Satsuma mandarins (*Citrus unshiu* Marc.) are the predominant cultivated citrus variety in Japan. Clarification of its origin would prove valuable for citrus taxonomy and mandarin breeding programs; however, current information is limited. We applied genome-wide genotyping using a 384 citrus single nucleotide polymorphism (SNP) array and MARCO computer software to investigate the satsuma mandarin parentage. Genotyping data from 206 validated SNPs were obtained to evaluate 67 citrus varieties and lines. A total of five parent–offspring relationships were newly found by MARCO based on the 206 SNP genotypes, indicating that ‘Kishuu mikan’ type mandarins (*Citrus kinokuni* hort. ex Tanaka accession ‘Kishuu mikan’ and ‘Nanfengmiju’) and ‘Kunenbo’ type mandarins (*Citrus nobilis* Lour. var. *kumip* Tanaka accession ‘Kunenbo’ and ‘Bendiguangju’) are possible parents of the satsuma mandarin. Moreover, cleaved amplified polymorphic sequences analysis showed that the genotypes of four regions in chloroplast DNA of ‘Kishuu mikan’ type mandarins were identical to that of the satsuma mandarin. Considering the historical background, satsuma mandarins may therefore derive from an occasional cross between a ‘Kishuu mikan’ type mandarin seed parent (derivative or synonym of ‘Nanfengmiju’) and a ‘Kunenbo’ type mandarin pollen parent (derivative or synonym of ‘Bendiguangju’).

**Key Words:** citrus, satsuma mandarin, parentage, genotype, origin.
(1997) reported that the mandarin variety ‘Bendiguangju’ resembled the satsuma mandarin. ‘Bendiguangju’ seed was originally brought by a Japanese monk from China, so could have been the origin of the satsuma mandarin.

Parental diagnosis using molecular and biochemical markers has advanced rapidly in humans, animals, and plants following the remarkable progress of genome sequencing. Citrus species are diploid with a basic chromosome number $x = 9$ (Krug 1943) and genome size of sweet orange ($C. sinensis$ Osbeck) and clementine ($C. clementina$ Hort. ex Tanaka) are 370 Mb and 367 Mb/haploid, respectively (Ollitrault et al. 2003). The genomes of sweet orange (diploid) and clementine mandarin (haploid) have been sequenced (Wu et al. 2014) and draft sequences are available in a public database, such as Pytozome (http://phytozome.jgi.doe.gov/pz/portal.html) and the Citrus Genome Database (https://www.citrusgenomedb.org/). Several attempts to explore the origin of citrus have been made based on molecular markers of nuclear DNA and chloroplast (cp) DNA (Curk et al. 2016, Gulsen and Roose 2001, Li et al. 2005, Coletta Filho et al. 2003), and the evaluation of genetic diversity of Chinese wild mandarins and separated them into two groups. While these reports provided valuable information for the organization of genetic resources, and the improvement of breeding, the origin of satsuma mandarins is still uncertain.

Numerous DNA markers, such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA, cleaved amplified polymorphic sequences (CAPS), and polymerase chain reaction (PCR)-RFLP analysis using chloroplast gene regions and chloroplast simple sequence repeat (cpSSR), have been developed and applied to mandarin cultivar identification (Matsuyama et al. 1992, Ninomiya et al. 2015, Omura et al. 2003, Sugawara et al. 2002, Ueda et al. 2003), and the evaluation of genetic diversity (Cheng et al. 2005, Coletta Filho et al. 1998, Li et al. 2006, Yamamoto et al. 2013). Yamamoto et al. (2013) evaluated the diversity of 103 species of mandarin and related fruits using cpDNA polymorphisms by CAPS analysis, and classified them into seven groups. Similarly, Li et al. (2006) reported the genetic diversity of Chinese wild mandarins using nuclear simple sequence repeat and cpSSR markers and separated them into two groups. While these reports provide valuable information for the organization of genetic resources and the improvement of breeding, the origin of satsuma mandarins is still uncertain.

Recently, Fujii et al. (2013) developed a 384 single nucleotide polymorphism (SNP) genotyping array using Illumina’s GoldenGate assay system to genotype a hybrid population of 88 progenies and 103 citrus accessions for Japanese breeding purposes. This is a useful tool to evaluate parentage because many of these markers have been mapped onto a citrus framework genetic map (Shimada et al. 2014) and provide a highly accurate diagnosis. Additionally, most SNPs derive from expression sequenced tags and are linked to the clementine genome sequence, which provides functional annotation and position information of clementine scaffolds ver.1.0. In an earlier study, these authors also developed MARCO computer software, which automatically performs calculations and estimates parentage based on DNA marker genotypes (Fujii et al. 2010). The application of these developments has enabled cultivar parentage to be evaluated, areas of confusion regarding certain cultivars to be resolved, and rights to be secured for citrus breeders. For example, the parentage of Japanese domesticated chestnuts was clarified based on SSR genotyping data (Nishio et al. 2014).

In the present study, 67 citrus varieties and lines, related to the satsuma mandarin derivation, underwent genome-wide genotyping using the citrus Illumina GoldenGate 384 SNP array. Based on validated data from the genotypes of 206 SNPs, four novel parent–offspring relationships were identified by MARCO. The possibilities of satsuma mandarin parentage are discussed with reference to cpDNA genotyping by CAPS analysis.

**Materials and Methods**

**Plant material and DNA preparation**

A total of 67 citrus varieties and lines related to the satsuma mandarin derivation were used for parental diagnosis (Table 1). Sample accession numbers and species names were based on the National Institute of Agrobiological Sciences (NIAS) Genbank. All plants were cultivated at Okitsu Citrus Research Station and Kuchinotsu Citrus Research Station of NIFTS in Japan. Genomic DNA was extracted from fresh and fully expanded leaves of these individuals and their parent cultivars, according to the method of Dellaporta et al. (1983). A total of 67 DNA samples were then adjusted to concentrations of 50 ng/µl using distilled water.

**Illumina GoldenGate assay**

Illumina’s GoldenGate Assay utilizing a bead array platform was carried out using the 384 SNP array for citrus genotyping (Fujii et al. 2013). The assay was performed according to the manufacturer’s protocol (Illumina, San Diego, CA) with help from the NIAS genome analysis support program to generate genotyping reports. Scores generated by the SNP signal calling system software were used to validate SNP genotyping for each combination of DNA sample and SNP according to the manufacturer’s description.

**Data analysis for putative parent–offspring relationships by MARCO**

Putative parent–offspring relationships were calculated using the parent calculation program MARCO (Fujii et al. 2010). MARCO detects possible parents from among the genotypes in a pair of varieties. Genotypes were considered to have a parent–offspring relationship if they shared at least one allele per SNP locus, with the exception that a discrepancy at a single SNP locus was permitted for possible genotyping errors, or the presence of null alleles or mutation, as previously proposed (Boursiquot et al. 2009, Cipriani et al.
Table 1. Cultivars and strains genotyped by 206 SNP markers

| No. | Plant name | Cultivar/Accession name | JP No. | Scientific name | Origin | Conservation in all SNPs |
|-----|------------|-------------------------|--------|----------------|--------|-------------------------|
| 1   | Sour orange | Kabasui, Shiitou, Daidai | 117365 | C. aurantium L. | Japan (Unknown) | O |
| 2   | Mediterranean mandarin | Chichikui mandarin, Meditteranen mandarin, | 117393 | C. deliciosa Tenore | China | O |
| 3   | Shaddock, Pommelo | Hirado buntan | 117507 | C. grandis (L.) Osbeck | Japan (Nagasaki) | O |
| 4   | Shaddock, Pommelo | Tanagawa buntan | 117433 | C. grandis Osbeck var. tanakawana hort. ex Tanaka | Japan (Kozen) | O |
| 5   | Juzu, Yuzu | Yuzu | 117380 | C. junos Siebold hort. ex Tanaka | Foreign (Unknown) | O |
| 6   | Juzu, Yuzu | Tadamisishi (Yuzu) | 113187 | C. junos Siebold hort. ex Tanaka | Japan (Tokushima) | O |
| 7   | Kinokuni | Kishu mikan | 171490 | C. kinokuni hort. ex Tanaka | China | 1 |
| 8   | Kinokuni | Sakurajima komikan | 117495 | C. kinokuni hort. ex Tanaka | Japan (Kagoshima) | 1 |
| 9   | Kinokuni | Harihakus | 117398 | C. kinokuni hort. ex Tanaka | Japan (Unknown) | 1 |
| 10  | Kinokuni | Harihakus | 117398 | C. kinokuni hort. ex Tanaka | Japan (Shiokawa) | 1 |
| 11  | Kinokuni | Makaku kishu | 117399 | C. kinokuni hort. ex Tanaka | Japan (Unknown) | 1 |
| 12  | Kinokuni | Taka mikan | 113181 | C. kinokuni hort. ex Tanaka | Japan (Wakayama) | O |
| 13  | Kinokuni | Nan-fen-mi-ji, Nanfengmu | 117731 | C. kinokuni hort. ex Tanaka | China | 1 |
| 14  | Kinokuni | Zuo ju, Soukitsu, Zaojio | 117400 | C. kinokuni hort. ex Tanaka var. subcompressa hort. ex Tanaka | China | O |
| 15  | Calamondin | Shikikitsu, Calamondin, | 117409 | C. malarensis Lour. | China | O |
| 16  | Calamondin | Calamondin, | 117386 | C. nobilis Lour. | USA | O |
| 17  | Calamondin, Kuneno | Kunenibo | 117387 | C. nobilis Lour. var. kuniop Tanaka | Foreign (Unknown) | O |
| 18  | Calamondin, Kuneno | Kunenbo, Kunenbo-Kagoshima | 117450 | C. nobilis Lour. var. kuniop Tanaka | Japan (Kagoshima) | 2 |
| 19  | Calamondin, Kuneno | Kunenbo-Kamikoshikijima | 117950 | C. nobilis Lour. var. kuniop Tanaka | Japan (Kagoshima) | 2 |
| 20  | Binkitsuka | Binkitsuka | 113168 | C. platymamma hort. ex Tanaka | China | O |
| 21  | Grapefruit | Duncan grapefruit | 116864 | C. paradisi Macf. | US | 3 |
| 22  | Grapefruit | Triumph grapefruit | 113255 | C. paradisi Macf. | USA | K |
| 23  | Ponkan | Ponkan F2428 | 113176 | C. reticulata Blanco | Taiwan | O |
| 24  | Ponkan | Idayu (ponkan) | 113179 | C. reticulata Blanco | Japan (Shiokawa) | 4 |
| 25  | Sweet orange | Touvita orang | 117254 | C. sinaensis (L.) Osbeck | USA | O |
| 26  | New Summer orange | Hyuganatsu, Konatsu | 117317 | C. tamurana hort. ex Tanaka | Japan (Myazaki) | O |
| 27  | Japanese mandarin, Miyagawa-wase | 117551 | C. unshiu Marcv. var. praecox Tanaka, C. unshiu | Japan (Fukuo) | O |
| 28  | Citrus | Hystoucan | 113365 | Citrus ampulacea hort. ex Tanaka | Japan (Unknown) | O |
| 29  | Citrus | Kawabata | 113344 | C. aurora hort. ex Tanaka | Japan (Kagoshima) | O |
| 30  | Citrus | Shiikusawa | 117406 | C. depressa Hayata | Japan (Okinawa) | O |
| 31  | Citrus | Kobeni mikan, Chu Sha Chu | 117397 | C. erythrosa hort. ex Tanaka | China | O |
| 32  | Citrus | Funadoko | 117372 | C. funadoko hort. ex Yu. Tanaka | Japan (Kouchi) | O |
| 33  | Citrus | Genshokan | 113199 | C. genshokan hort. ex Tanaka | China | O |
| 34  | Citrus | Kinukawa | 117278 | C. hershii hort. ex Tanaka | Japan (Okayama) | O |
| 35  | Citrus | Hassaku | 117286 | C. hassaku hort. ex Tanaka | Japan (Hiroshima) | O |
| 36  | Citrus | Yamamikan | 117539 | C. intermedi hort. ex Tanaka | Japan (Myazaki) | O |
| 37  | Citrus | Iyo, Iyokan, Miyauchi iyokan | 117373 | C. iyo hort. ex Tanaka | Japan (Yamaguchi) | O |
| 38  | Citrus | Iyo, Iyokan, Ootani iyokan | 115518 | C. iyo hort. ex Tanaka | Japan (Ehime) | O |
| 39  | Citrus | Kabuchi | 117390 | C. kerai hort. ex Tanaka var. kuchihii hort. ex Tanaka | Japan (Okayama) | O |
| 40  | Citrus | Kerai | 117389 | C. kerai hort. ex Tanaka | Japan (Kagoshima) | K |
| 41  | Citrus | Kisi | 113356 | C. leioarpa hort. ex Tanaka | Japan (Unknown) | O |
| 42  | Citrus | Kabosu | 117581 | C. shaenrcarpa hort. ex Tanaka | Japan (Oita) | O |
| 43  | Citrus | Ben di zao, Bendiuzao | 116116 | C. succosu hort. ex Tanaka | China | O |
| 44  | Citrus | Sudachi | 11383 | C. sudachi hort. ex Shira | Japan (Tokushima) | O |
| 45  | Citrus | Shiraikun | 113165 | C. suhuanensis hort. ex Tanaka | China | O |
| 46  | Citrus | Sanbukan | 117315 | C. suhuanensis hort. ex Tanaka | Japan (Wakayama) | O |
| 47  | Citrus | Sunzukan | 116117 | C. sunki (Hayata) hort. ex Tanaka | China (Shiokawa) | O |
| 48  | Citrus | Sunan ju, Sunkitsu, Sung ammunition | 117403 | C. sunki (Hataya) hort. ex Tanaka | China | O |
| 49  | Citrus | Taijihana | 117405 | C. tachihana (Makino) Tanaka | Japan (Unknown) | O |
| 50  | Citrus | Tachihana-Ishimami No.1 | 117880 | C. tachihana (Makino) Tanaka | Japan (Ishinami) | O |
| 51  | Citrus | Tachihana-Toshishi | 209687 | C. tachihana (Makino) Tanaka | Japan (Tosashi) | O |
| 52  | Citrus | Oobenimik | 117395 | C. tachihana (Makino) Tanaka | China | O |
| 53  | Citrus | Dancy tangerine, Dancy | 117396 | C. tangerina hort. ex Tanaka | Foreign (Unknown) | O |
| 54  | Citrus | Ujukisut | 115519 | C. ujukisut | Japan (Unknown) | O |
| 55  | Citrus | Yaunkuniba | 113383 | C. yunkuniba hort. ex Tanaka | Japan (Okinawa) | O |
| 56  | Citrus | Yatsuhiro | 117388 | C. yatsuhiro hort. ex Tanaka | Japan (Unknown) | O |
| 57  | Clementin, Hybrid | Clementine | 113161 | C. clementina hort. ex Tanaka, Mediterranean mandarin (C. deliciosa Tenore) × Sweet orange (C. sinaensis (L.) Osbeck) | Algeria | O |

Optimal SNP numbers:
- KP: 103 SNPs
- Barcoding: 48 SNPs
- Parentage: 54 SNPs

* O: Optima Research Station of NIFTS, L: Kuchinotsu Research Station of NIFTS.
* b: The same number shows that the genotype in all SNPs is the same.
* c: Author complemented.
* d: Mochida et. al. 2012.
Table 2. Primer sequence used for PCR amplification of 4 coding and non-coding regions in cpDNA

| Primer | Sequence (5’-3’) | Reference |
|--------|------------------|-----------|
| trnT(a)-F | CATTACAAATGGCATCCTCT | Taberlet et al. 1991 |
| trnL(b)-R | TCTACGGATTTGCGCATATC | |
| trnL(e)-F | GGTCTCAGTTCCTTCACCTCC | Taberlet et al. 1991 |
| Tmi(t)-R | AATTGAACTGTTGACAGGAC | |
| rbc-F | TTTGTGCAAAATAGTCCGAGGA | Cipriani and Morgante 1993 |
| rbc-R | TGTCTCAAAGTTGCCTCCAC | |
| matK-F | CCGAAATCTTGGTTCAAA | Penjor et al. 2013 |
| matK-R | GATGCCCTTAATGGCTTAC | |

Excluding 2010, Di Vecchi-Staraz et al. 2007, Lacombe et al. 2013. Among 384 SNPs, 206 were chosen as reliable for genotyping according to previously reported criteria (Fujii et al. 2013). A database of genotypes for 206 SNPs in 67 citrus varieties and lines was constructed (Supplemental Table 1). Genotypes with a discrepancy at none of the 206 SNPs were considered to represent putative parent–offspring pairs (comprising one parent and one offspring) or trios (comprising two parents and one offspring).

CAPS analysis using noncoding and coding regions in cpDNA

The noncoding region between trnL and trnF (trnL-trnF), the noncoding region between trnT and trnL (trnT-trnL), and coding regions rbcL and matK were PCR-amplified using primer sets described in the reports of Taberlet et al. (1991), Cipriani and Morgante (1993), and Penjor et al. (2013), and listed in Table 2. Amplification was performed in a total volume of 12.5 l containing 10 pg genomic DNA, 2.5 pmol of each primer, 2.5 mM dNTPs, 5 mM MgCl2, 0.2 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and PCR buffer. PCR was carried out using an ABI 9700 thermal cycler (Applied Biosystems) under the following conditions: preliminary denaturation for 10 min at 94°C; 35 cycles of 40 s at 94°C, 1 min at 52–62°C, and 2 min at 72°C; and an additional extension of 7 min at 72°C. PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer and the fragment pattern was visualized under UV light by ethidium bromide staining. For the detection of restriction site polymorphisms, PCR products were subjected to digestion using 16 restriction enzymes: MspI, HaeIII, HinII, RsaI, PvuII, StyI, HinfI, MuII, EcoRI, EcoRV, HhaI, Mbo I (NdeII), DraI, XbaI, HindIII, and BamHI.

Results

SNP genotyping related to satsuma mandarin derivation

A total of 67 citrus varieties and lines, including yuzu (Citrus junos Siebold hort. ex Tanaka) [5 (refer Table 1)] [6], Kinokuni mandarins (Citrus kinokuni hort. ex Tanaka) [7][8][9][10][11][12][13][14], Kuenbo mandarins (Citrus nobilis Lour. var. kunip Tanaka) [17][18][19], grapefruit (Citrus paradisi Macf.) [21][22], Ponkan mandarins (Citrus reticulata Blanco) [23][24], iyokan (Citrus iyo hort. ex Tanaka) [37][38], and tachibana (Citrus tachibana (Makino) Tanaka) [49][50][51], were genotyped for 206 SNPs by the GoldenGate Assay.

Seven lines of Kinokuni mandarins (‘Kishuu mikan’[7], ‘Sakurajima komikan’[8], ‘Hira-kishiu’[9], ‘Kishuunmikan-Iharaichiyoi’[10], ‘Mukaku kishiu’[11], ‘Taka mikan’[12], ‘Nanfengmiju’[13]) except ‘Zaoju’[14], three lines of Kuenbo mandarins (‘Kuenbo’[17], ‘Kuneno-Kagoshima’[18] and ‘Kuneno-Kamikoshikijima’[19]), two lines of grapefruit (‘Duncan’[21] and ‘Triumph’[22]), two lines of Ponkan mandarins (F2428 [23] and ‘Ideyu’[24]), and two lines of iyokan (‘Miyauchi’[37] and ‘Otanni’[38]), were found to have identical genotypes for all 206 SNPs (Supplemental Table 1). This suggests that they are synonyms, originate from nucellar seedlings, or are bud sport derivatives.

All genotypes were shared between Bendiguangjui (C. sp)[66] and the ‘Kuneno’ mandarin (C. nobilis Lour. var. kunip Tanaka) [17][18][19], as well as, between Oobenimikan (Citrus tangerina hort. ex Tanaka) [52] and Dancy tangerine (Citrus tangerina hort. ex Tanaka) [53]. These varieties are therefore also expected to be synonyms or spontaneously occurring derivatives. By contrast, lines of tachibana and yuzu showed different genotypes. Three tachibana lines [49][50][51] were subdivided into two groups by 11 SNP genotypes, and the genotypes of three SNPs differed among two yuzu lines [5][6]. Thus, a total of 53 independent genotypes were detected among the 67 citrus varieties and lines examined. In a previous study (Fujii et al. 2013), four satsuma mandarins, ‘Miyagawa-wase’ [27], ‘Okitsu-wase’, ‘Kawada unshiu’ and ‘Imamura unshiu’, were confirmed to share the same SNP genotypes, but there was no identical genotype with satsuma mandarin of ‘Miyagawa-wase’[27] in the examined citrus species of this study.

Parent–offspring relationship estimated by MARCO

Parental diagnosis was carried out for the 53 citrus varieties and lines with independent genotypes using MARCO computer software. The 206 SNP loci were covered on citrus major scaffold 01 to 09 of the clementine genome sequence that corresponded to the reference linkage map (Shimada et al. 2014) (Supplemental Table 2). Eight varieties of citrus had origin information for the parent–offspring relationship from previous research (Cameron et al. 1965, Frost 1935, Hodgson 1967, Nishiura et al. 1983, Ollitrault et al. 2012) (Table 3). There was no discrepancy in parent–offspring diagnosis in 8 combinations for the set of 206 SNP genotypes, including offspring ‘Kiyomi’[60] derived from the parent pair of a satsuma mandarin ‘Miyagawa wase’ (C. unshiu Marc)[27] and sweet orange ‘Trovia’ (Citrus sinensis (L.) Osbeck) [25]. The clementine mandarin [57] had previously been shown to derive from a cross between the Mediterranean mandarin (Citrus deliciosa Tenore) [2] and sweet orange [25] from comparative mapping analysis (Ollitrault et al. 2012). MARCO confirmed this parent–offspring relationship based on genotyping data from the 206 validated SNPs. These data were also used to calculate
Table 3. 8 citrus varieties with the information on parent–offspring relationship

| No. | Cultivar/Accession name | Combination of parents | Reference |
|-----|-------------------------|------------------------|-----------|
| 57  | Clementine              | Mediterranean mandarin (C. deliciosa Tenore) [2] × Sweet orange (C. sinensis (L.) Osbeck) [25] | Ollitrault et al. 2012 |
| 58  | Encore                  | King mandarin (C. nobilis Lour.) [16] × Mediterranean mandarin (C. deliciosa Tenore) [2] | Cameron et al. 1965 |
| 59  | Kara                    | Satsuma mandarin ‘Owari’ (C. unshiu Marc.) [27] × King mandarin [16] | Frost 1935 |
| 60  | Kiyomi                  | Satsuma mandarin ‘Miyagawa-wase’ (C. unshiu Marc.) [27] × Sweet orange ‘Trovita’ | Nishiyama et al. 1983 |
| 61  | Minneaora               | Grapefruit ‘Duncan’ (C. paradis Macf.) [21] × Dancy tangerine (C. tangerina v. Dancy) [53] | Hodgson 1967 |
| 62  | Orlando                 | Grapefruit ‘Duncan’ (C. paradis Macf.) [21] × Dancy tangerine (C. tangerina v. Dancy) [53] | Hodgson 1967 |
| 63  | Seminole                | Grapefruit ‘Duncan’ (C. paradis Macf.) [21] × Dancy tangerine (C. tangerina v. Dancy) [53] | Hodgson 1967 |
| 64  | Wilking                 | King mandarin (C. nobilis Lour.) [16] × Mediterranean mandarin (C. deliciosa Tenore) [2] | Frost 1935 |

*See Table 1.

Table 4. Parent–offspring relationships newly estimated by MARCO based on 206 SNP genotypes

| No. | Cultivar/Accession name | Candidate combination of parent varieties (No. × No.) |
|-----|-------------------------|------------------------------------------------------|
| 27  | Satsuma mandarin ‘Miyagawa-wase’ | Kishuu mikan (C. kinokuni hort. ex Tanaka) [7] × Kunenbo (C. nobilis Lour. var. kunip Tanaka) [17] |
| 14  | Zaoju                   | Kishuu mikan (C. kinokuni hort. ex Tanaka) [7] × Kobeni mikan (C. erythrosa hort. ex Tanaka) [31] |
| 39  | Kabuchi                 | Kunenbo (C. nobilis Lour. var. kunip Tanaka) [17] × Yatsushiro (C. yatsushiro hort. ex Tanaka) [56] |
| 40  | Keraji                  | Kunenbo (C. nobilis Lour. var. kunip Tanaka) [17] × Kabuchi (C. keraji hort. ex Tanaka var. kabuchii hort. ex Tanaka) [39] |
| 67  | Bakamikan               | Kunenbo (C. nobilis Lour. var. kunip Tanaka) [17] × Tachibana-Ishinami No.1 (C. tachibana (Makino) Tanaka) [50] |

*See Table 1.

a It is unclear which variety is seed parent or pollen parent.

parent–offspring relationships for the remaining six varieties of citrus.

Among the 53 varieties, MARCO also confirmed five new potential parent–offspring relationships based on the genotyping data, as summarized in Table 4. ‘Miyagawa wase’ [27], a typical cultivar of the satsuma mandarin, was possibly derived from a cross between ‘Kishuu mikan’ [7] and ‘Kunenbo’ mandarins [17]. ‘Zaoju’ (C. kinokuni hort. ex Tanaka var. subcompressa hort. ex Tanaka) [14] from ‘Kishuu mikan’ [7] and ‘Kobeni mikan’ (Citrus erythrosa hort. ex Tanaka) [31], ‘Kabuchi’ (C. keraji hort. ex Tanaka var. kabuchii hort. ex Tanaka) [39] from a cross between ‘Kunenbo’ [17] and ‘Yatsushiro’ (Citrus yatsushiro hort. ex Tanaka) [56], ‘Keraji’ [40] from ‘Kunenbo’ [17] and ‘Kabuchi’ [39] and ‘Bakamikan’ (C. sp) [67] from ‘Kunenbo’ [17] and ‘Tachibana-Ishinami No.1’ (C. tachibana (Makino) Tanaka) [50]. Interestingly, four parent–offspring relationship among the five newly suggested involved ‘Kunenbo’ [17] as a parent. This suggests that citrus germplasm might be derived from combination of relatively small number of ancestors, and ‘Kunenbo’ [17] is one of important ones.

Analysis of the cpDNA polymorphism by CAPS

Parental diagnosis by MARCO indicated that the satsuma mandarin was possibly derived from a cross between the ‘Kishuu mikan’ [7][8][9][10][11][12][13] and ‘Kunenbo’ types of mandarin [17][18][19][66]. To assign the seed parent from two parent varieties, the four coding and noncoding regions in cpDNA were amplified using sequence-tagged sites (STS) primers from 12 varieties as follows: seven ‘Kishuu mikan’ type mandarins (C. kinokuni hort. ex Tanaka) of ‘Kishuu mikan’ [7], ‘Sakurajima komikan’ [8], ‘Hirakishiu’ [9], ‘Kishuumikan-Iharaihijiyouji’ [10], ‘Mukaku kishiu’ [11], ‘Taka mikan’ [12] and ‘Nanfengmiju’ [13], and four ‘Kunenbo’ type mandarins of ‘Kunenbo’ [17], ‘Kunenbo-Kagoshima’ [18], ‘Kunenbo-Kamikoshikiijima’ [19], ‘Bendiguangiu’ [66], and ‘Miyagawa wase’ [27]. The approximate amplified fragment sizes of trnT-trnL, trnL-trnF, rbcL, and matK are 1.1 kb, 500 bps, 1.1 kb, and 800 bps, respectively (Fig. 1A). All fragment sizes were identical among the 12 varieties.

CAPS analysis was carried out for these four amplified regions using 16 restriction enzymes (Fig. 1B). In the trnT-trnL region, the Rsa I restriction site of ‘Kunenbo’ type mandarins varied compared with those of ‘Miyagawa wase’ [27], and ‘Kishuu mikan’ type mandarins [7][8][9][10][11][12][13]. In the trnL-trnF region, the Mbo I (NdeII) restriction site of ‘Kunenbo’ type mandarins [17][18][19][66] varied in comparison with those of other varieties. In the rbcL region, Rsa I and Hha I restriction sites of ‘Kunenbo’ type mandarins [17][18][19][66] differed from those of others. The HinI restriction site of ‘Kunenbo’ type mandarins [17][18][19][66] varied in comparison with others in the matK region. Any polymorphism was not detected in the four regions of cpDNA by the remaining restriction enzymes.
Identical genotypes were observed within ‘Kishuu mikan’ [7]-[9],[10],[11],[12],[13] and within ‘Kunenbo’ type mandarins [17],[18],[19],[66].

Penjor et al. (2013) previously sequenced the chloroplast matK gene of 135 citrus accessions and found that the genotype of the satsuma mandarin was identical to that of ‘Kinokuni’ (C. kinokuni hort. ex Tanaka) and ‘Mukaku kishu’ mandarins but differed from that of ‘Kunenbo’ mandarins. Yamamoto et al. (2013) evaluated the genetic diversity of 97 citrus varieties including local accessions from Japan, China, and Indonesia by the CAPS analysis of three interspecific regions in cpDNA. They found that the cpDNA genotype of the satsuma mandarin was similar to those of two lines of ‘Nanfengmiju’ sampled in Jiangxi and Guangxi in China, ‘Sakurajima komikan’, but was different to that of ‘Kunenbo’. In a study by Li et al. (2006), the cpDNA genotype of ‘Bendiguangju’ was shown to be different from that of the satsuma mandarin and ‘Nanfengmiju’. These results are in agreement with our own, indicating that the cpDNA genotype of the satsuma mandarin is identical to that of ‘Kishuu mikan’ type mandarins.
Parentage of satsuma mandarin (Citrus unshiu Marc.)

**Discussion**

Parentage diagnosis by MARCO based on 206 SNP genotypes and CAPS genotypes of cpDNA indicated that the seed parent of the satsuma mandarin is likely to be either of the ‘Kishuu mikan’ type mandarins [7][8][9][10][11][12][13], while the pollen parent is likely to be either of the ‘Kunenbo’ type mandarins [17][18][19][66]. Together with ‘Mukaku kishiu’, the satsuma mandarin has been used as a source of seedless fruits in citrus breeding in Japan. However, seedlessness occurs through physiologically different mechanisms between ‘Mukaku kishiu’ and the satsuma mandarin. ‘Mukaku kishiu’ is a seedless variety derived from a mutant of the native seeded variety ‘Kishuu mikan’. Its seedlessness is caused by an arrest in seed development at an early stage (Yamasaki and Kitajima 2007). By contrast, the seedlessness of the satsuma mandarin is mainly caused by cytoplasmic male sterility through pollen degradation (Nesumi et al. 1997) combined with unidentified nuclear factors that reduce the seed number. Genetic loci controlling the seedlessness of ‘Mukaku kishiu’ and anther development of the satsuma mandarin have previously been characterized by linkage analysis. The seedless locus of ‘Mukaku kishiu’ was mapped near Vsp015 and Edp005 in linkage group 9 (LG-09) of the AGI map (Shimada et al. 2014). The anther development locus (AD1) related to the satsuma mandarin regulating male sterility recessively was found to be located near the STS marker STS-D67-AD1 on linkage group 8 of the ‘Kiyomi’ × ‘Okitsu 41’ population (Nakano et al. 2003), which showed linkage map co-linearity with LG-08 of the AGI map. Moreover, a quantitative trait locus influencing fewer seed number derived from the ‘Miyagawa wase’ satsuma mandarin was located on LG-06 (Omura et al. 2003). This makes ‘Mukaku kishiu’ a less likely candidate for the seed parent of the satsuma mandarin, taking into consideration the inheritance of seedlessness from ‘Mukaku kishiu’.

In the GoldenGate assay of the present study, discrimination within ‘Kunenbo’ type mandarins [17][18][19][66] as well as within ‘Kishuu mikan’ types [7][8][9][10] [11][12][13] was not possible. Polymorphic differences within the satsuma mandarin varieties are generally limited in DNA markers because most varieties arose from spontaneous mutations during bud, limb, and nucellar embryogenesis. Indeed, an assessment of phylogenetic diversity in Citrus species by SSR, using highly polymorphic and reproducible co-dominant markers, also showed that satsuma mandarin varieties have a uniform genetic background (Golein et al. 2012).

The ‘Kishuu mikan’ mandarin is said to be a very old species of Chinese origin and one of the earliest introduced into Japan (Hodgson 1967) where it was commercially cultivated during the Edo period. ‘Nanfengmiju’ is a major variety cultivated in Jiangxi Province in China, which has several different lines that vary in seed number and fruit size. The level of genetic diversity among ‘Nanfengmiju’ lines is unclear, but origin histories suggest that ‘Nanfengmiju’ was the origin of ‘Kishuu mikan’ type mandarins or a synonym.

‘Kunenbo’ mandarins were introduced from Southeast Asia through the Ryukyu islands, and probably were the origin of many local citrus fruits on these islands (Yamamoto 2014). ‘Keraji’, ‘Kabuchii’, and ‘Oto’ (C. oto hort. ex Yu. Tanaka) appear to be closely related to ‘Kunenbo’ mandarins according to isozyme and DNA analyses (Yamamoto et al. 2011). Moreover, the cpDNA type of ‘Keraji’, ‘Kabuchii’, ‘Tarogayo’ (C. tarogayo hort. ex Tanaka), and ‘Oto’ is the same as that of ‘Kunenbo’ mandarins. These results agree with the parental diagnosis by MARCO that the ‘Kunenbo’ mandarin [17] is the parent variety of ‘Kabuchi’ [39] and ‘Keraji’ [40]. ‘Bendiguangu’ mandarins are thought to have been introduced to Japan from China by a Japanese envoy during the Tang dynasty (Xu 1997). These origin histories support the possibility that ‘Bendiguangu’ and ‘Kunenbo’ derive from the same progenitor individual of ‘Kunenbo’ type mandarins, which agrees with their identical SNP and CAPS genotypes.

Several hypotheses exist for the origin of satsuma mandarins based on morphological features. Ogaki (1979) supposed that they were chance seedlings from mandarins originating in China such as ‘Bendizao’, ‘Zaoju’, and ‘Manju’, while Xu (1997) proposed that ‘Bendiguangu’ was the original variety of the satsuma mandarin. Our SNP genotyping indicates that ‘Bendizao’ [43] and ‘Zaoju’ [14] would never generate the satsuma mandarin [27] genotype when hybridized with any other citrus variety genotype investigated. Although ‘Bendiguangu’ [66] was one of the candidate pollen varieties, it does not appear likely to be an original variety of satsuma mandarin as a polyembryonic derivative or synonym.

In conclusion, the satsuma mandarin appears to derive from an occasional cross between the seed parent of the ‘Kishuu mikan’ type mandarins [7][8][9][10][11][12][13] (derivative or synonym of ‘Nanfengmiju’ [13]) and the pollen parent of the ‘Kunenbo’ type mandarins [17][18][19] (derivative or synonym of ‘Bendiguangu’ [66]). Information about the parentage of the satsuma mandarin will further our understanding of citrus phylogeny, and help the production of a superior variety of satsuma mandarin by the cross hybridization of putative parents. Further research by comprehensive genome-wide genotyping using resequencing will be required to clarify the phylogenetic relationship between ‘Kishuu mikan’ type mandarins [7][8][9][10][11][12] and ‘Nanfengmiju’ [13], as well as ‘Kunenbo’ type mandarins [17][18][19] and ‘Bendiguangu’ [66].

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Literature Cited

Boursiquot, J.M., T. Lacombe, V. Laucou, S. Julliard, F.X. Perrin, N. Lanier, D. Legrand, C. Meredith and P. This (2009) Parentage of Merlot and related winegrape cultivars of southwestern France: discovery of the missing link. Aust. J. Grape Wine Res. 15: 144–155.

Cameron, J.W., R.K. Soost and H.B. Frost (1965) Encore and Pixie—two new mandarin hybrids with unusually late seasons of use. Calif. Agr. Exp. Sta. Bul. 814: 8 pp.

Cheng, Y., M. Carmen de Vicente, H. Meng, W. Guo, N. Tao and X. Deng (2005) A set of primers for analyzing chloroplast DNA diversity in Citrus and related genera. Tree Physiol. 25: 661–672.

Cipriani, G. and M. Morgante (1993) Evidence of chloroplast DNA variation in the genus Actinidia revealed by restriction analysis of PCR amplified fragments. J. Genet. Breed. 47: 319–326.

Cipriani, G., M.T. Marrazzo and E. Peterlunger (2010) Molecular characterization of the autochthonous grape cultivars of the region Friuli Venezia Giulia—North-Eastern Italy. Vitis 49: 29–38.

Colletta Filho, H.D., M.A. Machado, M.L.P.N. Targon, M.C.P.Q.D.G. Moreira and J. Pompeu Jr. (1998) Analysis of the genetic diversity among mandarins (Citrus spp.) using RAPD markers. Euphytica 102: 133–139.

Curk, F., F. Ollitrault, A. Garcia-Lor, F. Luro, L. Navarro and P. Ollitrault (2016) Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers. Ann. Bot. 117: 565–583.

Dellaporta, S.L., J. Wood and J.B. Hicks (1983) A plant DNA mini-preparation. Version II. Plant Mol. Biol. Rep. 1: 19–21.

Di Vecchi-Staraz, M., R. Bandinelli, M. Boselli, P. This, J.M. Boursiquot, V. Laucou, T. Lacombe and D. Vares (2007) Genetic structuring and parentage analysis for evolutionary studies in grapevine: kin group and origin of the cultivar Sangiovese revealed. J. Am. Soc. Hortic. Sci. 132: 514–524.

Frost, H.B. (1935) Four new citrus varieties—the Kara, Kinnow and Wiking mandarins and Trovita orange. Calif. Agr. Exp. Sta. Bul. 597: 14 pp.

Fujii, H., H. Yamashita, F. Hosaka, S. Terakami and T. Yamamoto (2013) High-throughput genotyping in citrus accessions using an SNP genotyping array. Tree Genet. Genomes 9: 1021–1030.

Golein, B., M. Nazeryan and B. Babakhani (2012) Assessing genetic variability in male sterile and low fertile citrus cultivars utilizing simple sequence repeat markers (SSRs). Afr. J. Biotechnol. 11: 145–153.

Golein, B., M. Nazeryan and B. Babakhani (2012) Assessing genetic variability in male sterile and low fertile citrus cultivars utilizing simple sequence repeat markers (SSRs). Afr. J. Biotechnol. 11: 1632–1638.

Gulsen, O. and M.L. Roose (2001) Chloroplast and nuclear genome analysis of the parentage of lemons. J. Am. Soc. Hortic. Sci. 126: 210–215.

Hodgson, R.W. (1967) Horticultural Varieties of Citrus, Chapter 4. In: Reuther, W., H.J. Webber and L.D. Buchanan (eds.) The Citrus Industry. University of California Press, Vol. 1, pp. 431–611.

Krug, C.A. (1943) Chromosome numbers in the subfamily Aurantioideae with special reference to the genus Citrus. Bot. Gaz. 104: 602–611.

Lacombe, T., J.M. Boursiquot, V. Laucou, M. Di Vecchi-Staraz, J.P. Peros and P. This (2013) Large-scale parentage analysis in an extended set of grapevine cultivars (Vitis vinifera L.). Theor. Appl. Genet. 126: 401–414.

Li, H., X. Yang, L. Zhu, H. Yi, L. Chai and X. Deng (2015) Parentage analysis of natural citrus hybrid ‘Zhelong Zhoupigan’ based on nuclear and chloroplast SSR markers. Sci. Hort. 186: 24–30.

Li, Y.Z., Y.J. Cheng, H.L. Yi and X.X. Deng (2006) Genetic diversity in mandarin landraces and wild mandarins from China based on nuclear and chloroplast simple sequence repeat markers. J. Hortic. Sci. Biotechnol. 81: 371–378.

Matsuyama, M., R. Motohashi, T. Akihama and M. Omura (1992) DNA fingerprinting in citrus cultivars. Breed. Sci. 42: 155–159.

Nakano, M., H. Nesumi, T. Yoshioka, M. Omura and T. Yoshiida (2003) Linkage analysis between male sterility of citrus and STS markers. Proc. Intl. Soc. Citricult. IX Congr. 2000: 179–180.

Nesumi, H., M. Nakano, T. Yoshiida, T. Yoshioka and Y. Ito (1997) Effect of plasmom on segregation of male sterile traits in citrus. J. Japan. Soc. Hort. Sci. 66 (Suppl. 2): 164–165.

Ninomiya, T., T. Shimada, T. Endo, K. Nonaka, M. Omura and H. Fujii (2015) Development of citrus cultivar identification by CAPS markers and parentage analysis. Hort. Res. 14: 127–133.

Ogaki, T. (1979) Chinese citrus, Chapter 1. In: Kondo, Y. and T. Ogaki (eds.) Chinese citrus. Sanbi Press, pp. 9–31.

Ollitrault, P., C. Jacquemond, C. Dubois and F. Luro (2003) Citrus. In: Hamon, P., M. Seguin, X. Perrier and J.C. Glasziou (eds.) Genetic diversity of cultivated tropical plants. CIRAD, Montpellier, 193–217.

Ollitrault, P., J. Terol, C. Chen, C.T. Federici, S. Lotfy, I. Hippolyte, F. Ollitrault, A. Berard, A. Chauveau, J. Cuenc et al. (2012) A reference genetic map of C. clementina hort. ex Tan.; citrus evolution inferences from comparative mapping. BMC Genomics 13: 593.

Omura, M., T. Ueda, T. Shimada, T. Endo, H. Fujii, H. Nesumi and T. Yoshiida (2003) Graphical genotype of citrus cultivars by co-dominant CAPS markers. Abst. Plant & Animal genome XI Conference.p.22. January 11–15, San Diego, CA.

Penjor, T., M. Yamamoto, M. Uehara, M. Ide, N. Matsumoto, R. Matsumoto and Y. Nagano (2013) Phylogenetic relationships of Citrus and its relatives based on matK gene sequences. PLoS ONE 8: e62574.

Shimada, T., H. Fujii, T. Endo, T. Ueda, A. Sugiyama, M. Nakano, M. Kita, T. Yoshioka, T. Shimizu, H. Nesumi et al. (2014) Construction of a citrus framework genetic map anchored by 708 gene-based markers. Tree Genet. Genomes 10: 1001–1013.

Sugawara, K., T. Wakizuka, A. Oowada, T. Moriguchi and M. Omura (2002) Histogenic identification by RAPD analysis of leaves and fruit of newly synthesized chimeric citrus. J. Am. Soc. Hortic. Sci. 127: 104–107.

Taberlet, P., L. Giely, G. Pautou and J. Bouvet (1991) Universal primers and parentage analysis. Hort. Res. 14: 127–133.
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history of admixture during citrus domestication. Nat. Biotechnol. 32: 656–662.

Xu, J. (1997) Origin of satsuma mandarin. In: Ren,Y. and S.Chen (eds.) In The Huangyan citrus history. Learning and Historical & Cultural Data Committee of CPPCC, Taizhou, pp. 276–280.

Yamamoto, M., R.Kouno, T.Nakagawa, T.Usui, T.Kubo and S. Tominaga (2011) Isozyme and DNA analyses of local *Citrus* germplasm on Amami islands, Japan. J. Japan. Soc. Hort. Sci. 80: 268–275.

Yamamoto, M., Y.Tsuchimochi, T.Ninomiya, T.Koga, A.Kitajima, A. Yamasaki, S.Inafuku-Teramoto, X.Yang, X.Yang, G.Zhong *et al.* (2013) Diversity of chloroplast DNA in various mandarins (*Citrus* spp.) and other citrus demonstrated by CAPS analysis. J. Japan. Soc. Hort. Sci. 82: 106–113.

Yamamoto, M. (2014) Citrus Genetic Resources Grown on the Ryukyu Islands, Japan. OCCASIONAL PAPERS No. 54: 9–15.

Yamasaki, A. and A. Kitajima (2007) Histological study of expression of seedlessness in *Citrus kinokuni* ‘Mukaku Kishu’ and its progenies. J. Am. Soc. Hortic. Sci. 132: 869–875.