Identification and Parentage Analysis of Citrus Cultivars Developed in Japan by CAPS Markers

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To protect the rights of breeders of the major citrus cultivars developed under breeding programs by the national institute of Japan, we developed a method of cultivar identification based on cleaved amplified polymorphic sequence (CAPS) markers, and used it to evaluate their identity and parentage. We selected 19 CAPS markers that had a single-locus origin and moderate polymorphism, and used them to construct genotyping data for 59 citrus cultivars (including American accessions), local varieties, and selections. Of the 19 CAPS markers, 8 were sufficient to discriminate among all accessions except ‘Mato’ buntan (Citrus grandis Osbeck) and ‘Hirado’ buntan (Citrus grandis Osbeck). Among the 33 Japanese cultivars, the parentage of 30 agreed with that reported, but ‘Setoka’, ‘Southern Red’, and ‘Reikou’ had discrepancies at one or more loci. Using 15 to 18 CAPS markers to validate the putative parentage revealed that the seed parent of ‘Setoka’ was ‘KyEn No. 4’, not ‘Tsunonozomi’, and the pollen parent of ‘Southern Red’ was ‘Osceola’, not ponkan (C. reticulate Blanco). The seed parent of ‘Reikou’ remains unknown.

Key Words: breeder rights, genotype, ‘Reikou’, ‘Setoka’, ‘Southern Red’.

Introduction

Citrus is one of the most important genera of fruit trees that are adapted to temperate and subtropical areas of Japan. The citrus cross-breeding program in Japan began in 1937 at the National Horticulture Research Station of the Ministry of Agriculture and Forestry, now the Okitsu Citrus Research Station of the National Agriculture and Food Research Organization Institute of Fruit Tree Science (NIFTS). The program has continued since 1964 at the NIFTS Okitsu and Kuchinotsu Citrus Research Stations. It is focused mainly on improving fruit quality, with the goals of a high sugar content, excellent flavor, seedlessness, ease of peeling, and presence of a thin locular membrane, which are characteristics specific to mandarins. The program released 41 new citrus cultivars by means of controlled cross-breeding between 1963 and 2014.

When we develop a new plant cultivar, we must simultaneously consider how to protect the rights of farmers and plant breeders from unwarranted damage concerning the outflow of a new cultivar overseas and the inflow of such products back to the host country. Therefore, it has become necessary to develop cultivar identification techniques (Kunihisa et al., 2003; Ninomiya et al., 2015). The rights of plant breeders are of increasing interest worldwide. The International Union for the Protection of New Varieties of Plants (UPOV; http://www.upov.int/index_en.html, June 14, 2016) has provided and promoted effective systems of plant variety protection. For example, UPOV’s working group on Biochemical and Molecular Techniques, and DNA-profiling in particular (BMT), has encouraged the use of DNA profiling techniques to protect the rights of plant breeders (Shoda et al., 2012).

To help identify citrus cultivars developed in Japan by using DNA-profiling techniques, several research groups have reported the development of DNA markers (Matsuyama et al., 1992; Omura et al., 2004; Ueda et al., 2003). However, information on these markers, their polymorphisms and their application to important commercial cultivars has not been made fully available.
to the public. Recently, Ninomiya et al. (2015) reported that 33 citrus cultivars or accessions, including 7 local varieties and 11 cultivars collected from abroad, could be identified from polymorphisms at 11 cleaved amplified polymorphic sequence (CAPS) markers. However, this information was not sufficient because the accessions they examined included only 8 of 41 cultivars developed by NIFTS that are commercially produced and frequently used as parents in citrus breeding programs in Japan.

In Citrus, a variety of DNA marker types have been developed. These include random amplified polymorphic DNA (RAPD), restriction-fragment-length polymorphism (RFLP), amplified-fragment-length polymorphism (AFLP), CAPS, and simple sequence repeat (SSR) markers (Kimura et al., 2003; Kitahara et al., 2005; Matsumoto et al., 1999d; Moriya et al., 2011; Ninomiya et al., 2015; Sawamura et al., 2004, 2008; Yamamoto et al., 2003). However, citrus cultivars developed in the NIFTS breeding program have generally not been investigated to confirm their parent–offspring relationships, even though they have frequently been used as parents in other breeding programs.

Here, we aimed at establishing a method for identification of the citrus cultivars developed by the citrus breeding program in Japan by using a subset of the CAPS markers developed by Shimada et al. (2014). We also aimed at confirming the parent–offspring relationships of these cultivars by adding related cultivars and selections in the analysis to increase the reliability of the identification. Using the results of this analysis, we investigated candidate parents of the cultivars that had discrepancies in the parent–offspring relationships.

**Materials and Methods**

**Plant material and DNA preparation**

We selected 59 citrus cultivars, local varieties and selections that were developed or used as parents in the NIFTS citrus breeding program (Table 1), for use in the CAPS analysis. ‘Okitsu-wase’, ‘Trovita’, and ‘Yoshida’ were chosen for the analysis as representative mutants of satsuma mandarin (Citrus unshiu Marc.), sweet orange (C. sinensis (L.) Osbeck), and ponkan (C. reticulata Blanco), respectively. All plant materials were obtained from the NIFTS collections at Kuchinotsu Citrus Research Station (Nagasaki, Japan) and Okitsu Citrus Research Station (Shizuoka, Japan). Total DNA was isolated from fully expanded fresh leaves by using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions.

**CAPS analysis**

The CAPS genotypes were identified by using 37 of the citrus CAPS markers developed by Shimada et al. (2014) that showed high versatility in the cultivars and selections used in this study (Table 2). Each CAPS amplification was conducted in 12.5 μL of 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.16 mM each dNTP, 10 pM each forward and reverse primers, 10 ng of genomic DNA, 1.25 units of AmpliTaq Gold DNA polymerase (Roche, Branchburg, NJ, USA), and the manufacturer’s PCR buffer for the polymerase. After 10 min of denaturation at 94.5°C, amplification was performed using 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 52 to 64°C (Table 2), and a 2 min extension at 70°C, followed by a 10-min final extension at 70°C. The PCR products were checked using a 200-bp ladder marker using 1.5% agarose gel electrophoresis.

The PCR products were digested with restriction enzymes (Takara Bio Inc., Shiga, Japan) (Table 2) under the following conditions. We mixed 4 μL of the PCR
Table 1. Citrus cultivars, local varieties, and other selections used in the present study. Cultivars 1 to 33 were developed by the citrus breeding program at the national institute of Japan and were used for parentage analysis.

| Code | Sample name | Described parentage (seed parent × pollen parent) or species | Type and Origin | Reference | Results for parentage analysis in the present study |
|------|-------------|-------------------------------------------------------------|-----------------|-----------|--------------------------------------------------|
| 1    | "Akami" | "Kiyomi" × "Semimole" | Cultivar, bred by NI in Japan | Yoshida et al. (2000b) | No discrepancy |
| 2    | "Amaka" | "Kiyomi" × "Encore" | Cultivar, bred by NI in Japan | Matsumoto et al. (2001) | No discrepancy |
| 3    | "Amakusa" | "KyoNo. 14" × "Page" | Cultivar, bred by NI in Japan | Matsumoto et al. (1999b) | No discrepancy |
| 4    | "Arike" | "Seike" × "Clementine" | Cultivar, bred by NI in Japan | Yamada et al. (1995a) | No discrepancy |
| 5    | "Asami" | "Okitu 46 gout × "Harumi" | Cultivar, bred by NI in Japan | Kita et al. (2012) | No discrepancy |
| 6    | "Benbabe" | "HF No. 9" × "Encore" | Cultivar, bred by NI in Japan | Takahara et al. (2006) | No discrepancy |
| 7    | "Benimaru" | "Mato" × "Hirodo" | Cultivar, bred by NI in Japan | Yamada et al. (1993) | No discrepancy |
| 8    | "Harihime" | "E-647" × "Miyagawa Wase" | Cultivar, bred by NI in Japan | Yoshida et al. (2005d) | No discrepancy |
| 9    | "Hareyaka" | "Encore" × "Nakano 3 gosanponkan" | Cultivar, bred by NI in Japan | Matsumoto et al. (1999d) | No discrepancy |
| 10   | "Harumi" | "Kiyomi" × "Ponkan F-2432" | Cultivar, bred by NI in Japan | Yoshida et al. (2000a) | No discrepancy |
| 11   | "Hayaku" | "Imamura" × "Nakano 3 gosanponkan" | Cultivar, bred by NI in Japan | Okiyama et al. (1991b) | No discrepancy |
| 12   | "Kankitsu Chukanbohon Nou 5 Gou" | "Lee" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005a) | No discrepancy |
| 13   | "Kankitsu Chukanbohon Nou 6 Gou" | "Kiyomi" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005b) | No discrepancy |
| 14   | "Miyagawa Wase" × "Trovia" | "Orai" | Cultivar, bred by NI in Japan | Nishimura et al. (1983a) | No discrepancy |
| 15   | "Miyaku" | "Kiyomi" × "Encore" | Cultivar, bred by NI in Japan | Nishimura et al. (2012) | No discrepancy |
| 16   | "Miyaku" | "KyoNo. 14" × "Encore" | Cultivar, bred by NI in Japan | Matsumoto et al. (1999c) | No discrepancy |
| 17   | "Nakakou" | "Kiyomi" × "Encore" | Cultivar, bred by NI in Japan | Okai et al. (1991a) | No discrepancy |
| 18   | "Nakakou" | "KyoNo. 5" × "Makiku" | Cultivar, bred by NI in Japan | Yoshida et al. (2009) | Unidentified |
| 19   | "Nishinokaori" | "EnOw No. 21" × "Youkou" | Cultivar, bred by NI in Japan | Yoshida et al. (2015) | No discrepancy |
| 20   | "Orito" | "KyoNo. 5" | Cultivar, bred by NI in Japan | Ueno et al. (1985) | No discrepancy |
| 21   | "Orito" | "KyoNo. 5" × "Makiku" | Cultivar, bred by NI in Japan | Yoshida et al. (2005b) | No discrepancy |
| 22   | "Okitsu 46 gousanponkan" | "Lee" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005a) | No discrepancy |
| 23   | "Okitsu 46 gousanponkan" | "Lee" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005b) | No discrepancy |
| 24   | "Okitsu 46 gousanponkan" | "Lee" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005a) | No discrepancy |
| 25   | "Okitsu 46 gousanponkan" | "Lee" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005b) | No discrepancy |
| 26   | "Okitsu 46 gousanponkan" | "Lee" × "Makiku kishu" | Cultivar, bred by NI in Japan | Yoshida et al. (2005a) | No discrepancy |

* Citrus species are based on Tanaka (1954).
* "Okitu Wase", "Trovia", and "Yoshida" were used in the analysis as representatives of mutants of satsuma mandarin, sweet orange, and ponkan, respectively.
* NI is abbreviation of the National Institute.
Table 2. Characteristics of the CAPS markers used in this study. The first 19 CAPS markers were used in the cultivar identification and parentage analysis for all 33 NIFTS cultivars; the next 18 markers were used to clarify any parentage discrepancies revealed by the first 19 markers.

| Marker name | Restriction enzyme | Annealing Temperature (°C) | PCR product size (bp) | Polymorphic fragment size (bp) | Position in the integration map (AGI map) | Linkage group | Scaffold | Start position | End position |
|-------------|-------------------|-----------------------------|-----------------------|-------------------------------|------------------------------------------|---------------|----------|---------------|-------------|
|             |                   |                             | a allele              | b allele                      | common fragment of both allele           |               |          |               |             |
| A0326       | NdeI              | 60                          | 600                   | 500                           | 300                                      | 200           | scaffold_3 | 6409311      | 6411254     |
| A0413       | MspI              | 56                          | 1100                  | 1100                          | 900                                      |               | scaffold_9 | 7579900      | 7583093     |
| A0636       | EcoRI             | 1400                        | 1200                  | 1200                          | 800                                      | 650           | scaffold_1 | 23776796     | 23782088    |
| B0028       | HaeIII            | 180                        | 655                   | 655                           | 600                                      |               | scaffold_2 | 35354776     | 35363062    |
| B0029       | SspI              | 2200                        | 2200                  | 2200                          | 1200                                     |               | scaffold_2 | 8683898      | 8685984     |
| B0036       | MspI              | 1400                        | 1400                  | 1400                          | 800                                      | 650           | scaffold_1 | 22840947     | 22844111    |
| B0145       | MspI              | 1800                        | 1200                  | 1200                          | 1000                                     |               | scaffold_8 | 22730839     | 22737521    |
| B0158       | PstII             | 600                         | 600                   | 600                           | 300                                      |               | scaffold_3 | 5253281      | 5254755     |
| Cp1624      | MspI              | 1000                        | 900                   | 900                           | 800                                      |               | scaffold_1 | 18086827     | 18070262    |
| Gn0043      | HincII            | 1100                        | 1100                  | 1100                          | 1000                                     |               | scaffold_8 | 21953129     | 21960709    |
| I0208       | HinfI             | 58                          | 550                   | 550                           | 300                                      | 250           | scaffold_5 | 40562844     | 40630241    |
| M0097       | DraI              | 400                          | 400                   | 400                           | 300                                      |               | scaffold_6 | 16712914     | 16716984    |
| T0062       | Rsal              | 900                          | 800                   | 800                           | 600                                      |               | scaffold_6 | 11826472     | 11830875    |
| T0079       | SspI              | 950                          | 950                   | 950                           | 700                                      |               | scaffold_3 | 7047024      | 7049430     |
| T0150       | HinfI             | 500                          | 500                   | 500                           | 350                                      |               | scaffold_8 | 22191056     | 22193554    |
| T0168       | Rsal              | 1200                        | 1100                  | 1100                          | 600                                      |               | scaffold_1 | 24898769     | 24905716    |
| T0235       | HaeIII            | 700                          | 650                   | 650                           | 450                                      |               | scaffold_6 | 17057658     | 17074575    |
| T0271       | Rsal              | 800                          | 700                   | 800                           | 400                                      |               | scaffold_6 | 11707593     | 11715646    |
| T0326       | Hhal              | 62                          | 1400                  | 1400                          | 800                                      | 750           | scaffold_7 | 2501520      | 2508737     |
| B0136       | HhaI              | 56                          | 1000                  | 1000                          | 750                                      |               | scaffold_6 | 15056834     | 15058632    |
| Cp0075      | HinfI             | 54                          | 1600                  | 850                           | 600                                      | 700           | scaffold_6 | 7924189      | 7928192     |
| Fn0059      | HincII            | 60                          | 1400                  | 850                           | 450                                      | 500           | scaffold_3 | 16223756     | 16233636    |
| Gn0040      | HaeIII            | 54                          | 1200                  | 1200                          | 550                                      | 800           | scaffold_6 | 25506466     | 2551133    |
| I0211       | PvuII             | 58                          | 600                   | 600                           | 400                                      |               | scaffold_8 | 6632695      | 6643448     |
| Ov0305      | MspI              | 58                          | 1250                  | 1250                          | 1000                                     |               | scaffold_6 | 16904992     | 16912088    |
| T0069       | MspI              | 64                          | 800                   | 800                           | 450                                      | 300           | scaffold_5 | 38566192     | 38571337    |
| T0165       | DraI              | 1100                        | 1100                  | 1100                          | 750                                      |               | scaffold_4 | 19605473     | 19608059    |
| T0300       | DraI              | 800                          | 800                   | 800                           | 500                                      |               | scaffold_3 | 43862440     | 43867443    |
| T0348       | HinfI             | 62                          | 800                   | 800                           | 450                                      | 350           | scaffold_8 | 11619894     | 11619437    |
| T0350       | Rsal              | 64                          | 900                   | 900                           | 600                                      | 250           | scaffold_3 | 8914744      | 8921411     |
| T0364       | HinfI             | 1000                        | 900                   | 900                           | 400                                      |               | scaffold_6 | 24606501     | 24612654    |
| T0386       | MspI              | 500                          | 500                   | 500                           | 300                                      |               | scaffold_6 | 23593516     | 23595492    |
| Wx0002      | MspI              | 1200                        | 1000                  | 1000                          | 600                                      | 400           | scaffold_5 | 37189553     | 37192231    |
| C0336       | PvuII             | 58                          | 1200                  | 1200                          | 600                                      |               | scaffold_6 | 37124440     | 37125036    |
| C2223       | NdeI              | 1400                        | 800                   | 800                           | 700                                      |               | scaffold_3 | 34883334     | 34856373    |

Note:
- * from Shimada et al. (2014).
- † from Shimada et al. (2014). Seed and pollen parents except T0250/HinfIII are ‘Okitsu 46 gou’ and ‘Kankitsu Chakanbohon Nou 5 Gou’. Segregation analysis of T0250/HinfIII and the values in parentheses of Fb0159/HinfII were analyzed on the basis of the offspring of the cross between ‘E-647’ and ‘Setoka’.
- a, b, c on the same line refer to the c, b, and a alleles, respectively.
- a, b, c in parentheses denote the c, b, and a alleles, respectively.
- a, b, c in parentheses show the fragment size of the c allele.

The annealing temperature was reduced by 2°C for every 2 cycles. When the annealing temperature reached 54°C, the amplification was continued at 54°C for 30 cycles.
product with 1.0 µL of the buffer and 2 to 3 units of the restriction enzyme, and then adjusted the volume to a total of 10 µL with sterilized water. After digestion at 37°C for more than 2 h, the segmentation patterns were analyzed by means of 1.5% agarose gel electrophoresis with ethidium bromide staining.

Of the 37 CAPS markers, 19 were used for the cultivar identification and parentage analysis (Table 3), and the other 18 for parentage analysis for cultivars that showed discrepancies in the previously reported parent–offspring relationship.

Identification of citrus cultivars and parentage analysis

We used MinimalMarker software (Fujii et al., 2013a) to select minimal CAPS marker subsets for identification of tested cultivars on the basis of the analysis results with the 19 markers. We then used the genotypes defined by the selected CAPS markers to identify 45 cultivars, 7 local varieties, and 7 selections.

We used MARCO software (Fujii et al., 2010) to identify the parent–offspring relationship on the basis of the inheritance of one allele at each CAPS locus being transmitted from the parents to the offspring. MARCO could arbitrarily set a cultivar as the “parent” or “offspring” from a supplied dataset. To confirm the assumed parent–offspring relationship, we defined the 33 cultivars developed in the citrus breeding program by the national institute of Japan as “offspring”, and the cultivars or selections reported as parents in previous records as “parents”. The software then revealed whether the inheritance pattern based on the genetic markers supported the assumed parentage or revealed a different one. We judged that a discrepancy in the parent–offspring relationship existed if neither of the two alleles in the offspring existed in the candidate parent.

Validation of the putative parent–offspring relationships using SNP genotyping data

We used the 116 SNPs randomly selected from the 268 reliable SNPs obtained from genotyping data of 103 citrus accessions analyzed by Fujii et al. (2013b) to validate the parent–offspring relationships and to identify the putative parentage predicted based on the CAPS marker analysis performed in MARCO. SNP genotyping data for accessions that were not analyzed by Fujii et al. (2013b) were acquired by using the same genotyping method (i.e., the GoldenGate assay).

Results

Selection and evaluation of 19 CAPS markers for cultivar identification and parentage analysis

We selected 19 CAPS markers from the frame markers in the linkage map of Shimada et al. (2014) and evaluated their use for cultivar identification and parentage analysis using the 59 accessions (Table 3). The sample genotypes for each CAPS marker were decided from the fragment patterns obtained from images of the electrophoresis results. Table 3 shows the observed heterozygosity ($H_o$) and polymorphism information content (PIC) of each CAPS marker, which could be used to evaluate their genetic diversities. $H_o$ was calculated as the percentage of individuals with heterogeneous genotypes that were actually observed in the 59 accessions, and PIC was calculated according to the method of Botstein et al. (1980). $H_o$ for the CAPS markers ranged from 0.246 to 0.820, and averaged 0.461. PIC ranged from 0.207 to 0.375, and averaged 0.325. These markers were assigned to the reference genetic map of the Clementine (C. clemetina Hort. ex Tanaka) scaffolds v. 1.0 developed by Ollitrault et al. (2012), and at least one marker was located in each of the nine scaffolds (Table 2). Segregation of the genotypes of the 19 markers in the F1 progeny is shown in Table 2. All markers except Bf0028/HaeIII showed Mendelian segregation which fitted the expected ratio deduced from the parent genotypes ($\chi^2$ test, $P > 0.05$), indicating that each CAPS marker was derived from one locus. The Bf0028/HaeIII marker showed a significant $\chi^2$ result ($P < 0.05$), but was nonetheless judged to be a suitable marker because allele inheritance based on it was evaluated using more than 10 combinations of parents and their offspring, and 1 allele was transmitted from the parents to their offspring without any discrepancies. Thus, we concluded that the 19 CAPS markers were each amplified from a single locus, and could be effectively used in cultivar identification and parentage analysis.

Cultivar identification

The genotyping data based on the 19 CAPS markers for the 59 accessions (Table 3) were analyzed with MinimalMarker software (Fujii et al., 2013a) to estimate the minimal number of markers that could be used to identify the 33 cultivars developed by the citrus breeding program in the national institute. We found a set of 8 CAPS markers that was sufficient to discriminate among 57 accessions, except for ‘Mato Buntan’ (C. grandis Osbeck) and ‘Hirado Buntan’ (C. grandis Osbeck), resulting in 7 subsets of the 19 CAPS markers:

Set 1: A10413/MspI, Bf0029/SylI, Bf0036/MspI, Cp1624/MspI, If0208/HinFI, Tf0062/RsaI, Tf0150/HinFI, Tf0326/HhaI

Set 2: A10413/MspI, Bf0028/HaeIII, Bf0036/MspI, Cp1624/MspI, Mf0097/DraI, Tf0150/HinFI, Tf0271/RsaI, Tf0326/HhaI

Set 3: A10413/MspI, Bf0028/HaeIII, Bf0036/MspI, Cp1624/MspI, Tf0150/HinFI, Tf0235/HaeIII, Tf0271/RsaI, Tf0326/HhaI

Set 4: A10413/MspI, Bf0036/MspI, Bf0158/PvuII, Cp1624/MspI, If0208/HinFI, Tf0062/RsaI, Tf0150/HinFI, Tf0326/HhaI

Set 5: A10413/MspI, Bf0036/MspI, Cp1624/MspI, If0208/HinFI, Tf0062/RsaI, Tf0079/SylI, Tf0150/HinFI, Tf0326/HhaI
Table 3. The genotypes for the 19 CAPS markers and the associated embryo types of the 59 cultivars and selections used in the parentage analysis. The underlined genotypes represent discrepancies with the assumed parent-offspring relationship.

| Code | Sample name          | Embryo type | Observed heterozygosity (Ho) | PIC |
|------|----------------------|-------------|------------------------------|-----|
|      |                      |             | CAPS marker                  |     |
|      |                      |             | A00326/Ax/II                 | 0.479/0.326 |
|      |                      |             | A00413/MspI                  | 0.508/0.358 |
|      |                      |             | Al0636/EcoRI                 | 0.599/0.322 |
|      |                      |             | Bf0028/HaeIII                | 0.576/0.326 |
|      |                      |             | Bf0029/StyI                  | 0.322/0.278 |
|      |                      |             | Bf0036/MspI                  | 0.492/0.364 |
|      |                      |             | Bf0145/MspI                  | 0.254/0.263 |
|      |                      |             | Bf158/PvuII                  | 0.559/0.322 |
|      |                      |             | Cp1624/MspI                  | 0.492/0.351 |

1. "Akemi"  p  aa  ab  ab  ab  aa  ab  aa  bb  ab
2. "Amaka"  m  aa  aa  bb  ab  aa  ab  aa  ab  bb
3. "Amakura" p  ab  ab  ab  aa  ab  ab  aa  ab  ab
4. "Ariake"  m  aa  ab  bb  aa  ab  ab  ab  ab  bb
5. "Asami"  m  ab  ab  ab  aa  aa  ab  ab  ab  aa
6. "Benibae" m  ab  ab  ab  aa  aa  aa  aa  bb  ab
7. "Benimados" m  aa  bb  ab  aa  bb  bb  bb  bb  aa
8. "Harimine" m  ab  ab  ab  ab  aa  ab  aa  ab  aa
9. "Haryuka" p  aa  aa  bb  ab  aa  bb  aa  bb  ab
10. "Harumi"  p  ab  ab  ab  ab  aa  ab  aa  ab  aa
11. "Hayaka"  p  aa  aa  bb  ab  aa  ab  aa  ab  bb
12. "Kanita Chukanteban Nou 5 Gou"  m  ab  ab  ab  ab  aa  ab  aa  ab  bb
13. "Kanita Chukanteban Nou 6 Gou"  m  aa  ab  bb  ab  aa  ab  aa  bb  bb
14. "Kiyomi"  m  ab  ab  ab  ab  ab  ab  ab  ab  ab
15. "May Pummmelo"  m  aa  bb  ab  aa  ab  bb  ab  ab  ab
16. "Mihaya"  m  ab  aa  bb  aa  aa  ab  ab  aa  bb
17. "Misho core"  p  ab  aa  bb  ab  aa  bb  aa  bb  bb
18. "Nankou"  m  ab  aa  bb  ab  ab  ab  aa  ab  bb
19. "Nishihon"  m  ab  ab  ab  aa  aa  ab  ab  aa  ab
20. "Rekou"  p  ab  ab  ab  ab  aa  ab  aa  ab  ab
21. "Seinnohiikari"  p  aa  aa  bb  ab  aa  ab  aa  ab  bb
22. "Seto"  p  aa  ab  ab  aa  ab  ab  ab  ab  bb
23. "Shirinami"  p  aa  ab  ab  aa  aa  aa  aa  ab  ab
24. "Southern Red"  m  aa  aa  bb  aa  aa  bb  aa  bb  bb
25. "Southern Yellow"  m  aa  ab  ab  ab  aa  bb  ab  ab  ab
26. "Summer Fresh"  p  aa  bb  bb  aa  ab  bb  ab  ab  ab
27. "Sweet Spring"  m  aa  ab  ab  ab  aa  ab  ab  ab  ab
28. "Tamami"  m  bb  aa  ab  ab  aa  aa  aa  ab  bb
29. "Tsunokagu"  m  ab  aa  ab  ab  aa  ab  aa  ab  ab
30. "Tsunobara"  p  ab  ab  ab  ab  ab  ab  ab  ab  ab
31. "Tsunozono"  m  ab  ab  ab  aa  aa  aa  aa  ab  ab
32. "Yellow Pummele"  m  ab  aa  ab  ab  aa  bb  ab  ab  bb
33. "Youkou"  p  aa  aa  ab  ab  aa  aa  aa  ab  ab
34. "Clementine"  m  ab  ab  ab  ab  ab  ab  ab  ab  ab
35. "E-447"  m  ab  bb  ab  aa  aa  ab  aa  ab  ab
36. "Encore"  m  ab  aa  bb  ab  aa  ab  aa  ab  bb
37. "Fu-Da No 21"  m  ab  aa  bb  ab  aa  bb  aa  bb  bb
38. "Hasaku"  m  aa  ab  ab  aa  ab  bb  ab  ab  ab
39. "HFW"  m  ab  ab  ab  ab  aa  ab  aa  ab  ab
40. "Hirado buntan"  m  aa  bb  ab  aa  bb  bb  bb  bb  aa
41. "Kara"  p  aa  aa  bb  ab  aa  bb  aa  ab  bb
42. "Kawano Natsudai"  p  aa  ab  ab  ab  ab  ab  ab  ab  bb
43. "King"  p  aa  ab  ab  ab  aa  ab  aa  ab  bb
44. "Ku-En No.5"  m  bb  aa  bb  ab  ab  ab  bb  ab  ab
45. "Ku-Da No.14"  m  ab  aa  ab  ab  ab  ab  ab  ab  ab
46. "Lee"  m  bb  ab  ab  aa  aa  aa  aa  ab  ab
47. "Mato buntan"  m  aa  bb  ab  aa  bb  bb  bb  bb  aa
48. "Murakami kishu"  m  aa  aa  bb  ab  ab  ab  ab  ab  ab
49. "Murcott"  p  aa  ab  bb  aa  ab  bb  ab  ab  ab
50. "Na-1408"  m  aa  ab  bb  ab  aa  bb  aa  bb  bb
51. "Okiitsu 46 gou"  m  ab  aa  ab  ab  ab  ab  ab  ab  ab
52. "Okiitsu Wase"  p  aa  aa  bb  ab  aa  bb  aa  ab  ab
53. "Page"  p  ab  ab  bb  ab  ab  ab  ab  ab  ab
54. "Semilole"  p  ab  ab  ab  ab  ab  ab  ab  ab  ab
55. "Takawara buntan"  m  aa  bb  ab  aa  ab  bb  bb  bb  ab
56. "Trovia orange"  p  ab  ab  ab  ab  ab  ab  ab  ab  ab
57. "Wilk"  m  ab  ab  bb  ab  aa  ab  aa  bb  ab
58. "Yoshida ponkan"  p  ab  ab  bb  ab  aa  ab  aa  ab  ab
59. "Uoceola"  m  aa  ab  bb  aa  aa  ab  aa  bb  bb

Embryo types: "p", polyembryony; "m", monoembryony.
### Table 3.  Continued.

| Code   | Sample name                                                                 | CAPS marker | Observed heterozygosity (Ho) | Polymorphic information content (PIC) |
|--------|-----------------------------------------------------------------------------|-------------|------------------------------|---------------------------------------|
|        |                                                                             | Gn0043/HincII | 0.559/0.356                 | 0.475/0.341                           |
| 1      | 'Akemi'                                                                     | ab           | bb                           | bb                                    |
| 2      | 'Amaka'                                                                     | ab           | bb                           | ab                                    |
| 3      | 'Amakuni'                                                                   | ab           | bb                           | ab                                    |
| 4      | 'Ariake'                                                                    | ab           | bb                           | ab                                    |
| 5      | 'Azumi'                                                                     | ab           | bb                           | ab                                    |
| 6      | 'Benihoe'                                                                   | aa           | bb                           | ab                                    |
| 7      | 'Benimadoko'                                                                | bb           | aa                           | bb                                    |
| 8      | 'Harehime'                                                                  | ab           | ab                           | ab                                    |
| 9      | 'Hareyaka'                                                                  | aa           | ab                           | ab                                    |
| 10     | 'Harumi'                                                                    | aa           | bb                           | ab                                    |
| 11     | 'Hayaka'                                                                    | ab           | bb                           | ab                                    |
| 12     | 'Kankitu Chukanshoku Nengeto 5 Gou'                                        | ab           | dd                           | ab                                    |
| 13     | 'Kankitu Chukanshoku Nengeto 6 Gou'                                        | ab           | bb                           | dd                                    |
| 14     | 'Kiyomi'                                                                    | ab           | ab                           | dd                                    |
| 15     | 'May Pummelo'                                                               | ab           | ab                           | dd                                    |
| 16     | 'Mihaya'                                                                    | aa           | bb                           | dd                                    |
| 17     | 'Milche-core'                                                               | aa           | bb                           | dd                                    |
| 18     | 'Nankou'                                                                    | ab           | dd                           | ab                                    |
| 19     | 'Nishihonokairo'                                                            | ab           | ab                           | dd                                    |
| 20     | 'Ruskin'                                                                    | aa           | bb                           | dd                                    |
| 21     | 'Seiunshihokari'                                                            | ab           | bb                           | dd                                    |
| 22     | 'Setoka'                                                                    | ab           | bb                           | dd                                    |
| 23     | 'Shiramahi'                                                                 | ab           | bb                           | dd                                    |
| 24     | 'Southern Red'                                                               | aa           | ab                           | dd                                    |
| 25     | 'Southern Yellow'                                                            | ab           | ab                           | dd                                    |
| 26     | 'Summer Fresh'                                                               | aa           | ab                           | dd                                    |
| 27     | 'Sweet Spring'                                                               | ab           | ab                           | dd                                    |
| 28     | 'Tamami'                                                                    | aa           | ab                           | dd                                    |
| 29     | 'Tsunokagayakio'                                                            | ab           | bb                           | dd                                    |
| 30     | 'Tsunokasiori'                                                              | aa           | ab                           | dd                                    |
| 31     | 'Tsunonozumi'                                                                | ab           | bb                           | dd                                    |
| 32     | 'Yellow Pummelo'                                                             | ab           | ab                           | dd                                    |
| 33     | 'Youkou'                                                                    | ab           | ab                           | dd                                    |
| 34     | 'Clementine'                                                                | aa           | ab                           | dd                                    |
| 35     | 'Encore'                                                                    | aa           | bb                           | dd                                    |
| 36     | 'EntDw No.21'                                                               | ab           | ab                           | dd                                    |
| 37     | 'Hassaku'                                                                   | ab           | ab                           | dd                                    |
| 38     | 'HFW'                                                                       | ab           | bb                           | dd                                    |
| 39     | 'Hirado' buntan                                                             | bb           | aa                           | dd                                    |
| 40     | 'Kara'                                                                      | ab           | bb                           | dd                                    |
| 41     | 'Kawano' Natsudaidai                                                        | ab           | ab                           | dd                                    |
| 42     | 'Lang'                                                                      | ab           | ab                           | dd                                    |
| 43     | 'King'                                                                       | ab           | bb                           | dd                                    |
| 44     | 'KyEn No.5'                                                                 | ab           | ab                           | dd                                    |
| 45     | 'KyDw No.14'                                                                | bb           | bb                           | dd                                    |
| 46     | 'Lee'                                                                       | ab           | ab                           | dd                                    |
| 47     | 'Mato' buntan                                                                | bb           | aa                           | dd                                    |
| 48     | 'Mukake kishu'                                                              | aa           | bb                           | dd                                    |
| 49     | 'Murcott'                                                                    | ab           | ab                           | dd                                    |
| 50     | 'No.1408'                                                                   | ab           | ab                           | dd                                    |
| 51     | 'Okitu 46 gyou'                                                              | ab           | ab                           | dd                                    |
| 52     | 'Okitu Wase'                                                                 | ab           | ab                           | dd                                    |
| 53     | 'Page'                                                                       | ab           | ab                           | dd                                    |
| 54     | 'Seminole'                                                                   | ab           | ab                           | dd                                    |
| 55     | 'Tanakawa' buntan                                                           | bb           | ab                           | dd                                    |
| 56     | 'Trovis' orange                                                              | ab           | ab                           | dd                                    |
| 57     | 'Wilking'                                                                    | aa           | bb                           | dd                                    |
| 58     | 'Yoshida' ponkan                                                             | aa           | bb                           | dd                                    |
| 59     | 'Osceola'                                                                    | aa           | ab                           | dd                                    |
‘Reikou’. In the case of ‘Reikou’, ‘Murcott’ was cor-
protect, and other developed pummelo cultivars such as
C. grandis ‘Benimadoka’ (Thermore, we included ‘KyEn No. 3’, ‘KyEn No. 4’,
data from the 19 CAPS markers.

Both ‘Mato’ buntan and ‘Hirado’ buntan are pumme-
lo (C. grandis Osbeck) local varieties and have the same genotypes at all 19 CAPS markers. Pummelo has high homozygosity at most loci (Moore, 2001), so it may be difficult to find polymorphisms among pumme-
lo cultivars. ‘Mato’ buntan and ‘Hirado’ buntan were not distinguished by the seven subsets of the CAPS markers, but this may not be important because both cultivars are local varieties with no breeders’ rights to protect, and other developed pummelo cultivars such as ‘Benimadoka’ (C. grandis Osbeck) could be distin-
guished from them by the marker sets.

Parentage analysis

We compared previously reported parent–offspring relationships with the parentage predicted using the 19 CAPS markers within the 33 citrus cultivars in MARCO software. The reported relationships were validated for 30 of the cultivars, but not for ‘Setoka’, ‘Southern Red’, or ‘Reikou’ (Table 1). Every allele at the 19 CAPS marker loci within the 30 cultivars was the previously reported parent allele. However, ‘Setoka’, ‘Southern Red’, and ‘Reikou’ had a discrep-
ant allele at one or more loci compared with their repor-
ted parents: there were discrepancies at Bf0029/StyI in ‘Setoka’; at If0208/HinfI in ‘Southern Red’; and at Al0636/EcoRI, Bf0036/MspI, and Tfo326/HhaI in ‘Reikou’. In the case of ‘Reikou’, ‘Murcott’ was cor-
rectly described as the pollen parent, but the discrepan-
cy at Tfo326/HhaI showed that ‘KyEn No. 5’ was not correctly described as the seed parent. However, it was not clear which parent was descendant in ‘Setoka’ and ‘Southern Red’ solely on the basis of the genotyping data from the 19 CAPS markers.

To solve this problem, we applied an additional 15 CAPS markers to the parentage analysis for ‘Southern Red’ and ‘Reikou’, and an additional 18 (including the aforementioned 15 markers) for ‘Setoka’ (Table 2). Furthermore, we included ‘KyEn No. 3′, ‘KyEn No. 4′, ‘KyEn No. 86′, ‘Minneola’, and ‘Osceola’ as potential true parents, since these cultivars were commonly util-
ized as parents in the cross-breeding program at the time when the three discrepant cultivars were produced. ‘KyEn No. 3′, ‘KyEn No. 4′, ‘KyEn No. 5′, and ‘KyEn No. 86′ were full siblings of ‘Tsunonozomi’ (that is, they were selected from seedlings that resulted from a cross between ‘Kiyomi’ and ‘Encore’).

The genotyping data in the parentage analysis for ‘Setoka’ and ‘Southern Red’ are summarized in Tables 4 and 5, respectively. ‘Setoka’ is registered as being derived from a cross between the seed parent

‘Tsunonozomi’ and the pollen parent ‘Murcott’. In this parent–offspring relationship, there were discrepancies at C0313/STS, C0223/NdeI, and T0250/HinII (Fig. 1), and the result for C0223/NdeI suggested that ‘Tsunonozomi’ could not be the seed parent. ‘Murcott’ was compatible with being the pollen parent because both alleles at all 37 CAPS marker loci were trans-
mitted without any discrepancies (Table 4). Among the candidate parent accessions, ‘KyEn No. 4′ appears more likely to be the seed parent than ‘Tsunonozomi’.

‘Southern Red’ is registered as being derived from a cross between the seed parent ‘Kara’ and the pollen par-
ent ponkan. In this parent–offspring relationship, there were discrepancies at Bfo159/HinII, Gmo071/PvuII, and If0211/PvuII (Fig. 2), and the result for Bfo159/
HinII indicated that ponkan could not be the pollen par-
ent. ‘Kara’ was compatible with being the seed parent because at least one of the two alleles for each of the 34 CAPS markers loci was transmitted without any discrepancies (Table 5). Among the candidate parent ac-
cessions, ‘Osceola’ was predicted to be the pollen parent instead of ponkan.

In contrast, we could not confirm the seed parent for ‘Reikou’, which was registered as being derived from a cross between the seed parent ‘KyEn No. 5′ and the pollen parent ‘Murcott’, because there were discrepan-
cies at A10636/EcoRI, Bf0036/MspI, Tfo326/HhaI, and Tfo300/DraI (Table 6). ‘Murcott’ was compatible with being the pollen parent because at least one of the two alleles for each of the 34 CAPS markers’ loci was trans-
mitted without any discrepancies (Table 6).

Validation of the putative parent–offspring relationships using SNP genotyping data

To further evaluate the putative parentage of ‘Setoka’ and ‘Southern Red’, we used 116 reliable SNPs from the analysis by Fujii et al. (2013b). We obtained the genotyping data for ‘KyEn No. 4’, which was not included in their analysis, by following their method; thus, we used genotype data from a total of 104 citrus accessions for the parentage validation.

We confirmed discrepancies in the parentage of ‘Setoka’ at six SNP markers (SI116, SI145, SI209, SI269, SI282, and SI363), and the ‘Tsunonozomi’ alleles had discrepancies at three of these (SI145, SI209, and SI282; Table 4). In contrast, there were no discrepan-
cies in the inheritance of the alleles at any marker when the parents for ‘Setoka’ were ‘KyEn No. 4′ and ‘Murcott’. Furthermore, when ‘Murcott’ was fixed as the pollen parent, MARCO indicated ‘KyEn No. 4′ as the only seed parent. These results strongly support our finding that ‘Setoka’ is the offspring of a cross between ‘KyEn No. 4′ and ‘Murcott’.

We also confirmed discrepancies in the parentage of ‘Southern Red’, which was reported as being the result of a cross between ‘Kara’ and ponkan, at seven SNP markers (SI173, SI199, SI228, SI270, SI322, SI348,
Table 4.  CAPS and SNP genotypes for the reported and putative parents of ‘Setoka’. The underlined CAPS genotypes represent discrepancies between the reported parent–offspring relationship, in which ‘Setoka’ resulted from a cross between ‘Tsunonozomi’ and ‘Murcott’, and the marker results. A discrepancy exists if neither of the two alleles in the offspring exist in the candidate parent. The first 19 CAPS markers were used in the overall parentage analysis for all 33 NIFTS cultivars; the next 18 markers were used to clarify any parentage discrepancies revealed by the first 19 markers. SNP marker results are shown only for loci with discrepancies.

| Marker name | Cultivar | 'Setoka' | 'Tsunonozomi' | 'Murcott' | 'KyEn No. 4' |
|-------------|----------|----------|---------------|-----------|--------------|
| Al0326/NdeII | aa | ab | aa | aa |
| Al0413/MspI | ab | ab | ab | ab |
| Al0636/EcoRI | ab | ab | bb | ab |
| Bf0028/HaeIII | aa | aa | aa | ab |
| Bf0029/StyI | ab | aa | aa | ab |
| Bf0036/MspI | ab | aa | bb | ab |
| Bf0145/MspI | ab | aa | ab | aa |
| Bf0158/PvuII | bb | bb | bb | bb |
| Cp1624/MspI | ab | ab | bb | ab |
| Gn0043/HincII | ab | ab | ab | aa |
| If0208/HinfI | bb | bb | bb | bb |
| Mt0097/DraI | bb | bb | ab | bb |
| T0062/RsaI | aa | aa | ab | aa |
| T0079/StyI | ab | ab | ab | ab |
| T0150/HinfI | bb | ab | bb | bb |
| T0168/RsaI | aa | aa | ab | ab |
| T0235/HaeIII | aa | aa | ab | aa |
| T0271/RsaI | aa | aa | ab | aa |
| T0326/HhaI | aa | ab | ab | aa |
| Bf0136/HhaI | bb | bb | ab | bb |
| Cp0975/HinfI | ab | ab | ab | ab |
| Fb0159/HincII | bc | ab | ac | ab |
| Gn0040/HaeIII | bb | bb | ab | bb |
| Gn0071/PvuII | bb | bb | bb | bb |
| If0211/PvuII | aa | aa | aa | aa |
| Ov0305/MspI | ab | aa | bb | aa |
| T0069/MspI | bb | ab | ab | ab |
| T0165/DraI | bb | bb | bb | bb |
| T0300/DraI | ab | ab | bb | aa |
| T0348/HinfI | ab | ab | ab | ab |
| T0350/DraI | ab | ab | ab | ab |
| T0364/HinfI | ab | aa | ab | aa |
| T0386/MspI | ab | ab | ab | ab |
| Wy0003/MspI | aa | ab | ab | ab |
| Cp0336/STS | ab | aa | aa | ab |
| Cp2223/NdeII | aa | bb | ab | ab |
| T0250/HindIII | ab | aa | aa | ab |

SNP marker

| Marker name | Cultivar | 'Setoka' | 'Tsunonozomi' | 'Murcott' | 'KyEn No. 4' |
|-------------|----------|----------|---------------|-----------|--------------|
| SI116       | CG | GG | GG | CG |
| SI145       | TT | AA | AT | AT |
| SI209       | GG | AA | AG | GG |
| SI269       | AG | AA | AA | AG |
| SI282       | AA | GG | AG | AG |
| SI363       | AT | AA | AA | AT |
Table 5. CAPS and SNP genotypes for the reported and putative parents of ‘Southern Red’. The underlined CAPS genotypes represent discrepancies between the reported parent–offspring relationship, in which ‘Southern Red’ resulted from a cross between ‘Kara’ and ponkan, and the marker results. A discrepancy exists if neither of the two alleles in the offspring exist in the candidate parent. The first 19 CAPS markers were used in the overall parentage analysis for all 33 NIFTS cultivars; the next 15 markers were used to clarify any parentage discrepancies revealed by the first 19 markers. SNP marker results are shown only for loci with discrepancies.

| Marker name | Cultivar              |
|-------------|-----------------------|
|             | ‘Southern Red’ | ‘Kara’ | Ponkan | ‘Osceola’ |
| CAPS marker |            |        |        |          |
| Al0326/NdeII | aa         | aa     | ab     | aa       |
| Al0413/MspI  | aa         | aa     | ab     | ab       |
| Al0636/EcoRI | bb         | bb     | bb     | bb       |
| Bf0028/HaeIII | aa       | ab     | ab     | aa       |
| Bf0029/StyI  | aa         | aa     | aa     | aa       |
| Bf0036/MspI  | bb         | bb     | ab     | ab       |
| Bf0145/MspI  | aa         | aa     | aa     | aa       |
| Bf0158/PvuII | bb         | ab     | ab     | bb       |
| Cpi1624/MspI | bb         | bb     | ab     | bb       |
| Gm0043/HincII | aa   | ab     | aa     | aa       |
| Fo208/HinfI  | ab         | bb     | bb     | aa       |
| Mf0097/DraI  | bb         | bb     | bb     | bb       |
| Tf0062/KsaI  | ab         | ab     | aa     | ab       |
| Tf0079/StyI  | aa         | aa     | aa     | aa       |
| Tf0150/HinfI | bb         | ab     | bb     | bb       |
| Tf0168/KsaI  | ab         | ab     | ab     | aa       |
| Tf0235/HaeIII | aa       | aa     | aa     | aa       |
| Tf0271/KsaI  | aa         | aa     | aa     | aa       |
| Tf0326/HhaI  | ab         | aa     | bb     | ab       |
| Bf0136/AlaI  | bb         | bb     | bb     | bb       |
| Cpi975/Hinfl | ab         | bb     | ab     | ab       |
| Fb0159/Hinfl | bb         | ab     | aa     | bb       |
| Gn0040/HaeIII | bb  | bb     | bb     | bb       |
| Gm0071/PvII  | ab         | bb     | bb     | ab       |
| Fo211/PvIll | ab         | aa     | aa     | bb       |
| Ov0305/MspI  | ab         | ab     | ab     | aa       |
| Tf0069/MspI  | ab         | ab     | ab     | aa       |
| Tf0165/DraI  | bb         | bb     | bb     | bb       |
| Tf0300/DraI  | ab         | bb     | bb     | bb       |
| Tf0348/HinfI | ab         | ab     | bb     | bb       |
| Tf0350/DraI  | ab         | ab     | aa     | ab       |
| Tf0364/HinfI | bb         | ab     | ab     | ab       |
| Tf0386/MspI  | ab         | ab     | ab     | aa       |
| Wy0003/MspI  | bb         | ab     | bb     | bb       |

SNP marker

| Marker name | Cultivar |
|-------------|----------|
| SI173       | AG       |
| SI199       | AC       |
| SI228       | CG       |
| SI270       | GG       |
| SI322       | AT       |
| SI348       | AA       |
| SI363       | AT       |
| SI270       | AA       |
| SI322       | AA       |
| SI348       | AA       |
| SI363       | AT       |

and SI363), and the ponkan alleles had discrepancies at two of these (SI270 and SI348; Table 5). In contrast, there were no discrepancies in the inheritance of the alleles at any marker when the parents for ‘Southern Red’ were ‘Kara’ and ‘Osceola’. Furthermore, when ‘Kara’ was fixed as the seed parent, MARCO predicted ‘Osceola’ as the only possible pollen parent. These results strongly suggest that ‘Southern Red’ is the offspring of a cross between ‘Kara’ and ‘Osceola’.

We also confirmed discrepancies in the parentage of ‘Reikou’, which was reported as being the result of a cross between ‘KyEn No. 5’ and ‘Murcott’, at nine SNP markers (SI124, SI156, SI158, SI190, SI220, SI251, SI313, SI334, and SI373), and the ‘KyEn No. 5’ alleles had a discrepancy at one of these (SI190; Table 5). However, when ‘Murcott’ was fixed as the pollen parent, MARCO could not select a compatible seed parent with no discrepancies.

Discussion

Intellectual property protection for plant breeders has become an important issue, and techniques for cultivar identification and parentage confirmation based on molecular markers have been developed to solve this problem in various plants. In Japanese citrus breeding, Ninomiya et al. (2015) developed genotyping data based on nine CAPS markers for 33 representative cultivars and selections, and used this to develop protection for citrus cultivars developed in the Ehime Prefecture breeding program. Our result complements this previous work. However, additional genotyping data will be required to protect breeders’ rights for the major citrus cultivars distributed in Japan. This is because the CAPS markers we used are mainly bi-allelic markers and have a lower PIC than would be provided by multi-allelic markers such as SSRs. In addition, superior cultivars, their offspring, and their full siblings are frequently used as breeding parents in the current breeding program. This makes it difficult to develop methods for cultivar identification and to find discrepancies in the reported parentage, since the developed cultivars and selections have similar genetic backgrounds.

In strawberry breeding (Fragaria × ananassa Duch.), CAPS markers have contributed greatly to preventing infringement of breeders’ rights (Kunihisa, 2011). Packed strawberry fruits imported from Korea that were labeled as ‘Nyohou’ were identified as a mix of ‘Redpearl’ and ‘Sachinoka’ by using CAPS markers (Kunihisa et al., 2005). After warnings and legal action against the brokers, the amount of illegally imported strawberry fruits decreased sharply. In other infringement cases, DNA profiling methods were developed for the sweet cherry (Prunus avium L.), rush (Juncus effusus L.), kidney beans (Phaseolus vulgaris L.), and adzuki beans (Vigna angularis (Willd.) Ohwi & Ohashi), among others (Shoda et al., 2012). Our results
show that it is possible to identify 59 citrus cultivars, local varieties, and selections, including new cultivars recently developed by the citrus breeding program in the national institute of Japan and that are now commercially produced and frequently used as citrus breeding parents in Japan, on the basis of their genotypes by using eight selected CAPS markers. Therefore, a cultivar identification method based on these CAPS markers and genotype data will contribute greatly to protecting breeders of the major citrus cultivars in Japan.

These results also show that the actual parentage of ‘Setoka’, ‘Southern Red’, and ‘Reikou’ differed from their reported parentage, and that the candidate true parents of ‘Setoka’ and ‘Southern Red’ could be estimated by using DNA markers. Since citrus cultivars have great diversity in their morphological and physiological characteristics, an incorrect recording of the parentage of a cultivar may be detected from phenotypic data. For example, the parentage of ‘Southern Red’ appeared for a long time to be incorrect on the basis of its morphological traits and disease resistance. ‘Southern Red’ has monoembryony and high susceptibility to citrus canker disease (Kobayashi, 1995b), but the offspring of ponkan frequently exhibit polyembryony (Parlevliet and Cameron, 1959) and relatively low susceptibility to citrus canker disease (Matsumoto and Okudai, 1990). On the other hand, the parentage of ‘Setoka’ was not suspected as being incorrect on the basis of its morphological traits or its disease resistance. This is reasonable because the putative seed parent, ‘KyEn No. 4’, is part of the full-sib family of ‘Tsunonozomi’, which was previously reported as the seed parent, and has a similar genetic background to ‘Tsunonozomi’.

Genetic assessment is important to validate the pedigree of the promising cultivar ‘Setoka’ because it has superior fruit quality and has been frequently used as a parent in citrus breeding programs. However, the correct parentage of ‘Reikou’ could not be estimated in this study. It is difficult to preserve all cultivars and selections used as parents in fruit tree breeding programs owing to the high labor costs and limitations on the amount of usable land in Japan. Therefore, it is possible
Table 6. CAPS and SNP genotypes for the reported parents of ‘Reikou’. The underlined CAPS genotypes represent discrepancies between the reported parent–offspring relationship, in which ‘Reikou’ resulted from a cross between ‘KyEn No. 5’ and ‘Murcott’, and the marker results. A discrepancy exists if neither of the two alleles in the offspring exist in the candidate parent. The first 19 CAPS markers were used in the overall parentage analysis for all 33 NIFTS cultivars; the next 15 markers were used to clarify any parentage discrepancies revealed by the first 19 markers. SNP marker results are shown only for loci with discrepancies.

| Marker name | Cultivar       | ‘Reikou’ | ‘KyEn No. 5’ | ‘Murcott’ |
|-------------|---------------|----------|--------------|-----------|
| CAPS marker |               |          |              |           |
| Al0326/NdeII| ab            | bb       | aa           |           |
| Al0413/MspI | ab            | aa       | ab           |           |
| Al0636/EcoRI| ab            | bb       | bb           |           |
| Bd0208/HaeIII| ab       | ab       | aa           |           |
| Bd0209/StyI | aa            | ab       | aa           |           |
| Bd0306/MspI | ab            | bb       | bb           |           |
| Bd1415/MspI | aa            | aa       | ab           |           |
| Bd1518/PvuII| ab            | ab       | bb           |           |
| Cp1624/MspI | bb            | bb       | bb           |           |
| Gn0043/HincII| aa        | aa       | ab           |           |
| Ho208/HinfI | bb            | ab       | bb           |           |
| Mf0097/DraI | bb            | ab       | ab           |           |
| T0062/RsaI  | aa            | aa       | ab           |           |
| T0079/StyI  | ab            | aa       | ab           |           |
| T0150/HinfF | bb            | ab       | ab           |           |
| T0168/RsaI  | aa            | ab       | ab           |           |
| T0235/HaeIII| aa            | ab       | ab           |           |
| T0271/RsaI  | aa            | aa       | ab           |           |
| T0326/HhaI  | bb            | aa       | ab           |           |
| Cps0975/HinfI| ab        | bb       | ab           |           |
| Fb0159/HinfI| aa            | ab       | ac           |           |
| Gn0040/HaeIII| ab        | ab       | ab           |           |
| Gn0071/PvuII| bb            | bb       | bb           |           |
| Ho2111/PvuII| aa            | ab       | aa           |           |
| Ov0305/MspI | ab            | ab       | bb           |           |
| T0069/MspI  | bb            | ab       | ab           |           |
| T0165/DraI  | bb            | ab       | bb           |           |
| T0300/DraI  | ab            | bb       | bb           |           |
| T0348/HinfI | ab            | ab       | ab           |           |
| T0350/DraI  | ab            | aa       | ab           |           |
| T0364/HinfF | aa            | aa       | ab           |           |
| T0386/MspI  | ab            | ab       | ab           |           |
| SNP marker  |               |          |              |           |
| SI124       | AG            | AA       | AA           |           |
| SI156       | AG            | GG       | GG           |           |
| SI158       | AC            | AA       | AA           |           |
| SI190       | CC            | AA       | CC           |           |
| SI220       | AG            | GG       | GG           |           |
| SI251       | CG            | CC       | CC           |           |
| SI313       | AG            | AA       | AA           |           |
| SI334       | AG            | GG       | GG           |           |
| SI373       | AT            | AA       | AA           |           |

that the actual parent of ‘Reikou’ has already been fell to make room for new accessions.

Analysis based on the CAPS markers that we have developed will be useful because it does not demand expensive devices such as DNA sequencers; accessions can be genotyped by means of electrophoresis. However, there are advantages and disadvantages for each type of DNA marker (Kumar et al., 2009). In order to make DNA markers available in a range of situations, genotyping data for the DNA markers must be accumulated. CAPS marker data for the 59 cultivars and selections analyzed here will help us to protect breeders’ rights and to better understand the parentage, genetic diversity, and origins of the cultivars. Together, these benefits will help us to conduct more efficient citrus breeding programs.

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