BB	AA
Hassaku (hassaku)	AB	BB	AB	AB	AA	AB
"Trovita" orange (sweet orange)	AB	BB	AB	AB	AB	AB
"Ohta ponkan" (ponkan)	AB	BB	AB	AB	BB	AA
Clementine mandarin	BB	AB	AA	AB	AB	AA
"Ehime Kashi No. 28" [Benimadonna [c]]	BB	AB	AB	AB	AB	AA
"Tsunonozomi"	BB	AB	AB	BB	BB	AA
"Duncan" grapefruit (grapefruit)	BB	BB	AB	AB	AB	AB

[a]Refer to Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) for clementine genome information, [b]The registered trademark of the Federation of Kumamoto Prefectural Fruit Agriculture Cooperatives, [c]The registered trademark of the National Federation of Agricultural Cooperative Associations

"Asumi" could be identified from the other 32 citrus cultivars by genotypes at CI-IND2 (AB) and CI-IND12 (AB), whereas genotypes could identify "Setoka" at the following two subsets: subset 1: CI-IND1 (AB), CI-IND4 (AB), and CI-IND8 (BB) and subset 2: CI-IND4 (AB), CI-IND8 (BB), and IND10 (BB). Information on the minimal marker subset to identify the patent cultivar will facilitate inspection for cultivar identification.

Discussion

A concern of the agricultural product export strategy promoted by the Japanese government has been the infringement of breeder's rights for new Japanese citrus cultivars. The scions of newly registered cultivars are illegally shipped overseas, and the reverse import of pirated fruits and processed products into Japan is a serious problem. A cultivar identification system using CAPS and TaqMan-MGB SNP markers has been developed to inspect suspicious nursery trees and fruits in the markets (Fujii et al. 2019, Endo et al. 2020), which would also protect the breeder's rights of Japanese citrus cultivars from illegal infringement. These systems could precisely prove whether suspicious nursery trees or fruits infringed upon the breeder's right at the DNA level. Recently, DNA diagnosis has been demanded several purposes in addition to the infringement of rights, such as the prevention of incorrect scion distribution, confirmation of parentage for breeding materials, and confirmation of raw materials for processed products. For these purposes, a more convenient DNA diagnosis system with a simple experimental procedure is required. InDel maker analysis is a very simple experimental procedure involving PCR and agarose gel electrophoresis. The enzymatic reaction of PCR amplicons, expensive chemical reagents, and special analysis instruments is not required; therefore, it is a more convenient DNA diagnosis system than CAPS and SNP markers. In the newly developed system, only InDel markers with biallelic PCR fragment patterns were selected for cultivar identification because they are ideal for the inheritance of DNA markers that can be traced in cultivars with parent–offspring relationships. Validation tests should be restarted to check for contradictions in allele inheritance from parent cultivars to offspring. We confirmed that all alleles of the InDel markers were observed in 24 citrus cultivars derived from the 14 ancestral varieties and other extra alleles were not observed in 14 ancestral varieties.

Considering the validation test results for the parentage analysis and the sequence comparison with citrus genome sequences, the two alleles in each InDel marker are supposed to be derived from the same locus. All alleles detected in the cultivar by InDel marker can be traced back to those in 14 ancestral varieties. The average values of He, Ho, and PIC values are 0.41, 0.50, and 0.33, respectively, for CAPS markers (Fujii et al. 2019) and 0.45, 0.50, and 0.35, respectively, for SNP markers (Endo et al. 2020), which are similar to those of the InDel markers. The values of He, Ho, and PIC indicate the genetic diversity level and DNA polymorphism level of the molecular marker, and high values of those indexes implicate high level in genetic diversity and

Table 5. Minimal marker subset to identify the registered cultivar with valid breeder's right among 33 citrus cultivars and ancestral varieties in Table 1

Cultivar	Expired date of patent	Number of minimal InDEL marker for identification	Example of minimal marker subset	Total number of minimal marker subsets comprising different InDEL marker combination
"Reikou"	December in 2035	3	CI-IND 1 CI-IND 3 CI-IND 4	30
"Tsunokagayaki"	March in 2039	4	CI-IND 3 CI-IND 6 CI-IND 9 CI-IND10	2
"Seinannohikari"	March in 2039	3	CI-IND 1 CI-IND 2 CI-IND 5	11
"Tsunonozomi"	May in 2041	2	CI-IND 4 CI-IND 8	3
"Haruhi"	March in 2041	2	CI-IND 3 CI-IND 9	3
"Setoka"	October in 2026	3	CI-IND 1 CI-IND 4 CI-IND 8	2
"Harumi"	Nobember in 2024	3	CI-IND 1 CI-IND 2 CI-IND 4	9
"Harehime"	June in 2029	2	CI-IND 1 CI-IND 5	5
"Rinoka"	March in 2045	2	CI-IND10 CI-IND11	1
"Mihaya"	September in 2044	2	CI-IND 3 CI-IND 6	5
"Asumi"	September in 2044	2	CI-IND 2 CI-IND12	1
"Asuki"	N/A	3	CI-IND 6 CI-IND 9 CI-IND11	4
"Kanpei"	August in 2037	2	CI-IND 5 CI-IND 9	1
"Ehime Kashi No. 28 gou"[Benimadonna [a]]	March in 2035	3	CI-IND 2 CI-IND 4 CI-IND 8	6

[a] The registered trademark of the National Federation of Agricultural Cooperative Associations

DNA polymorphism. Ho value shows experimentally obtained heterozygosity, and the degree of inbreeding can be evaluated by comparing the difference between He and Ho values. Therefore, the selected InDel markers possess enough ability to detect DNA polymorphism as do the CAPS and SNP markers and could be a useful complement to those markers in cultivar identification. The Ho values tended to be higher than the He values, indicating that heterozygosity among Japanese citrus cultivars would be maintained in the progress of the repetitive crossbreeding within the limited germplasms. Thus, the newly developed cultivar identification system using 12 InDel markers is a superior and convenient DNA diagnosis system that will save time and costs during the inspection process.

Here, we confirmed that 12 InDel markers could be used for genotyping fresh fruit samples. We confirmed that approximately 650 bp of the PCR fragment could be stably amplified from fresh fruit samples from the previous study (Endo et al. 2021). Because the deletion or insertion generates DNA polymorphism of the InDel marker in the PCR amplicon, a difference of at least 50 bp between two alleles must obtain a clear PCR fragment pattern in 2% agarose gel electrophoresis. Because small PCR amplicons of less than 150 bp are used as DNA markers in the analysis of degraded DNA samples from canned tuna, bonito, pears, and citrus (Ram et al. 1996, Yamamoto et al. 2006, Endo et al. 2020) and comparatively large fragments are inserted in the 12 InDel markers, it is difficult to apply the newly developed system to processed products. PCR amplicons could not be amplified from DNA samples isolated from straight juice, dry fruit, canned fruit, and jam (data not shown). Of the 12 selected InDel markers, 5 were derived from scaffold 2, and 3 were from scaffold 1. The unweighted marker origins of scaffolds 1 and 2 were directly reflected because almost half of the InDels used in the screening were derived from scaffolds 1 and 2 (Fang et al. 2018, Kawahara et al. 2020).

In conclusion, we developed a cultivar identification system using 12 InDel markers for reliable and reproducible identification of citrus cultivars. The genotype information of 33 citrus cultivars and ancestral varieties reported here covers more than 94% of all citrus fruit shipments produced in Japan and is an essential reference source for citrus cultivar identification. The developed system is superior as a convenient DNA diagnosis to save time and costs during the inspection process. The cultivar identification system reported here is expected to play an important role in reinforcing registered cultivar breeder’s rights.

Acknowledgement

This work was partially supported by the MAFF-commissioned project study on “Cultivar Identification Technology,” No. 2-1 (Next Generation Breeding & Health Promotion Project) (Project ID : K027324).

References

Britten, R. J. et al. (2003) Majority of divergence between closely related DNA samples is due to InDels. *Proc. Natl. Acad. Sci. U.S.A*, **100**, 4661-4665.

Endo, T. et al. (2020) TaqMan-MGB SNP genotyping assay to identify 48 citrus cultivars distributed in the Japanese market. *Breed. Sci*., **70**, 363-372.

Endo, T. et al. (2021) Development of DNA diagnosis specific to new citrus cultivars, ‘Asumi,’ ‘Mihaya,’ ‘Rinoka’ and ‘Asuki.’ *DNA Polymorphism*, **29**, 43-49 [In Japanese].

Fang, Q. et al. (2018) Development of species-specific InDel markers in Citrus. *Plant Mol. Biol. Report*, **36**, 653-662.

Fujii, H. et al. (2008) MarkerToolKit: An analysis program for data sets consists of DNA maker types obtained from various varieties. *DNA Polymorphism*, **16**, 103-107.

Fujii, H. et al. (2010) Development of a software to presume the parent–child relationship using the result of DNA marker typing. *J. Jpn. Soc. Hortic. Sci*., **9**, 34 [In Japanese].

Fujii, H. et al. (2013) MinimalMarker: An algorithm and computer program for the identification of minimal sets of discriminating DNA markers for efficient variety identification. *J. Bioinform. Comput. Biol*., **11**, 1250022.

Fujii, H. et al. (2019) CAPS markers with stability and reproductivity for discriminating major citrus cultivars in Japan. *DNA Polymorphism*, **27**, 71-79.

García-Lor, A. et al. (2012) Comparative use of InDel and SSR markers in deciphering the interspecific structure of cultivated citrus genetic diversity: A perspective for genetic association studies. *Mol. Genet. Genomics*, **287**, 77-94.

Kawahara, Y. et al. (2020) Mikan genome database (MiGD): Integrated database of genome annotation, genomic diversity, and CAPS marker information for mandarin molecular breeding. *Breed. Sci*., **70**, 200-211.

Noda, T. et al. (2020) Development of Indel markers for the selection of satsuma mandarin (*Citrus unshiu* Marc.) hybrids that can be used for low-cost genotyping with agarose gels. *Euphytica*, **216**, 115.

Ollitrault, F. et al. (2012) Development of InDel markers from *Citrus clementina* (Rutaceae) BAC-end sequences and interspecific transferability in *Citrus*. *Am. J. Bot*., **99**, e268-e273.

Ram, J. L. et al. (1996) Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *J. Agric. Food. Chem*., **44**, 2460-2467.

Tanaka, T. (1969) Misunderstanding with regards *Citrus* classification and nomenclature. *Bull. Univ. Osaka Pref. Ser. B, Agr. Biol.,* **21**, 139-145.

Wang, X. et al. (2017) Genomic analyses of primitive, wild and cultivated citrus provide insights into asexual reproduction. *Nat. Genet*., **49**, 765-772.

Wu, G. A. et al. (2014) Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. *Nat. Biotechnol.*, **32**, 656-662.

Yamamoto, T. et al. (2006) DNA profiling of fresh and processed fruits in pear. *Breed. Sci.*, **56**, 165-171.