

Diversity of Chloroplast DNA in Various Mandarins (*Citrus* spp.) and Other Citrus Demonstrated by CAPS Analysis

Masashi Yamamoto^{1*}, Yui Tsuchimochi¹, Takanori Ninomiya¹, Takanori Koga¹, Akira Kitajima², Atsu Yamasaki^{3**}, Sayuri Inafuku-Teramoto⁴, Xuehu Yang^{5***}, Xiaoling Yang⁶, Guangyan Zhong^{7****}, Nasril Nasir⁸, Tatsuya Kubo¹ and Shigeto Tominaga¹

¹Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan

²Experimental Farm, Graduate School of Agriculture, Kyoto University, Takatsuki 569-0096, Japan

³Okitsu Citrus Research Station, NARO Institute of Fruit Tree Science, Shizuoka 424-0292, Japan

⁴Faculty of Agriculture, University of The Ryukyus, Nishihara, Okinawa 903-0213, Japan

⁵United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan

⁶School of Live Science & Technology, Tongji University, Shanghai 200092, China

⁷Citrus Research Institute, Southwest University, Chongqing 400712, China

⁸Faculty of Mathematics and Natural Science, Andalas University, Padang 25163, Indonesia

The diversity of chloroplast DNA (cpDNA) of citrus was revealed by means of a cleaved amplified polymorphic sequence (CAPS) using 97 *Citrus*, including many local accessions from Japan, China, and Indonesia, four *Fortunella*, and two *Poncirus*. All accessions were classified into seven types based on the results of polymorphic bands in all primer/enzyme combinations. The seven types were identified as follows: Type 1 (38 accessions): *C. macroptera*, *C. hystrix*, *C. aurantifolia*, *C. medica*, *C. limon*, *C. grandis*, *C. aurantium*, *C. myrtifolia*, *C. bergamia*, *C. rokugatsu*, *C. sinensis*, *C. sphaerocarpa*, *C. nobilis* (Kunenbo), *C. keraji*, *C. oto*, *C. tarogayo*, *C. suavissima*, mandarins of Yunnan and Guangxi, China, miscellaneous of Yunnan, China, *C. madurensis*, and *Fortunella*; Type 2 (one accession): *C. latipes*; Type 3 (two accessions): *C. ichangensis* and *C. junos*; Type 4 (six accessions): *C. tachibana* and *C. depressa*; Type 5 (18 accessions): *C. jambhiri*, *C. tankan*, *C. sunki*, *C. reshni*, *C. depressa*, mandarins of Kagoshima, Japan and Yunnan and Guangxi, China; Type 6 (36 accessions): *C. nobilis* (King), *C. unshiu*, *C. reticulata*, *C. genshokan*, *C. clementina*, *C. succosa*, *C. suhuensis*, *C. tardiferax*, *C. erythra*, *C. kinokuni*, *C. oleocarpa*, *C. leiocarpa*, mandarins of Kagoshima, Japan, Guangxi, Zhejiang, Yunnan and Guangdong, China, and mandarins of West Sumatra, Indonesia; Type 7 (Two accessions): *Poncirus*. The differentiation of cpDNA in citrus was discussed in accordance with the results.

Key Words: cpDNA, cytoplasmic genome, differentiation, evolution, polymorphism.

Introduction

Citrus is one of the most important fruit crops globally. It is cultivated between the latitudes of 40° north and

south, and the main growing area is the sub-tropical region. There are various accessions/cultivars adapted to different areas with great diversity.

Despite its economic importance, *Citrus* taxonomy has been difficult to characterize due to wide cross-compatibility, polyembryony (apomixis), the high frequency of bud mutation and a long history of cultivation; however, recent DNA analysis revealed that citron (*C. medica*), pummelo (*C. grandis*, synonym: *C. maxima*), and mandarin (*C. reticulata*) are the basic species of *Citrus*. Other species, such as sweet orange (*C. sinensis*), grapefruit (*C. paradisi*), and lemon

Received; June 14, 2012. Accepted; January 3, 2013.

* Corresponding author (E-mail: yamasa@agri.kagoshima-u.ac.jp).

** Present address: Grape and Persimmon Research Station, NARO Institute of Fruit Tree Science.

*** Present address: Yunnan Agricultural University.

**** Present address: Institute of Fruit Tree Research, Guangdong Academy of Agricultural Science.

(*C. limon*), are of hybrid origin (Barkley et al., 2006; Federici et al., 1998; Nicolosi et al., 2000; Pang et al., 2007). In addition, several studies have suggested that genetic variations within the mandarin are larger than those in citron and pummelo (Hirai and Kajiura, 1987; Hirai et al., 1986; Li et al. 2006; Yamamoto and Tominaga, 2003).

Among the various types of DNA analysis, chloroplast (cp) DNA analysis is useful for phylogenetic and evolutionary studies. Green et al. (1986) and Yamamoto and Kobayashi (1996) demonstrated the usefulness of cpDNA analysis of *Citrus* by restriction fragment length polymorphism (RFLP). This is a very time-, labor-, and cost-consuming method but has high reproducibility and reliability. As a recent development of the PCR technique, the cleaved amplified polymorphic sequence (CAPS) using universal primers, which is a simple and reliable method for cpDNA analysis, was developed (Arnold et al., 1991; Taberlet et al., 1991), and important results were obtained for *Citrus* using this approach (Asadi Abkenar et al., 2004a; Nicolosi et al., 2000); however, the materials used in these studies were mainly major accessions, although many different accessions are grown in various citrus cultivation areas.

Therefore, in the present study, various types of local citrus, especially mandarin accessions whose DNA profile has not been well clarified, were collected from the Ryukyu Islands of Japan, Yunnan and Guangxi of China, and West Sumatra of Indonesia to elucidate their diversity of cpDNA. Here we report the polymorphism of cpDNA revealed by CAPS analysis of citrus and discuss their phylogenetic relationships.

Materials and Methods

Ninety-seven *Citrus*, four *Fortunella*, and two *Poncirus* accessions were used in this study (Table 1). The sources of the materials are shown in Table 1. Total DNA was extracted from leaves using Isoplant II (Nippon Gene, Tokyo, Japan).

Three regions of cpDNA, *rbcL*-ORF106, *trnL-trnF*, and *trnF-trnVr* (Table 2), were amplified using universal primers (Arnold et al., 1991; Dumolin-Lapegue et al., 1997; Taberlet et al., 1991). The PCR reaction mixture of 15 μ L consisted of 10 ng template DNA, 10 pmol of each primer, 2 \times Ampdirect Plus, and 0.375 units of Nova Taq Hot Start DNA polymerase (Shimadzu, Kyoto, Japan). PCR reactions were performed in a PC320 (Astec, Fukuoka, Japan) thermal cycler programmed as follows: initial heating at 94°C for 1 min, 35 cycles of denaturing at 94°C for 1 min, 1 min at the annealing temperature (Table 2), extension at 72°C for 2 min, and a final extension of 10 min at 72°C. Aliquots of amplified products were digested with 5 units of the following enzymes: *rbcL*-ORF106 products were digested with *HinfI* or *HhaI*, *trnL-trnF* products were digested with *Sau3AI*, and *trnF-trnVr* products were digested with *TaqI*. These combinations of primers and enzymes

demonstrated polymorphisms in citrus (Asadi Abkenar, personal communication; Asadi Abkenar et al., 2004a; Urasaki et al., 2005). The digested products were electrophoresed on 1.5% agarose gels (Seakem GTG Agarose; Takara Bio, Shiga, Japan), and detected by staining with Mupid-Stain Eye (Advance, Tokyo, Japan).

Results

Each primer pair generated a single monomorphic fragment, and the sizes were 3100, 450, and 3000 bp for *rbcL*-ORF106, *trnL-trnF*, and *trnF-trnVr*, respectively. Polymorphic bands were obtained in all four primer/enzyme combinations. Citrus accessions used in this study were classified into three types in the combinations of *rbcL*-ORF106/*HinfI* or *HhaI* and *trnF-trnVr/TaqI* and two types in the combination of *trnL-trnF/Sau3AI* (Fig. 1, Table 3). In each primer/enzyme combination, accessions were classified as follows (Tables 3 and 4):

rbcL-ORF106/*HinfI* (type a: papeda, *C. aurantifolia*, *C. medica*, *C. limon*, *C. grandis*, *C. natsudaoidai*, sour and sweet oranges, and their relatives, except for *C. tankan*, *C. sphaerocarpa*, *C. nobilis* (Kunenbo), *C. keraji*, *C. oto*, *C. tarogayo*, *C. suavissima*, mandarins of Yunnan and Guangxi, China, miscellaneous of Yunnan, China, *C. madurensis*, and *Fortunella*), (type b: *C. jambhiri*, *C. tankan*, *C. ichangensis*, *C. junos*, mandarins, and its relatives except for *C. nobilis* (Kunenbo), *C. keraji*, *C. oto*, *C. tarogayo*, and *C. suavissima*, mandarins of Kagoshima, Japan, Guangxi, Zhejiang, Yunnan and Guangdong, China, and mandarins of West Sumatra, Indonesia), and (type c: *Poncirus*);

rbcL-ORF106/*HhaI* (type a: the accessions belonged “type a” in *rbcL*-ORF106/*HinfI*, *C. jambhiri*, *C. tankan*, *C. ichangensis*, *C. junos*, *C. tachibana*, *C. sunki*, *C. reshni*, *C. depressa*, mandarins of Kagoshima, Japan and Yunnan and Guangxi, China), (type b: *C. nobilis* (King), *C. unshiu*, *C. reticulata*, *C. genshokan*, *C. clementina*, *C. succosa*, *C. suhuensis*, *C. tardiferax*, *C. erythora*, *C. kinokuni*, *C. oleocarpa*, *C. leiocarpa*, mandarins of Kagoshima, Japan, Guangxi, Zhejiang, Yunnan and Guangdong, China, and mandarins of West Sumatra, Indonesia), and (type c: *Poncirus*);

trnL-trnF/Sau3AI (type a: the accessions belonged “type a” in *rbcL*-ORF106/*HinfI*, *C. ichangensis*, *C. junos*, *C. tachibana*, *C. depressa* (four accessions), *Poncirus*) and (type b: *C. jambhiri*, *C. tankan*, *C. nobilis* (King), *C. unshiu*, *C. reticulata*, *C. genshokan*, *C. clementina*, *C. succosa*, *C. suhuensis*, *C. tardiferax*, *C. erythora*, *C. kinokuni*, *C. sunki*, *C. reshni*, *C. depressa* (ten accessions), *C. oleocarpa*, *C. leiocarpa*, mandarins of Kagoshima, Japan, Guangxi, Zhejiang, Yunnan and Guangdong, China, and mandarins of West Sumatra, Indonesia);

trnF-trnVr/TaqI (type a: the accessions belonged “type a” in *rbcL*-ORF106/*HinfI* except for *C. latipes*, *C. ichangensis*, *C. junos*, the accessions belonged “type b” in *trnL-trnF/Sau3AI*, *Poncirus*), (type b: *C. latipes*),

Table 1. The materials used in this study, their distribution and source, and type of cpDNA.

No.	Common name	Latin name		Distribution	Source ^x	Type of cpDNA ^w
		Tanaka system ^z	Swingle system ^y			
Archicitrus						
Papeda						
1	Melanesian papeda	<i>Citrus macroptera</i> Mont.	<i>C. macroptera</i> Mont.	Southeastern Asia	1	1
2	Mauritius papeda	<i>C. hystrix</i> DC.	<i>C. hystrix</i> DC.	Southeastern Asia	1	1
3	Khasi papeda	<i>C. latipes</i> (Swingle) Tanaka	<i>C. latipes</i> (Swingle) Tanaka	India	1	2
Lime and its relatives						
4	Mexican lime	<i>C. aurantifolia</i> (Cristm.) Swingle	<i>C. aurantifolia</i> (Cristm.) Swingle	East Indian Archipelago	1	1
Citron and its relatives						
5	Marubusshukan	<i>C. medica</i> L.	<i>C. medica</i> L.	India	1	1
6	Xiangyuan (Jianshui)	<i>C. medica</i> L.	<i>C. medica</i> L.	Yunnan, China	2	1
7	Xiangyuan (Shiping)	<i>C. medica</i> L.	<i>C. medica</i> L.	Yunnan, China	2	1
8	Allen Eureka	<i>C. limon</i> (L.) Burm. f.	<i>C. limon</i> (L.) Burm. f.	India	1	1
9	Rough Lemon	<i>C. jambhiri</i> Lush.	<i>C. limon</i> relative	India	1	5
Pummelo and its relatives						
10	Benimadoka	<i>C. grandis</i> (L.) Osbeck	<i>C. grandis</i> (L.) Osbeck	Japan	1	1
11	Chandler	<i>C. grandis</i> (L.) Osbeck	<i>C. grandis</i> (L.) Osbeck	U. S. A.	1	1
12	Pomelo Ratu	<i>C. grandis</i> (L.) Osbeck	<i>C. grandis</i> (L.) Osbeck	Indonesia	3	1
13	Pomelo Raja	<i>C. grandis</i> (L.) Osbeck	<i>C. grandis</i> (L.) Osbeck	Indonesia	3	1
14	Beniamanatsu	<i>C. natsudaoidai</i> Hayata	<i>C. grandis</i> hybrid	Japan	1	1
Sour and sweet oranges and their relatives						
15	Sour orange 'Kabusu'	<i>C. aurantium</i> L.	<i>C. aurantium</i> L.	Japan	1	1
16	Chinotto	<i>C. myrtifolia</i> Rafin.	<i>C. aurantium</i> relative	Europe	1	1
17	Bergamot	<i>C. bergamia</i> Risso et Poit.	<i>C. aurantium</i> relative	Europe	1	1
18	Rokugatsumikan	<i>C. rokugatsu</i> hort. ex Yu. Tanaka	<i>C. aurantium</i> hybrid	Kyushu, Japan	4	1
19	Sweet orange 'Hamlin'	<i>C. sinensis</i> (L.) Osbeck	<i>C. sinensis</i> (L.) Osbeck	U. S. A.	1	1
20	Sweet orange 'Jemsri Jali'	<i>C. sinensis</i> (L.) Osbeck	<i>C. sinensis</i> (L.) Osbeck	Indonesia	3	1
21	Tankan 'Tarumizu 1 gou'	<i>C. tankan</i> Hayata	<i>C. sinensis</i> hybrid	Guangdong, China	1	5
Metacitrus						
Ichang papeda, Yuzu and their relatives						
22	Ichang papeda	<i>C. ichangensis</i> Swingle	<i>C. ichangensis</i> Swingle	Yunnan, China	1	3
23	Yuzu	<i>C. junos</i> Siebold. ex Tanaka	<i>C. ichangensis</i> hybrid	China and Japan	1	3
24	Kabosu	<i>C. sphaerocarpa</i> hort. ex Tanaka	<i>C. junos</i> hybrid	Oita, Japan	1	1
Mandarin and its relatives						
25	Kunenbo	<i>C. nobilis</i> Lour.	<i>C. reticulata</i> Blanco	Indo-China	1	1
26	King	<i>C. nobilis</i> Lour.	<i>C. reticulata</i> Blanco	Indo-China	1	6
27	Shagan	<i>C. nobilis</i> Lour.	<i>C. reticulata</i> Blanco	China	5	6
28	Satsuma mandarin 'Original strain'	<i>C. unshiu</i> Marcov.	<i>C. reticulata</i> Blanco	Kagoshima, Japan	1	6
29	Wenzhougan	<i>C. unshiu</i> Marcov.	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
30	Keraji	<i>C. keraji</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Kikaijima, Japan	4	1
31	Kikai mikan	<i>C. keraji</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Kikaijima, Japan	4	1
32	Kabuchi	<i>C. keraji</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Okinawa, Japan	4	1
33	Oto	<i>C. oto</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Okinawa, Japan	4	1
34	Tarogayo	<i>C. tarogayo</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Okinawa, Japan	4	1
35	Unju	<i>C. tarogayo</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Okinawa, Japan	4	1
36	Ponkan 'Yoshida ponkan'	<i>C. reticulata</i> Blanco	<i>C. reticulata</i> Blanco	Japan	1	6
37	Genshokan	<i>C. genshokan</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Taiwan	1	6
38	Clementine	<i>C. clementina</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Algeria	1	6
39	Bendizao	<i>C. succosa</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Zejiang, China	5	6
40	Sihuigan	<i>C. suhuiensis</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	China	5	6
41	Manju	<i>C. tardiferax</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Zejiang, China	5	6
42	Ougan	<i>C. suavissima</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Zejiang, China	5	1
43	Tachibana	<i>C. tachibana</i> (Makino) Tanaka	<i>C. tachibana</i> (Makino) Tanaka	Japan	1	4
44	Tachibana 'Okinawa strain'	<i>C. tachibana</i> (Makino) Tanaka	<i>C. tachibana</i> (Makino) Tanaka	Okinawa, Japan	4	4
45	Zhuhongju	<i>C. erythraea</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	China	5	6
46	Kinokuni 'Sakurajima komikan'	<i>C. kinokuni</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Japan	1	6
47	Nanfengmiju	<i>C. kinokuni</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Jiangxi, China	5	6
48	Nanfengmiju	<i>C. kinokuni</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
49	Miju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
50	Nianju	<i>C. oleocarpa</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Guangdong, China	5	6
51	Sunki	<i>C. sunki</i> (Hayata) hort. ex Tanaka	<i>C. reticulata</i> Blanco	Guangdong, China	1	5
52	Suanju	<i>C. sunki</i> (Hayata) hort. ex Tanaka	<i>C. reticulata</i> Blanco	Guangdong, China	5	5
53	Cleopatra	<i>C. reshni</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	India	1	5
54	Shiikuwasha 'Katsuyama kuganii'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	1	4
55	Shiikuwasha 'Ogimi kuganii'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	4	4

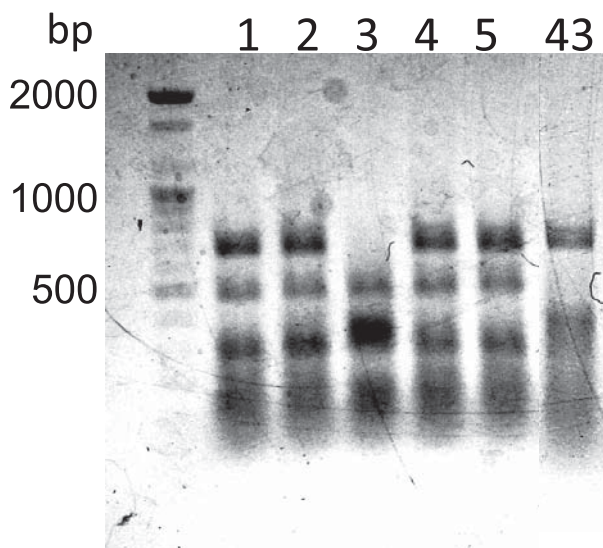
Table 1. Continued

No.	Common name	Latin name		Distribution	Source ^x	Type of cpDNA ^w
		Tanaka system ^z	Swingle system ^y			
56	Shiikuwasha 'Izumi kuganii'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	4	4
57	Shiikuwasha 'Few seeds strain'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	4	4
58	Shiikuwasha 'Okitsu strain'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	1	5
59	Shiikuwasha 'Kabishi'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	1	5
60	Shiikuwasha 'Ishikunibu'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinawa, Japan	4	5
61	Shiikuwasha 'Shiikunin (Ama)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Tokunoshima, Japan	4	5
62	Shiikuwasha 'Shiikunin (Kara)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Tokunoshima, Japan	4	5
63	Shiikuwasha 'Shiikuribu (Yakomo)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinoerabujima, Japan	4	5
64	Shiikuwasha 'Shiikuribu (Kamishiro)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinoerabujima, Japan	4	5
65	Shiikuwasha 'Shiikuribu (Masana)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinoerabujima, Japan	4	5
66	Shiikuwasha 'Shiikuribu (Amata)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinoerabujima, Japan	4	5
67	Shiikuwasha 'Shiikuribu (Saodsu)'	<i>C. depressa</i> Hayata	<i>C. tachibana</i> relative	Okinoerabujima, Japan	4	5
68	Koji	<i>C. leiocarpa</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Japan	1	6
69	Shimamikan (Nagashima)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Kagoshima, Japan	1	6
70	Shimamikan (Amami Oshima)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Kagoshima, Japan	4	6
71	Kuroshimamikan (Yakushima)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Kagoshima, Japan	4	6
72	Kuroshimamikan (Kuroshima)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Kagoshima, Japan	4	5
73	Shiyueju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Zhejiang, China	5	6
74	Gaojintou	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	1
75	Huangguo (Jianshui)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	1
76	Huangguo (near Jianshui)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	6
77	Huangguo (Wenshan)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	1
78	Huangguo (Mile)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	1
79	Baiju (near Jianshui)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	6
80	Baiju (Jianshui)	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	6
81	Fengdongju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	6
82	Suanjuzi	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	5
83	Tangfangju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Yunnan, China	2	6
84	Gonggan	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
85	Huapigan	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	1
86	Chuaxingan	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
87	Yeju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	5
88	Ningmingju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
89	Sihumiju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangdong, China	7	6
90	Shaju	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Guangxi, China	6	6
91	Jeruk Brastagi	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	West Sumatra, Indonesia	3	6
92	Jeruk Keprok	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	West Sumatra, Indonesia	3	6
93	Jeruk Siem	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	West Sumatra, Indonesia	3	6
94	Limau Talang Babungo	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	West Sumatra, Indonesia	3	6
95	Crifta	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	West Sumatra, Indonesia	3	6
Miscellaneous						
96	Suanganzi	<i>C. sp.</i>	<i>C. sp.</i>	Yunnan, China	2	1
Kumquat relatives						
97	Calamondin	<i>C. madurensis</i> Lour.	<i>C. reticulata</i> relative	China	1	1
Kumquat						
Protocitrus						
98	Kinzu	<i>Fortunella hindsii</i> (Champ. ex Benth.) Swingle	<i>F. hindsii</i> (Champ. ex Benth.) Swingle	China	1	1
Eufortunella						
99	Oval kumquat	<i>F. margarita</i> (Lour.) Swingle	<i>F. margarita</i> (Lour.) Swingle	China	1	1
100	Round kumquat	<i>F. japonica</i> (Thumb.) Swingle	<i>F. japonica</i> (Thumb.) Swingle	China	1	1
101	Meiwa kumquat	<i>F. crassifolia</i> Swingle	<i>F. sp.</i>	China	1	1
Trifoliolate orange						
102	Rubidoux	<i>Poncirus trifoliata</i> (L.) Raf.	<i>P. trifoliata</i> (L.) Raf.	China	1	7
103	Flying Dragon	<i>P. trifoliata</i> (L.) Raf.	<i>P. trifoliata</i> (L.) Raf.	China	1	7

^z Latin name by Tanaka's system (1969, 1977).^y Latin name by Swingle and Reece (1967).^x 1: Faculty of Agriculture, Kagoshima University, 2: Exploration in Yunnan, China, 3: Exploration in West Sumatra, Indonesia, 4: Farmer's orchard in Ryukyu islands, Japan, 5: Citrus Research Institute, Chinese Academy of Agricultural Sciences, 6: Exploration in Guangxi, China, 7: Exploration in Guangdong, China.^w See Table 4.

Table 2. Pairs of cpDNA primers used for PCR amplification in this study.

Primer	Sequence	T _m (°C)	Reference
<i>rbcL</i>	5'-ATGTCACCACAAACAGAACTAAAGCAAGT-3'	55	Arnold et al., 1991
ORF106	5'-ACTACAGATCTCATACTACCCC-3'		
<i>trnL</i>	5'-GGTTC AAGTCCCTCTATCCC-3'	55	Taberlet et al., 1991
<i>trnF</i>	5'-ATTTGAACTGGTGACACGAG-3'		
<i>trnF</i>	5'-CTCGTGTACCAGTTCAAAT-3'	57.5	Dumolin-Lapegue et al., 1997
<i>trnVr</i>	5'-CCGAGAAGGTCTACGGTTCG-3'		

**Fig. 1.** Restriction pattern obtained after digestion of *trnF-trnVr* amplified products with *TaqI*. 1: *C. macroptera* (type a), 2: *C. hystrix* (type a), 3: *C. latipes* (type b), 4: *C. aurantifolia* (type a), 5: *C. medica* (type a), 43: *C. tachibana* (type c).**Table 3.** Polymorphic bands that appeared in each primer and enzyme combination.

Primers	Enzyme	Polymorphic band (bp) ^z	Type
<i>rbcL</i> -ORF106	<i>HinfI</i>	900	a
		600	b
		950	c
	<i>HhaI</i>	1200, 600	a
		1200, 900	b
		1250, 600	c
<i>trnL-trnF</i>	<i>Sau3AI</i>	450	a
		250, 200	b
<i>trnF-trnVr</i>	<i>TaqI</i>	700, 500	a
		500, 400	b
		700, 400	c

^z Common bands are excluded.

and (type c: *C. tachibana*, *C. depressa* (four accessions)).

All accessions were classified into seven types based on the results of polymorphic bands in all primer/enzyme combinations (Tables 1 and 4) shown as follows:

Type 1 (38 accessions): *C. macroptera*, *C. hystrix*, *C. aurantifolia*, *C. medica*, *C. limon*, *C. grandis*, *C. aurantium*, *C. myrtifolia*, *C. bergamia*,

C. rokugatsu, *C. sinensis*, *C. sphaerocarpa*, *C. nobilis* (Kunenbo), *C. keraji*, *C. oto*, *C. tarogayo*, *C. suavissima*, mandarins of Yunnan and Guangxi (four and one accessions, respectively), China, miscellaneous of Yunnan, China, *C. madurensis*, and *Fortunella*;

Type 2 (one accession): *C. latipes*;

Type 3 (two accessions): *C. ichangensis* and *C. junos*;

Type 4 (six accessions): *C. tachibana* and *C. depressa* (four accessions);

Type 5 (18 accessions): *C. jambhiri*, *C. tankan*, *C. sunki*, *C. reshni*, *C. depressa* (ten accessions), mandarins of Kagoshima, Japan and Yunnan and Guangxi, China (each one accession);

Type 6 (36 accessions): *C. nobilis* (King), *C. unshiu*, *C. reticulata*, *C. genshokan*, *C. clementina*, *C. succosa*, *C. suhuensis*, *C. tardiferax*, *C. erythora*, *C. kinokuni*, *C. oleocarpa*, *C. leiocarpa*, mandarins of Kagoshima, Japan (three accessions), Guangxi, Zhejiang, Yunnan and Guangdong (four, one, five and one accessions, respectively), China, and mandarins of West Sumatra, Indonesia;

Type 7 (two accessions): *Poncirus*.

Discussion

The combinations of primers and restriction enzymes used in the present CAPS analysis were established in previous studies that analyzed citrus cpDNA (Asadi Abkenar, personal communication; Asadi Abkenar et al., 2004a; Urasaki et al., 2005). The sizes of the amplified products and the digested fragments in the present and previous studies were identical; however, small bands were not detected well in our results because Mupid-Stain Eye (Advance, Tokyo, Japan) was used as the staining reagent. Mupid-Stain Eye is not hazardous but its resolution is not enough at low molecular range compared with ethidium bromide. Although we could not determine the accurate size of small bands and showed only clear polymorphic bands in Table 3, each type in all combinations of primer and restriction enzyme could be distinguished easily.

In the three basic species of *Citrus*, *C. medica*, and *C. grandis* were not distinguished from each other and both belonged to type 1 in the present study, although previous CAPS analyses using many probes and restriction enzymes (Asadi Abkenar et al., 2004a; Nicolosi et al., 2000) reported a difference of cpDNA

Table 4. Types of cpDNA of accessions used in this study.

Type	<i>rbcL</i> -ORF106 ^z		<i>trnL</i> - <i>trnF</i>	<i>trnF</i> - <i>trnVr</i>	Accession ^x
	<i>Hinf</i> I ^y	<i>Hha</i> I	<i>Sau</i> 3A1	<i>Taq</i> I	
1	a ^w	a	a	a	1 2 4 5 6 7 8 10 11 12 13 14 15 16 17 18 19 20 24 25 30 31 32 33 34 35 42 74 75 77 78 85 96 97 98 99 100 101
2	a	a	a	b	3
3	b	a	a	a	22 23
4	b	a	a	c	43 44 54 55 56 57
5	b	a	b	a	9 21 51 52 53 58 59 60 61 62 63 64 65 66 67 72 82 87
6	b	b	b	a	26 27 28 29 36 37 38 39 40 41 45 46 47 48 49 50 68 69 70 71 73 76 79 80 81 83 84 86 88 89 90 91 92 93 94 95
7	c	c	a	a	102 103

^z Primers.^y Restriction enzyme.^x See Table 1.^w Each type is shown in Table 3.

between them. On the other hand, mandarins (*C. reticulata*) showed considerable diversity in their banding patterns and belonged to types 1, 4, 5, and 6.

C. aurantium, *C. sinensis*, and *C. limon* belonged to the same type as *C. grandis*, which is consistent with previous studies (Araújo et al., 2003; Asadi Abkenar et al., 2004a; Tshering Penjor et al., 2010). This result indicates that *C. medica* or *C. grandis* was the ancestral female parent of hybrid accessions belonging to type 1 because cpDNA was maternally inherited (Asadi Abkenar et al., 2004b). Japanese mandarins such as *C. keraji*, *C. oto*, and *C. tarogayo* were considered to be derived from *C. nobilis* (Kunenbo) and to possess the same type as cpDNA of *C. sinensis* (Yamamoto et al., 2010, 2011). Some of the Chinese mandarins cultivated in Yunnan and Guangxi belonging to type 1 may also be progeny of *C. sinensis*.

Among the papeda accessions, *C. macroptera* and *C. hystrix*, and *C. latipes* belonged to types 1 and 2, respectively; The resemblance of cpDNA of those species to that of *C. grandis* was reported by Nicolosi et al. (2000) and Asadi Abkenar et al. (2004a). On the other hand, cpDNA of *C. ichangensis* was different from both *C. grandis*/*C. medica* and mandarin. The unique taxonomic form of *C. ichangensis* was also reported by cpDNA analysis of Cheng et al. (2005). *C. junos* possessed the same type of cpDNA as *C. ichangensis*. This result supports the concept of genetic relatedness between the two species (Hirai and Kajiuira, 1987; Yamamoto et al., 2007).

The cpDNA of mandarins could be classified into three types: types 4, 5, and 6. Type 4 consists of *C. tachibana* and *C. depressa*, mandarins native to Japan. Since this type of cpDNA was not found in mandarins originating from other areas and other *Citrus* species, these Japanese mandarins, *C. tachibana* and *C. depressa*, are considered to be differentiated from other *Citrus* species. Some Japanese, Chinese, and Indian mandarins belonged to type 5. *C. sunki*, *C. reshni*, and *C. depressa* are small-fruit mandarins mainly used as rootstock. Yeju,

a wild mandarin found in China, also belonged to type 5. *C. depressa* belonged to both type 4 and type 5. The cpDNA divergence of this species was also reported by Urasaki et al. (2005). These results seemed to indicate a polyphyletic origin of *C. depressa*. This divergence was discovered in *C. depressa* of Okinawa but not in that of Amami islands (Tokunoshima and Okinoerabujima) because all *C. depressa* of Amami islands showed type 5 cpDNA. Various accessions with characteristic morphological traits are cultivated in Okinawa (Kinjo, 2007). The diversity of *C. depressa* in Okinawa is probably higher than that of the Amami islands based on these results. Froelicher et al. (2011) reported that the mitochondrial (mt) DNA of *C. sunki*, *C. reshni*, *C. depressa*, and *C. jambhiri* was distinct from that of other mandarins such as *C. reticulata*, *C. unshiu*, and *C. clementina*. This result completely agrees with that of the present study using cpDNA analyses and provides evidence of the differentiation of the cytoplasmic genome in mandarins. Although *C. tankan* was possibly derived from the cross-combination of *C. reticulata* and *C. sinensis* (Yamada, 1994), the types of cpDNA of all three species were different, and thus this hypothesis is rejected by our results. Mandarins such as *C. unshiu*, *C. reticulata*, *C. clementina*, and others whose fruits are consumed fresh or processed generally belonged to type 6. Many mandarins from various regions belonged to this group. Almost all local mandarin accessions cultivated in Yunnan and Guangxi in China and those in Indonesia showed cpDNA of type 6. Mandarins of type 6, which were collected in Japan, are considered to have arisen from mandarins in other areas because indigenous mandarins (Tanaka, 1936) are type 4 or 5. On the other hand, the relationship between the collected region and the type of cpDNA was ambiguous in Chinese mandarin. Type 6 is predominant but some mandarins of type 5 were discovered in Guanxi, Yunnan, and Guangdong.

Fortunella resembles *Citrus* in terms of its general morphological traits, although some of its characteristics

such as the number of ovules in each locule and the flowering period differ from those of *Citrus*. *Fortunella* was not distinguished from *Citrus* since all the fragment patterns of *Fortunella* were the same as those of type 1, which includes *C. grandis*, *C. medica*, and some others (Type 1) in the present study. This result agrees with those of previous studies (Bayer et al., 2009; Froelicher et al., 2011; Nicolosi et al., 2000; Yamamoto et al., 1993), which showed no significant differences between *Fortunella* and *Citrus*.

Although *Poncirus* is cross-compatible with *Citrus*, its various characteristics, deciduous, flowering period, and trifoliate leaves, differ from those of *Citrus*. The genetic distance between *Poncirus* and *Citrus* has already been reported in studies using cp and mtDNA analyses (Bayer et al., 2009; Froelicher et al., 2011; Jung et al., 2005; Yamamoto et al., 1993). In this study, *Poncirus* belonged to Type 7, which is consistent with the results of the above-mentioned studies.

Simple sequence repeat (SSR) (Kitajima, personal communication), inter-simple sequence repeat (ISSR) (Yamamoto et al., 2010), and sequence-related amplified polymorphism (SRAP) (Yamamoto et al., 2012) analyses revealed that some accessions collected in Japan, China, and Indonesia showed identical DNA profiles. The accessions which were not distinguished on the basis of these DNA analyses are the following: (*C. unshiu*, No. 28 and 29), (*C. tarogayo*, No. 34 and 35), (*C. kinokuni*, No. 46, 47, and 48), (*C. depressa*, No. 61 and 65), (*C. depressa*, No. 62–64 and 67), (Chinese mandarin Huangguo, No. 75, 77, and 78), (Chinese mandarin Baiju, No. 79 and 80), and (Indonesian mandarin, No. 91–94). These probably arose from bud sports or nucellar seedlings and belonged to the same type of cpDNA in this study.

The differentiation of the chloroplast genome of citrus is assumed on the basis of the present cpDNA data. *Poncirus* and *Citrus/Fortunella* were differentiated from an ancestral species. Among types 1 to 6, type 1 is considered to be an ancestral type of cpDNA because several genera and species, including papeda, which is probably the most primitive form of *Citrus* (Tanaka, 1969), belonged to this type. Both type 2 and type 3 were derived from type 1. In our hypothesis, *C. latipes* seemed not to be one of the most primitive species (Tanaka, 1969). An ancestral species of mandarin probably also derived from type 1, and then the mandarins native to Japan (type 4: *C. tachibana* and *C. depressa*) differentiated. In terms of the main differentiation route, it seems that type 5 (*C. sunki*, *C. reshni*, and *C. depressa*) arose first and was followed by type 6 (*C. reticulata*, *C. unshiu*, *C. clementina*, and so on), which developed from type 5. From this point of view, mandarins belonging to type 6 possessed the most differentiated cpDNA compared with that of *C. grandis/C. medica*.

In the present study, we could reveal the diversity of

cpDNA of *Citrus* and its related genera using more than 100 accessions, including various local Japanese, Chinese, and Indonesian mandarins, by means of CAPS analysis. We demonstrated that cpDNA of mandarin could be distinguished from pummelo and citron among the three basic *Citrus* species. Furthermore, divergence of cpDNA within the mandarin was clarified and the differentiation of mandarins native to Japan was elucidated. Recent advances in DNA sequencing techniques have allowed the extensive use of short DNA fragment sequences in the study of phylogenetic relationships. A large body of useful information on *Citrus* phylogeny was obtained from the sequencing of chloroplast genomes (Araújo et al., 2003; Bayer et al., 2009; Jung et al., 2005; Tshering Penjor et al., 2010). Studies like ours that use many accessions are necessary to obtain further information on citrus phylogeny.

Acknowledgements

We thank Prof. Ryoji Matsumoto and Dr. Asad Asadi Abkenar of the Faculty of Agriculture, Saga University, for providing valuable advice.

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