

Research Paper

Parental diagnosis of satsuma mandarin (*Citrus unshiu* Marc.) revealed by nuclear and cytoplasmic markers

Hiroshi Fujii¹*, Satoshi Ohta¹, Keisuke Nonaka¹, Yuichi Katayose², Toshimi Matsumoto², Tomoko Endo¹, Terutaka Yoshioka¹, Mitsuo Omura³ and Takehiko Shimada¹

¹ NARO Institute of Fruit Tree Science (NIFTS), National Agriculture and Bio-oriented Research Organization (NARO), Shizuoka, Shizuoka 424-0292, Japan

² Agronomic Research Center, National Institute of Agrobiological Sciences (NIAS), Tsukuba, Ibaraki 305-8602, Japan

³ Faculty of Agriculture, Shizuoka University, Shizuoka, Shizuoka 422-8529, Japan

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. *kunip* 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.

Introduction

Citrus fruits are the most commonly grown fruits worldwide, with more than 25 species cultivated in Japan. Of these, satsuma mandarin (*Citrus unshiu* Marc.) is particularly suited to the Japanese climate where it has been cultivated for more than 100 years. Its fruits offer many favorable characteristics, including the fact that they are seedless, easy to peel, mature early, and show disease resistance and a high productivity. More than 200 cultivars have been registered for cultivar license since 1981 in Japan. The satsuma mandarin is still the main cultivated variety of citrus, accounting for 62.5% (45.5 kha) of all citrus acreage (72.6 kha) in 2014 in Japan. While its origins remain unclear, clarification of these would provide valuable information for understanding genetic diversity and aiding the

breeding of superior cultivars.

Satsuma is the name of a former province, now Kagoshima Prefecture, on the southern tip of Kyushu Island, where satsumas were originally described by Siebold (Hodgson 1967). During prehistoric and historical ages, many types of citrus were introduced, or naturally migrated, to Japan, or occurred as natural hybrids. It is likely that the parents or original varieties of satsuma mandarin were among these.

The highly distinctive satsuma mandarin is considered to have been cultivated in Japan prior to 1600 A.D., which is the time of its earliest known reference. Ogaki (1979) previously described the existence of many mandarin varieties similar to the satsuma mandarin in China. Because the satsuma mandarin has never been found in China and as its Japanese name Unshiu is thought to be a corruption of Wenchow, an ancient Chinese province, it seems likely that it originated as a chance seedling from a fruit or form imported from China, possibly from Wenchow Province. According to a report by Tanaka (1927), it may have derived from Bendizao, Zaoju, or Manju mandarin varieties, which share similar morphological characteristics. However, Xu

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*Corresponding author (e-mail: hfujii@affrc.go.jp)

(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 Phytozome (<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.* 2015). This progress has extended not only to advances in citrus molecular breeding and functional genomics, but also to phylogenetic studies involving the organization of multiple citrus varieties.

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 au-

thors 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) Genebank. 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 ^a	Cultivar/Accession name ^a	JP No. ^a	Scientific name ^a	Origin ^a	Conser- vation ^b	Common genotypes in all SNPs ^c
1	Sour orange	Kabusu, Shiutou, Daidai	117365	<i>C. aurantium</i> L.	Japan (Unknown)	O	
2	Mediterranean mandarin	Chichukai mandarin, Mediterranean mandarin, Willow Leaf Mandarin	117393	<i>C. deliciosa</i> Tenore	China	O	
3	Shaddock, Pummelo	Hirado buntan	171507	<i>C. grandis</i> (L.) Osbeck	Japan (Nagasaki)	O	
4	Shaddock, Pummelo	Tanigawa buntan	117433	<i>C. grandis</i> Osbeck var. tanikawana hort. ex Tanaka	Japan (Shizuoka)	O	
5	Juzu, Yuzu ^d	Yuzu	117380	<i>C. junos</i> Siebold hort. ex Tanaka	Foreign (Unknown)	O	
6	Juzu, Yuzu ^d	Tadanishiki (Yuzu) ^d	113187	<i>C. junos</i> Siebold hort. ex Tanaka	Japan (Tokushima)	O	
7	Kinokuni	Kishuu mikan	171490	<i>C. kinokuni</i> hort. ex Tanaka	China	O	1
8	Kinokuni	Sakurajima komikan	117495	<i>C. kinokuni</i> hort. ex Tanaka	Japan (Kagoshima)	O	1
9	Kinokuni	Hirakishiu	117398	<i>C. kinokuni</i> hort. ex Tanaka	Japan (Unknown)	O	1
10	Kinokuni	Kishuumikan-Iharaichijouji	117948	<i>C. kinokuni</i> hort. ex Tanaka	Japan (Shizuoka)	O	1
11	Kinokuni	Mukaku kishiu	117399	<i>C. kinokuni</i> hort. ex Tanaka	Japan (Unknown)	O	1
12	Kinokuni	Taka mikan	113181	<i>C. kinokuni</i> hort. ex Tanaka	Japan (Wakayama)	O	1
13	Kinokuni	Nan-fen-mi-jie, Nanfengmiju ^d	117731	<i>C. kinokuni</i> hort. ex Tanaka	China	O	1
14	Kinokuni	Zao ju, Soukitsu, Zaoju ^d	117400	<i>C. kinokuni</i> hort. ex Tanaka var. subcompressa hort. ex Tanaka	China	O	
15	Calamondin	Shikikitsu, Calamondin, Toukinkan	117409	<i>C. madurensis</i> Lour.	China	O	
16	King orange, King mandarin ^d	King mandarin	117386	<i>C. nobilis</i> Lour.	USA	O	
17	King orange, Kunenbo ^d	Kunenibo	117387	<i>C. nobilis</i> Lour. var. kunip Tanaka	Foreign (Unknown)	O	2
18	King orange, Kunenbo ^d	Kunenbo-Kagoshima	247450	<i>C. nobilis</i> Lour. var. kunip Tanaka	Japan (Kagoshima)	O	2
19	King orange, Kunenbo ^d	Kunenbo-Kamikoshikijima	117950	<i>C. nobilis</i> Lour. var. kunip Tanaka	Japan (Kagoshima)	O	2
20	Binkitsu	Binkitsu	113168	<i>C. platymamma</i> hort. ex Tanaka	China	O	
21	Grapefruit	Duncan grapefruit	168864	<i>C. paradisi</i> Macfad.	USA	O	3
22	Grapefruit	Triumph grapefruit	113255	<i>C. paradisi</i> Macfad.	USA	K	3
23	Ponkan	Ponkan F2428	113176	<i>C. reticulata</i> Blanco	Taiwan	O	4
24	Ponkan	Ideyu (ponkan) ^d	113179	<i>C. reticulata</i> Blanco	Japan (Shizuoka)	O	4
25	Sweet orange	Trovita orange	172154	<i>C. sinensis</i> (L.) Osbeck	USA	O	
26	New Summer orange	Hyuganatsu, Konatsu	117317	<i>C. tamurana</i> hort. ex Tanaka	Japan (Miyazaki)	O	
27	Japanese mandarin, Satsuma, Satsuma mandarin ^d	Miyagawa-wase	117351	<i>C. unshiu</i> Marcov. var. praecox Tanaka, <i>C. unshiu</i> Marcov. ^d	Japan (Fukuoka)	O	
28	Citrus	Hyoukan	113365	<i>Citrus ampullacea</i> hort. ex Tanaka	Japan (Unknown)	O	
29	Citrus	Kawabata	113344	<i>C. aurea</i> hort. ex Tanaka	Japan (Kagoshima)	O	
30	Citrus	Shiikuwasha	117406	<i>C. depressa</i> Hayata	Japan (Okinawa)	O	
31	Citrus	Koben mikan, Chu Sha Chu	117397	<i>C. erythroa</i> hort. ex Tanaka	China	O	
32	Citrus	Funadoko	117372	<i>C. funadoko</i> hort. ex Yu. Tanaka	Japan (Kouchi)	O	
33	Citrus	Genshokan	113159	<i>C. genshokan</i> hort. ex Tanaka	China	O	
34	Citrus	Kinukawa	117278	<i>C. glaberima</i> hort. ex Tanaka	Japan (Okayama)	O	
35	Citrus	Hassaku	117286	<i>C. hassaku</i> hort. ex Tanaka	Japan (Hiroshima)	O	
36	Citrus	Yamamikan	117359	<i>C. intermedia</i> hort. ex Tanaka	Japan (Miyazaki)	O	
37	Citrus	Iyo, Iyokan, Miyauchi iyokan ^d	117373	<i>C. iyo</i> hort. ex Tanaka	Japan (Yamaguchi)	O	5
38	Citrus	Iyo, Iyokan, Ootani iyokan ^d	115518	<i>C. iyo</i> hort. ex Tanaka	Japan (Ehime)	O	5
39	Citrus	Kabuchi	117390	<i>C. keraji</i> hort. ex Tanaka var. kabuchii hort. ex Tanaka	Japan (Okayama)	O	
40	Citrus	Keraji	117389	<i>C. keraji</i> hort. ex Tanaka	Japan (Kagoshima)	K	
41	Citrus	Kouji	113156	<i>C. leiocarpa</i> hort. ex Tanaka	Japan (Unknown)	O	
42	Citrus	Kabusu	117381	<i>C. shaerocarpa</i> hort. ex Tanaka	Japan (Ooita)	O	
43	Citrus	Ben di zao, Bendizao ^d	116116	<i>C. succosa</i> hort. ex Tanaka	China	O	
44	Citrus	Sudachi	117383	<i>C. sudachi</i> hort. ex Shirai	Japan (Tokushima)	O	
45	Citrus	Shikaikan	113165	<i>C. suluensis</i> hort. ex Tanaka	China	O	
46	Citrus	Sanboukan	117315	<i>C. sulcata</i> hort. ex I. Takah.	Japan (Wakayama)	O	
47	Citrus	Nian jie, Nianju ^d	116117	<i>C. sunki</i> (Hayata) hort. ex Tanaka	China	O	
48	Citrus	Sunan ju, Sankitsu, Sunki, Suanju ^d	117403	<i>C. sunki</i> (Hayata) hort. ex Tanaka	China	O	
49	Citrus	Tachibana	117405	<i>C. tachibana</i> (Makino) Tanaka	Japan (Unknown)	O	
50	Citrus	Tachibana-Ishinami No.1	117880	<i>C. tachibana</i> (Makino) Tanaka	Japan (Ishinami)	O	6
51	Citrus	Tachibana-Toshashi	209687	<i>C. tachibana</i> (Makino) Tanaka	Japan (Tosashi)	O	6
52	Citrus	Oobenimikan	117395	<i>C. tangerina</i> hort. ex Tanaka	India	O	7
53	Citrus	Dancy tangerine, Dancy	117396	<i>C. tangerina</i> hort. ex Tanaka	Foreign (Unknown)	O	7
54	Citrus	Ujukitsu	115519	<i>C. ujukitsu</i> hort. ex Tanaka	Japan (Unknown)	O	
55	Citrus	Yuukunibu	113383	<i>C. yanbaruensis</i> Tanaka	Japan (Okinawa)	O	
56	Citrus	Yatsuhoro	117388	<i>C. yatsushiro</i> hort. ex Tanaka	Japan (Unknown)	O	
57	Clementin, Hybrid ^d	Clementine	113161	<i>C. clementina</i> hort. ex Tanaka, Mediterranean mandarin (<i>C. deliciosa</i> Tenore) × Sweet orange (<i>C. sinensis</i> (L.) Osbeck) ^{d,e}	Algeria	O	
58	Hybrid	Encore mandarin	117421	<i>C. sp.</i> , King mandarin (<i>C. nobilis</i> Lour.) × Mediterranean mandarin (<i>C. deliciosa</i> Tenore) ^d	USA	O	
59	Hybrid	Kara	113158	<i>C. sp.</i> , Satsuma mandarin (<i>C. unshiu</i> Marc.) × King mandarin (<i>C. nobilis</i> Lour.) ^d	USA	O	
60	Hybrid	Kiyomi	115521	<i>C. sinensis</i> × <i>C. reticulata</i> , (Satsuma mandarin (<i>C. unshiu</i> Marc.) × Sweet orange (<i>C. sinensis</i> (L.) Osbeck) ^d	Japan (Shizuoka)	O	
61	Hybrid	Minneola	113377	<i>C. tangerina</i> × <i>C. grandis</i> , Duncan grapefruit (<i>C. paradisi</i> Macf.) × Dancy tangerine (<i>C. tangerina</i> v. Dancy) ^d	USA	O	
62	Hybrid	Orlando	113327	<i>C. tangerina</i> × <i>C. grandis</i> , Duncan grapefruit (<i>C. paradisi</i> Macf.) × Dancy tangerine (<i>C. tangerina</i> v. Dancy) ^d	USA	O	
63	Hybrid	Seminole	113348	<i>C. tangerina</i> × <i>C. grandis</i> , Duncan grapefruit (<i>C. paradisi</i> Macf.) × Dancy tangerine (<i>C. tangerina</i> v. Dancy) ^d	USA	O	
64	Hybrid	Wilking mandarin	117425	<i>C. sp.</i> , King mandarin (<i>C. nobilis</i> Lour.) × Mediterranean mandarin (<i>C. deliciosa</i> Tenore) ^d	USA	K	
65	Hybrid	Murcott	113374	<i>C. reticulata</i> Blanco × <i>C. sinensis</i> (L.) Osbeck	USA	O	
66	Hybrid	Bendi guanguju, Bendiguanguju ^d	113459	<i>C. sp.</i>	China	O	2
67	Hybrid	Baka mikan	117473	<i>C. sp.</i>	Japan (Shizuoka)	O	

^a Description by NIAS Genebank (http://www.gene.affrc.go.jp/databases-plant_search.php).^b O: Okitsu Research Station of NIFTS, K: Kuchinotsu Research Station of NIFTS.^c The same number shows that the genotype in all SNPs is the same.^d Author complemented.^e Ollitrault *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	CATTACAAATGCGATGCTCT	Taberlet <i>et al.</i> 1991
trnL(b)-R	TCTACCGATTTCGCCATATC	
trnL(e)-F	GGTTCAAGTCCCTCTATCCC	Taberlet <i>et al.</i> 1991
TrnF(f)-R	ATTTGAAGTGGTGACACGAG	
rbcL-F	TTGGCAGCATCCGAGTAA	Cipriani and Morgante 1993
rbcL-R	TGTCCTAAAGTTCTCCAC	
matK-F	CCGAAATCTTGGTTCAA	Penjor <i>et al.</i> 2013
matK-R	GATGCCCTAATGCGTTAC	

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 four coding and noncoding 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 µl 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 94.5°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*, *HincII*, *RsaI*, *PvuII*, *SlyI*, *HinfI*, *MulI*, *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], Kunenbo mandarins (*Citrus nobilis* Lour. var. *kunip* Tanaka) [17][18][19], grapefruit (*Citrus paradisi* Macfad.) [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], ‘Kishuumikan-Iharaichijyoji’ [10], ‘Mukaku kishiu’ [11], ‘Taka mikan’ [12], ‘Nanfengmiju’ [13]) except ‘Zaoju’ [14], three lines of Kunenbo mandarins (‘Kunenbo’ [17], ‘Kunenbo-Kagoshima’ [18] and ‘Kunenbo-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 Bendiguangju’ (*C. sp*) [66] and the ‘Kunenbo’ mandarin (*C. nobilis* Lour. var. *kunip* Tanaka) [17][18][19], as well as, between Oobeni-mikan (*C. tangerina* hort. ex Tanaka) [52] and Dancy tangerine (*C. 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. ^a	Cultivar/ Accession name ^a	Combination of parents	Reference
57	Clementine	Mediterranean mandarin (<i>C. deliciosa</i> Tenore) [2] × Sweet orange (<i>C. sinensis</i> (L.) Osbeck) [25]	Ollitrault <i>et al.</i> 2012
58	Encore	King mandarin (<i>C. nobilis</i> Lour.) [16] × Mediterranean mandarin (<i>C. deliciosa</i> Tenore) [2]	Cameron <i>et al.</i> 1965
59	Kara	Satsuma mandarin ‘Owari’ (<i>C. unshiu</i> Marc.) [27] × King mandarin [16]	Frost 1935
60	Kiyomi	Satsuma mandarin ‘Miyagawa-wase’ (<i>C. unshiu</i> Marc.) [27] × Sweet orange ‘Trovita’ (<i>C. sinensis</i> (L.) Osbeck) [25]	Nishiura <i>et al.</i> 1983
61	Minneora	Grapfruit ‘Duncan’ (<i>C. paradisi</i> Macf.) [21] × Dancy tangerine (<i>C. tangerina</i> v. Dancy) [53]	Hodgson 1967
62	Orlando	Grapfruit ‘Duncan’ (<i>C. paradisi</i> Macf.) [21] × Dancy tangerine (<i>C. tangerina</i> v. Dancy) [53]	Hodgson 1967
63	Seminole	Grapfruit ‘Duncan’ (<i>C. paradisi</i> Macf.) [21] × Dancy tangerine (<i>C. tangerina</i> v. Dancy) [53]	Hodgson 1967
64	Wilking	King mandarin (<i>C. nobilis</i> Lour.) [16] × Mediterranean mandarin (<i>C. deliciosa</i> Tenore) [2]	Frost 1935

^a See **Table 1**.**Table 4.** Parent–offspring relationships newly estimated by MARCO based on 206 SNP genotypes

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

^a See **Table 1**.^b 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 erythroasa* 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 par-

ent 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-Iharaichijyouji’ [10], ‘Mukaku kishiu’ [11], ‘Taka mikan’ [12] and ‘Nanfengmiju’ [13], and four ‘Kunenbo’ type mandarins of ‘Kunenbo’ [17], ‘Kunenbo-Kagoshima’ [18], ‘Kunenbo-Kamikoshikijima’ [19], ‘Bendiguangju’ [66], and ‘Miyagawa wase’ [27]. The approximate amplified fragment sizes of *trnT-trnL*, *trnL-trnF*, *rbcL*, and *matK* are 1.1 kbs, 500 bps, 1.1 kbs, 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 (*Nde*II) 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 *Hinf* I 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.

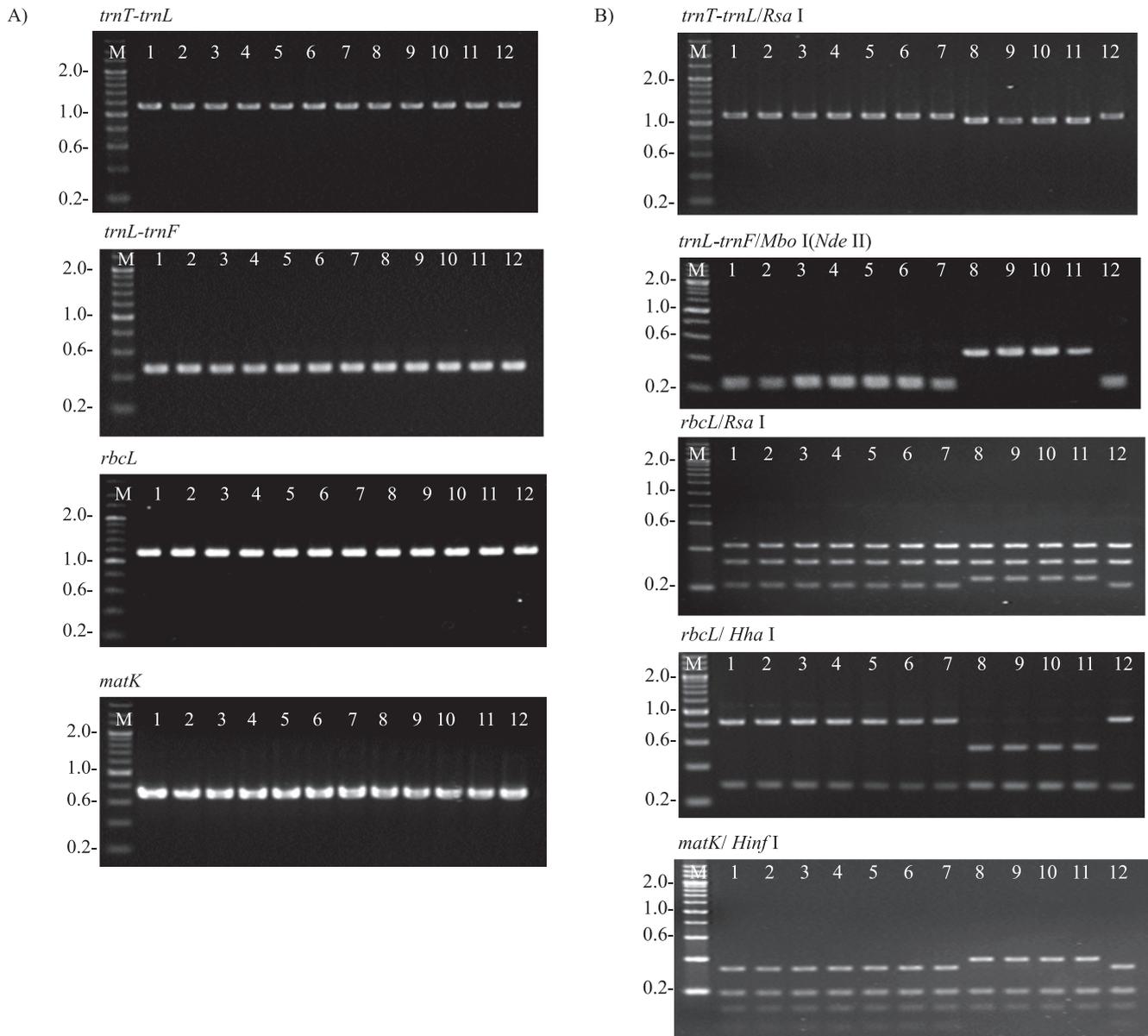


Fig. 1. PCR products of *trnT-trnL*, *trnL-trnF*, *rbcL*, and *matK* regions in cpDNA (A) and CAPS patterns (B). M: 200base DNA ladder of size marker, 1: ‘Mukaku kishiu’ [11]; 2: ‘Kishuu mikan’ [7]; 3: ‘Hira kishiu’ [9]; 4: ‘Sakurajima komikan’ [8]; 5: ‘Kishuu mikan-Iharaichijyouji’ [10]; 6: ‘Taka mikan’ [12]; 7: ‘Nanfengmiju’ [13]; 8: ‘Kunenbo’ [17]; 9: ‘Kunenbo-Kagoshima’ [18]; 10: ‘Kunenbo-Kamikoshikijima’ [19]; 11: ‘Bendiguangju’ [66]; 12: Satsuma mandarin (‘Miyagawa wase’) [27]. ‘Kyshuu mikan’ type mandarins including ‘Nanfengmiju’ [13] are lane 1–7 and ‘Kunenbo’ type mandarins including ‘Bendiguangju’ [66] are lane 8–11. Positions of size markers are indicate (Kb, kilobase pairs) on the left side.

Identical genotypes were observed within ‘Kishuu mikan’ [7][8][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.

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 Vs0015 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 to 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’ type mandarins [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 un-

clear, 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]. ‘Bendiguangju’ 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 ‘Bendiguangju’ 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 ‘Bendiguangju’ 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 ‘Bendiguangju’ [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] (derivative or synonym of ‘Nanfengmiju’ [13]) and the pollen parent of the ‘Kunenbo’ type mandarins [17][18][19] (derivative or synonym of ‘Bendiguangju’ [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 ‘Bendiguangju’ [66].

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