J. Japan. Soc. Hort. Sci. 79 (1): 16–22. 2010.
Available online at www.jstage.jst.go.jp/browse/jjshs1

Triploid and Aneuploid Hybrids from Diploid-diploid Intergeneric Crosses between *Citrus* Cultivar 'Kiyomi' Tangor and Meiwa Kumquat (*Fortunella crassifolia* Swingle) for Seedless Breeding of Kumquats

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In order to produce new seedless kumquat cultivars, we carried out an intergeneric cross between 'Kiyomi' tangor [*Citrus unshiu* Marcow. × *C. sinensis* (L.) Osbeck] and Meiwa kumquat (*Fortunella crassifolia* Swingle), obtaining 2 normal seeds and 7 undeveloped seeds. These seeds were cultivated on Murashige and Tucker medium, and the 2 normal seeds germinated and developed. The results of genome size analysis by flow cytometry revealed that both seedlings were triploids and that the difference in genome size corresponded to more than one chromosome in the 2 seedlings. Chromosome observation confirmed diploid (2n = 2x = 18) in both parents, aneuploid with 28 chromosomes (2n=28) for one of the seedlings, and triploid (2n=3x=27) for the other seedling. Random amplified polymorphic DNA (RAPD) and cleaved amplified polymorphic sequence (CAPS) analyses proved that the seedlings were intergeneric hybrids between 'Kiyomi' tangor and Meiwa kumquat, with the maternal organelle genome. These hybrids have the potential to be released as a cultivar after further tree and fruit evaluations, and for use as cross-parents in seedless kumquat breeding.

Key Words: aneuploidy, flow cytometry, genome size analysis, 'Kiyomi' tangor, Meiwa kumquat.

Introduction

The kumquat (*Fortunella*) is closely related to *Citrus* and *Poncirus* in the subfamily Aurantioideae (Citroideae) of the family Rutaceae (Swingle and Reece, 1967). Species in this genus are important as genetic resources for *Citrus* breeding because they have cold hardiness, pest tolerance, small tree size, and small fruit with an edible peel. They have been cultivated for both as table fruit and for processing in mainly Japan, China, and Peninsular Malaysia. In Japan, the most cultivated kumquat is the Meiwa kumquat (*F. crassifolia* Swingle), which has the best eating quality in this genus; it has also been used widely for candied fruits, jam, marmalade, and fruit wine in addition to table use.

So far, only 4 kumquat varieties have been registered under the Plant Variety Protection and Seed Act in Japan (Ministry of Agriculture, Forestry and Fisheries, http//www.hinsyu.maff.go.jp, April 26, 2009): 'Konta' (registration no. 10249), 'Yumichan-no-hoppe' (registration no. 14785), 'Yubeni' (registration no. 14416), and 'Puchimaru' (registration no. 10379).

'Yubeni' is a ploidy periclinal chimera derived from bud mutation in the Meiwa kumquat (Yasuda et al., 2008). 'Puchimaru' is an interspecific hybrid derived from a cross between an oval kumquat [*F. margarita* (Lour.) Swingle] and an autotetraploid Meiwa kumquat (Kawase et al., 2005; Yoshida et al., 2003). 'Konta' sets fruits with an extremely low acidity, 'Yumichan-no-hoppe' is an early maturing cultivar, and 'Yubeni' has large fruits with a small number of seeds caused by ploidy periclinal chimera. 'Puchimaru' is a triploid cultivar produced for the purpose of breeding seedless cultivars, but the cultivar is not completely seedless because the fruits often contain a few seeds and/or undeveloped seeds (Yoshida et al., 2003). Although seedlessness is a highly desirable trait in the breeding of kumquats, no seedless kumquat cultivar has yet been

Received; February 7, 2009. Accepted; July 24, 2009.
This research was supported by KAKENHI (No. 20580034).
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produced.

'Kiyomi' tangor [*C. unshiu* Marcow. × *C. sinensis* (L.) Osbeck] is a superior citrus cultivar with undeveloped anthers caused by cytoplasmic-genic male sterility and strong parthenocarpy which are very important traits for the production of seedless fruits (Nakano et al., 2001; Nishiura et al., 1983; Yamamoto et al., 1997). Moreover, the cultivar has seed monoembryony, which make it suitable for breeding as a seed parent. To date, a great number of high quality cultivars with male sterility and parthenocarpy for seedlessness, such as 'Tsunokaori' and 'Shiranui', have been produced from crosses with 'Kiyomi' tangor as the seed parent (Yoshida, 2003). Therefore, we proposed the production of intergeneric hybrids between 'Kiyomi' tangor as a seed parent and kumquats, as a new breeding method for seedless kumquat cultivars.

There is a high intergeneric cross compatibility between *Citrus* and *Fortunella*. Intergeneric hybrids such as limequats [*Citrus aurantifolia* (Cristm.) Swingle × *Fortunella* sp.] are documented to have arisen as chance seedlings (Swingle and Reece, 1967). Furthermore, Iwamasa et al. (1988) successfully produced hybrids between the oval kumquat and the sour orange (*C. aurantium* L.) in a study on cross compatibility among Aurantioideae. In the present study, we attempted crosses between 'Kiyomi' tangor and 6 *Fortunella* species, and performed ploidy and hybridity analyses by flow cytometry analysis, chromosome observation, random amplified polymorphic DNA (RAPD) analysis and cleaved amplified polymorphic sequence (CAPS) analysis for cytoplasmic DNA. We report here that triploid and aneuploid hybrids were obtained from diploid-diploid intergeneric crosses between 'Kiyomi' tangor and Meiwa kumquat.

Materials and Methods

Plant materials

For crossing in the present study, we used 'Kiyomi' tangor as a seed parent and the 6 *Fortunella* species established by Tanaka (1933) were used as pollen parents: the oval kumquat, round kumquat [*F. japonica* (Thunb.) Swingle], Meiwa kumquat, Malayan kumquat [*F. polyandra* (Ridl.) Tanaka], Changshou kumquat (*F. obovata* hort. ex Tanaka), and Hongkong kumquat [*F. hindsii* (Champ. ex Benth.) Swingle var. *chintou*]. The 'Kiyomi' tangor used in this study was conserved at the Kumamoto Prefectural Agricultural Research Center in Japan. Pollens were collected from the flowers of the 6 *Fortunella* species maintained in a greenhouse at Tokai University, Kumamoto, Japan.

Intergeneric crosses

The cross combinations are shown in Table 1. The pollens of the 6 *Fortunella* species were collected and stored at −40°C for one year until crossing, because the flowering period varies widely between *Citrus* (May) and *Fortunella* (July to August). Pollen fertility was evaluated by percent pollen germination on 1% agar medium containing 10% sucrose. The randomly-selected anthers were rubbed on the agar medium, and the slides were then incubated for 10 h in a moistened chamber at 25°C in the dark. Three hundred pollen grains were observed per slide, and the percent pollen germination was determined on three slides for each sample. The flowers were pollinated immediately after emasculation and covered with paraffin paper bags. Seeds were collected from the fruits of all crosses at maturity and were classified into three groups (normal, small, and undeveloped) based on their size and shape. Small and undeveloped seeds were defined as seed 1/2 to 1/6 the size of normal seeds and empty seeds, respectively. Developed seeds included both normal and small seeds. Both developed and undeveloped seeds were then cultured on Murashige and Tucker (MT) medium (Murashige and Tucker, 1969) containing 500 mg·L^{-1} malt extract, 30 g·L^{-1} sucrose, and 2 mg·L^{-1} gellan gum at 25°C under continuous illumination (38 μmol·m^{-2}·s^{-1}). After germination, the 2 seedlings obtained from the cross were transplanted to vermiculite in pots and were

Table 1. Fruit set, seed contents, and number of seedlings in the crosses between 'Kiyomi' tangor and the 6 *Fortunella*.

Cross combination		No. of flowers pollinated	No. of fruits set	% of fruits set	Av. fruit wt. (g)	No. of seeds			No. of developed seeds per fruit[w]	% of germinated seeds[v]	No. of seedlings
Seed parent	Pollen parent[z]					Normal	Small[y]	Undeveloped[x]			
'Kiyomi' tangor	Open pollination	—	10	—	371.6	70	0	24	7.0	—	—
	Oval kumquat	30	7	23.3	262.9	2	0	0	0.3	100	2
	Round kumquat	20	5	25.0	384.2	0	0	0	0	—	—
	Meiwa kumquat	20	5	25.0	370.8	2	0	7	0.4	100	2
	Malayan kumquat	22	6	27.3	358.2	76	0	11	13.0	93.4	64
	Changshou kumquat	19	3	15.8	334.7	12	0	0	4.0	83.3	8
	Hongkong kumquat	20	8	40.0	329.0	0	0	0	0	—	—

[z] The pollen germination percentages on 1% agar medium contained 10% sucrose were 12.9, 1.2, 19.0, 5.2, 13.2, and 2.0% in oval, round, Meiwa, Malayan, Changshou, and Hongkong kumquats, respectively (storage at −40°C for one year).

[y] 1/2 to 1/6 the size of normal seeds.

[x] Empty seeds.

[w] Developed seeds included both normal and small seeds.

[v] % of germinated seeds = (No. of seedlings/No. of developed seeds) × 100.

transferred to a greenhouse, where they were grafted onto trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and cultivated at the Kibana Agriculture Science Station of the University of Miyazaki, Japan.

Genome size analysis

Completely mature leaf segments of approximately 1 cm^2 were collected from each of the seedlings and their parents, and chopped with a razor blade. These samples were treated for 5 min in 1 mL buffer solution containing 1.0% (v/v) Triton X-100 (Nacalai Tesque, Inc., Kyoto, Japan), 140 mM mercaptoethanol, 50 mM Na_2SO_3 and 50 mM Tris-HCl at pH 7.5, prior to preparation following the method described by Yahata et al. (2005). Crude samples were filtered at 550 μL through Miracloth (Merck KGaA, Darmstadt, Germany) and stained with 25 $\mu g \cdot L^{-1}$ propidium iodide (PI) (Nacalai Tesque). The relative fluorescence of the total DNA was measured for each nucleus using a flow cytometry system (EPICS XL; Beckman Coulter, Inc., Fullerton, CA, USA) equipped with an argon laser (488 nm, 15 mW). To calculate the relative nuclear DNA content of the obtained seedlings, the nuclei of a haploid pummelo [*C. maxima* (Burm.) Merr.; 2n = x = 9] were used as an internal standard (Toolapong et al., 1996). A reliable value for the haploid pummelo (391 Mbp/2C) was determined by comparison with Tahiti lime (*C. aurantifolia* Swing.; 1.17 pg/2C, 2n = 3x = 27) using 30 samples (Ollitrault et al., 1994). The relative nuclear DNA content of each sample was estimated using the following formula: [(sample G_1 peak position)/(standard peak position)] × standard DNA content (pg/2C), and was measured three times for statistical analysis (Tukey's multiple range test). These data were then converted to the putative nuclear genome size at (0.978×10^9) bp to 1 pg (Dolezel et al., 2003).

Chromosome observation

Young leaves (approximately 3–5 mm long) were excised from 'Kiyomi' tangor, Meiwa kumquat and the seedlings, immersed in 2 mM 8-hydroxyquinoline for 10 h at 4°C, and fixed in a mixed solution of ethanol and acetic acid (3 : 1) for 12 h at 4°C. Enzymatic maceration and air-drying were performed following the method described by Fukui (1996) with some modifications. The young leaves were washed in distilled water to remove the fixative and then macerated in an enzyme mixture containing 2.0% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 1.0% (w/v) Macerozyme (MP Biomedicals, Inc., Irvine, CA, USA), 0.3% Pectolyase Y-23 (w/v) (Kyowa Chemical Products Co., Ltd., Osaka, Japan) and 200 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 40 min. The chromosomes were stained with 2.0% Giemsa solution (Merck) in 1/30 M phosphate buffer (pH 6.8) for 30 min. They were then rinsed with distilled water, air dried, and observed under an optical microscope. After confirming the position on the slide, the chromosomes were de-stained with 70% methanol, re-stained with 0.1 $mg \cdot L^{-1}$ propidium iodide (Nacalai Tesque) and observed under a fluorescence microscope (Olympus Co., Ltd., Tokyo, Japan) with a green (G) filter cassette.

Molecular marker analyses

Total DNA was extracted from young leaves of each plant following the method described by Doyle and Doyle (1987). The total DNA was used for RAPD and CAPS analyses.

RAPD analysis of nuclear DNA was performed by a modified version of the methods described by Williams et al. (1990). Polymerase chain reaction (PCR) was carried out with Operon random 10-mer primers (Operon Technologies Inc., Alameda, CA, USA) using the Astec Program Control System PC-700 (Astec Co., Ltd., Fukuoka, Japan). The reaction products were electrophoresed on 1.5% agarose gels containing 25 $\mu l \cdot L^{-1}$ SYBR Safe™ (Life Technologies Japan Ltd., Tokyo, Japan) and subsequently photographed under ultraviolet light (360 nm). For each combination of sample and primers, PCR was carried out twice, and only stable polymorphisms were analyzed.

CAPS analysis was performed for several chloroplastic (cp) and mitochondrial (mt) non-coding regions. For cpDNA analysis, three primer pairs of *trnK-3914F-trnK-2R*, *rbcL-psaI*, and *trnD-trnT* were used for amplification following the methods of Cheng et al. (2002) and Ureshino and Miyajima (2002). For mtDNA analysis, five primer pairs of *18SrRNA-5SrRNA*, *nad4exon1-nad4exon2*, *nad5/1-nad5/2r*, *nad7/1-nad7/2r*, and *nad1/4-nad1/5r* were used for amplification following the methods of Cheng et al. (2002) and Dumolin-Lapegue et al. (1997). The PCR products were digested with several restriction endonucleases and then electrophoresed under the same protocol as that used in our RAPD analysis.

Results and Discussion

We made crosses between 'Kiyomi' tangor and the 6 *Fortunella* species (Table 1). All pollens stored at –40°C for one year had maintained sufficient fertility for crossing (Data shown in the footnote for Table 1). Of the 6 cross combinations, seeds were successfully obtained from the crosses with the oval, Meiwa, Malayan and Changshou kumquats.

The numbers of seeds obtained from the crosses with the Malayan kumquat and the Changshou kumquat were larger than those obtained from the other cross combinations. The Malayan and Chanshou kumquats are presumed to be intergeneric hybrids of *Citrus* with *Fortunella* (Swingle and Reece, 1967). The relatively large number of seeds obtained from the crosses with these species in the present study might be explained by this assumption.

No seeds were obtained from the crosses with the

round or Hongkong kumquats. Both of these kumquats showed lower pollen germination percentages (1.2% for the round kumquat, 2.0% for the Hongkong kumquat) on 1% agar medium containing 10% sucrose than the other species, which may have caused the failure to hybridize. In addition, the Hongkong kumquat belongs to *Protocitrus*, which does not include the other 5 species (Swingle and Reece, 1967; Tanaka, 1933); this might have caused stronger reproductive isolation with *Citrus*.

When the Meiwa kumquat was used as a pollen parent, 2 normal seeds and 7 undeveloped seeds were obtained. All of these seeds were cultured on MT medium. Only the 2 normal seeds germinated normally, but the seedlings (identified as H15-701 and H15-702) then showed poor growth *in vitro* (Fig. 1A, 1B). However, they recovered and grew vigorously during the early stage after being transplanted to soil (Fig. 1C). At the same time, 2, 64, and 8 seedlings were obtained from the crosses with the oval, Malayan, and Changshou kumquats, respectively. Ploidy analyses and confirmation of hybridism are presently being carried out on these seedlings (data not shown). In the present study, we focused on H15-701 and H15-702 obtained from the cross between 'Kiyomi' tangor and the Meiwa kumquat.

H15-701 and H15-702 were analyzed for ploidy levels by flow cytometry analysis. In this analysis, both parents showed diploidy peaks, while both seedlings showed unexpected triploidy peaks (Fig. 2). Furthermore, the putative genome sizes of each sample were also obtained from the relative nuclear DNA content using flow cytometry. The putative genome sizes of H15-701, H15-702, and their parents were investigated using the haploid pummelo as an internal standard (Fig. 3). The putative genome sizes of 'Kiyomi' tangor and the Meiwa kumquat were 781 Mbp/2C and 774 Mbp/2C, respectively, while those of H15-701 and H15-702 were 1234 Mbp/2C and 1164 Mbp/2C, respectively. Interestingly, the significant difference in genome size corresponded to more than one chromosome represented in the 2 seedlings, suggesting the possibility of an aneuploid.

The chromosome count of young leaves in 'Kiyomi' tangor and the Meiwa kumquat used as cross parents confirmed eudiploidy ($2n = 2x = 18$; Fig. 4A, 4B). Chromosome observation also revealed that H15-701 and H15-702 were an aneutriploid ($2n = 28$) and a eutriploid ($2n=3x=27$), respectively (Fig. 4C, 4D). Flow cytometry analysis has been used to screen for aneuploidy in several plant species. The addition of a larger number of chromosomes can be determined based on differences in peak positions (Kopecky and Vagera,

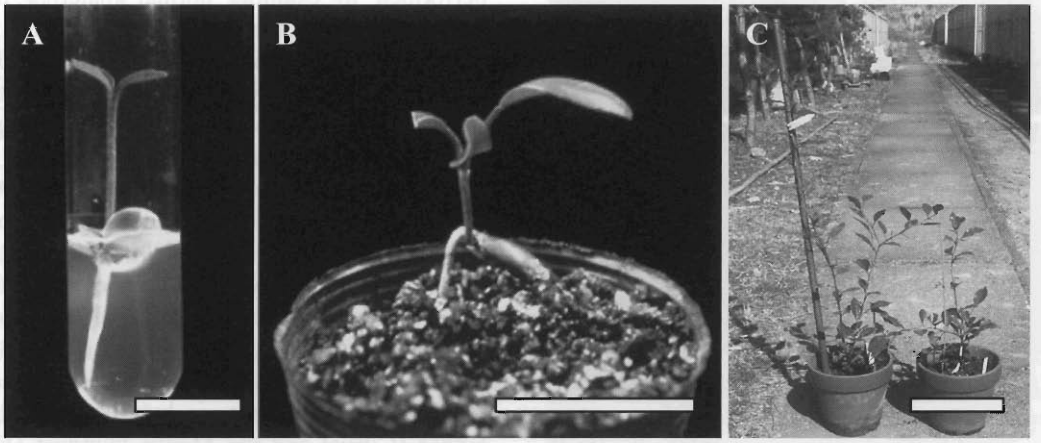

Fig. 1. Photographs of the 2 seedlings obtained from a cross between 'Kiyomi' tangor and the Meiwa kumquat. A: a seedling germinated *in vitro* (Bar = 1.5 cm), B: a seedling soon after acclimatization (Bar = 3.0 cm), C: growth and development of the seedlings during the early stage (Bar = 30 cm).

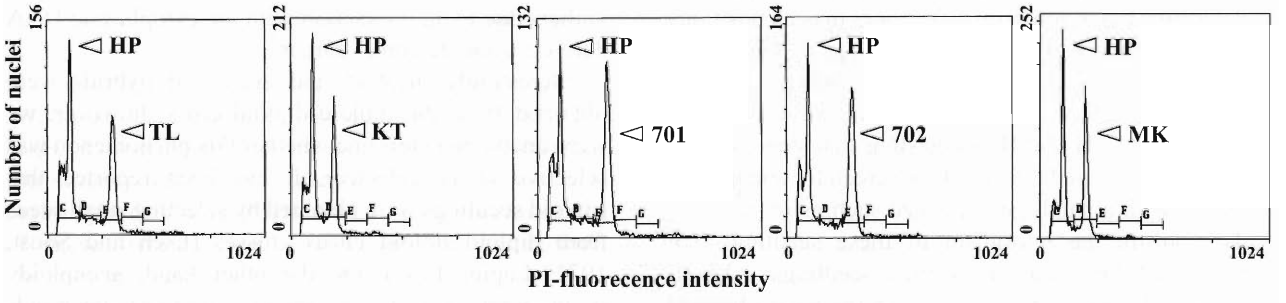

Fig. 2. Histograms of the flow cytometry analysis of mature leaves of 'Kiyomi' tangor, the Meiwa kumquat, and the 2 seedlings obtained from the cross. The haploid pummelo was used as an internal standard. HP: haploid pummelo, TL: Tahiti lime, KT: 'Kiyomi' tangor, 701: H15-701, 702: H15-702, MK: Meiwa kumquat.

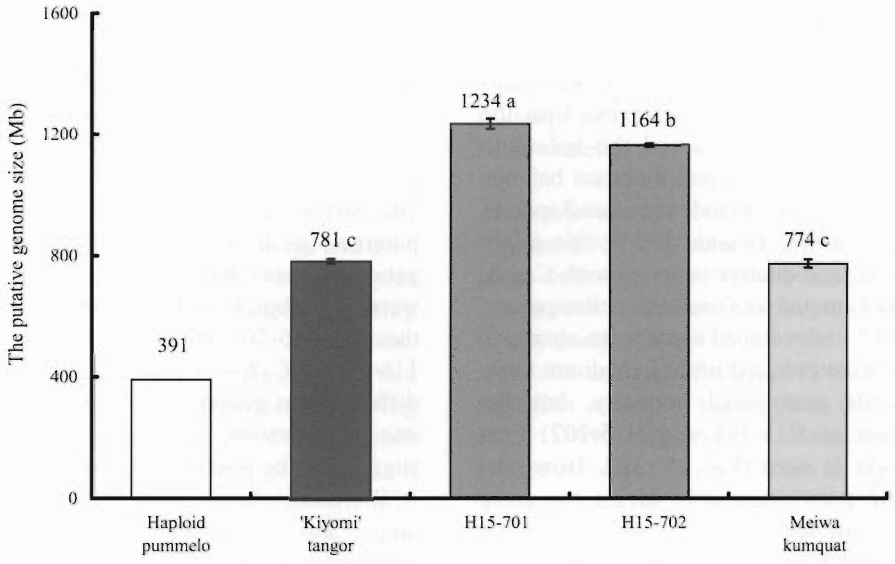

Fig. 3. The putative genome size of 'Kiyomi' tangor, the Meiwa kumquat, and the 2 seedlings, H15-701 and H15-702, obtained from the cross determined by comparison with the haploid pummelo as an internal standard. Different letters represent significant differences in Tukey's multiple range test, 1% level (n = 5). The nuclear genome size of the haploid pummelo was determined by comparison with the Tahiti lime (n = 30).

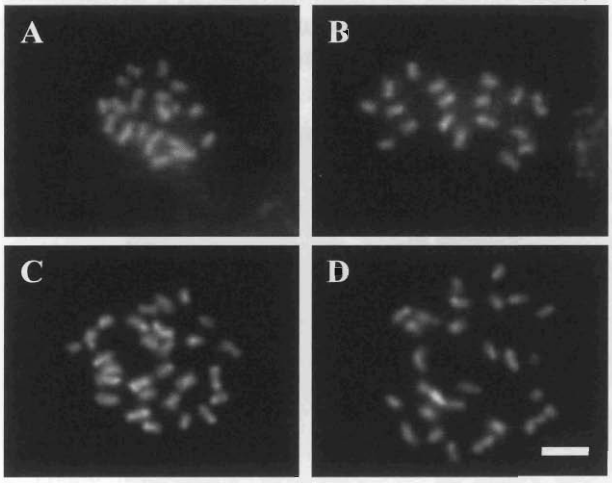

Fig. 4. Somatic chromosomes at metaphase in young leaves of 'Kiyomi' tangor, the Meiwa kumquat, and the 2 seedlings obtained from the cross. A: 'Kiyomi' tangor (2n = 2x = 18), B: Meiwa kumquat (2n = 2x = 18), C: H15-701 (2n = 28), D: H15-702 (2n = 3x = 27). The bar in D represents 5.0 μm for all figures.

2005; Nakamura et al., 2005). However, it is impossible to determine based solely on differences in peak positions exactly whether a given species has one or two added chromosomes. The results of the genome size analysis in the present study clearly revealed the addition of one chromosome in H15-701, suggesting that genome size analysis may be used effectively to screen for aneuploidy in plants with a small genome size such as citrus.

To confirm the hybridism of these seedlings, we carried out RAPD analysis on the 2 seedlings and both parents. Polymorphism banding patterns were obtained in 8 of 20 random primer pairs used for the experiment. As shown in Figure 5, both seedlings yielded bands specific to both parents, confirming the hybridism of the seedlings.

CAPS analysis for cytoplasmic DNA regions was then performed to examine normal maternal inheritance. While every primer pair amplified the bands satisfactorily, none revealed any polymorphism on the agarose gels. When the PCR products were digested with 4 restriction endonucleases, cpDNA polymorphism was observed in 2 primer/enzyme combinations: *rbcL-psaI*/*Hinf*I and *trnD-trnT*/*Mbo*I (Fig. 6A). MtDNA polymorphism was observed in 3 primer/enzyme combinations: *nad7/1-nad7/2r*/*Alu*I, *18SrRNA-5SrRNA*/*Hae*III, and *Mbo*I (Fig. 6B). These seedlings showed uniform bands that were identical to those of the seed parents indicating that their cytoplasmic DNA was of maternal origin.

Thus, RAPD and CAPS analyses confirmed that the seedlings were intergeneric hybrids between 'Kiyomi' tangor (*Citrus*) and the Meiwa kumquat (*Fortunella*). The undeveloped anthers of 'Kiyomi' tangor were caused by cytoplasmic-genic male sterility (Yamamoto et al., 1997). If the nuclear genes that regulate male sterility are successfully inherited, the hybrids show undeveloped anthers due to an interaction with the cytoplasmic DNA derived from 'Kiyomi' tangor.

Interestingly, triploid and aneuploid hybrids were obtained from the diploid-diploid cross; however, we were unable to determine whether this phenomenon was selective or nonselective. It has been reported that triploid seedlings were obtained by selecting small seeds from diploid-diploid citrus crosses (Esen and Soost, 1971; Lapin, 1937). On the other hand, aneuploidy occurs with a very low frequency, and most aneuploids never develop to adulthood (Krug and Bacchi, 1943; Sharma and Bal, 1957; Wakana et al., 1981).

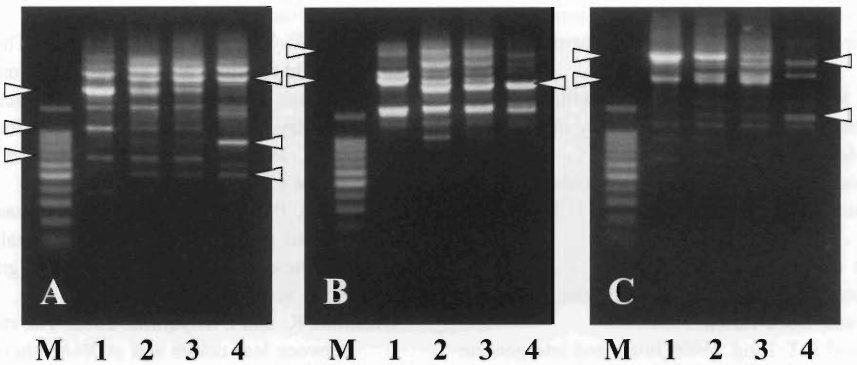

Fig. 5. RAPD analysis of the seedlings obtained from the cross between 'Kiyomi' tangor and the Meiwa kumquat. A: OPA-9 primer, B: OPA-12 primer, C: OPA-19 primer. Arrowheads indicate the bands specific to each parent. M: 100-bp ladder marker, 1: 'Kiyomi' tangor, 2: H15-701, 3: H15-702, 4: Meiwa kumquat.

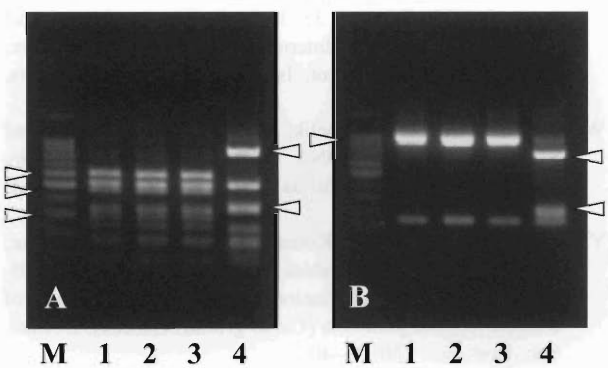

Fig. 6. A: restriction pattern of the *Hinf*I-digested *PSAI-rbcL* regions of chloroplast genomes. B: restriction pattern of the *Alu*I-digested *nad7/1-nad2r* regions of mitochondrial genomes. Arrowheads indicate the bands specific to each parent. M: 100-bp ladder marker, 1: 'Kiyomi' tangor, 2: H15-701, 3: H15-702, 4: Meiwa kumquat.

Aneutriploidy and eutriploidy would also induce the depression of pollen fertility due to meiotic abnormality.

In fact, a large number of fruits including completely seedless fruits set on the present hybrids (Fig. 7), suggesting that they might have extremely low or no pollen fertility and parthenocarpy. The morphological characteristics, male fertility, and fruits traits including sugar and organic acid contents in these fruits are under investigation.

In conclusion, the present cross between 'Kiyomi' tangor and the Meiwa kumquat produced an aneuploid intergeneric hybrid seedling and a eutriploid intergeneric hybrid seedling. These hybrids have the potential for use as cross-parents in seedless kumquat breeding. To determine the genomic constitutions of these hybrids, further investigation is necessary, including Chromomycin A3 (CMA) karyotype analysis, genomic *in situ* hybridization (GISH) of the somatic chromosomes of these hybrids, and observation of the meiotic chromosome behavior of both parents.

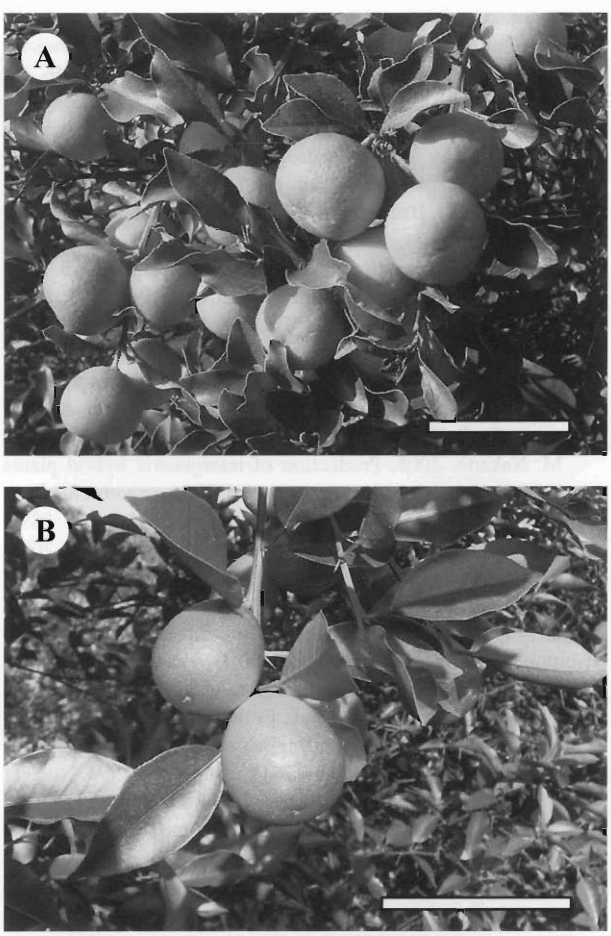

Fig. 7. Photographs of the fruits of the intergeneric hybrids between 'Kiyomi' tangor and the Meiwa kumquat. A: H15-701, B: H15-702. (Bars = 5.0 cm)

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