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| メタデータ | 言語: English<br>出版者:<br>公開日: 2020-01-09<br>キーワード (Ja):<br>キーワード (En): Chromomycin A3(CMA), Chromosome, Citrus, Flow cytometry, Polyploidy breeding<br>作成者: Nukaya, Tsunaki, Sudo, Miki, Yahata, Masaki, Nakajo, Yoshiyuki, Ohta, Tomohiro, Yasuda, Kiichi, Tominaga, Akiyoshi, Mukai, Hiroo, Kunitake, Hisato<br>メールアドレス:<br>所属: |
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| URL | http://hdl.handle.net/10297/00027010 |

**Characteristics in Autotetraploid Kumquats (*Fortunella* spp.) Induced by Colchicine Treatment to Nucellar Embryos and Their Utilization for Triploid Breeding**

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Received; 28 August, 2018. Accepted; 8 October, 2018.

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**ABSTRACT**

Three *Fortunella* (kumquat) species, the Meiwa kumquat (*F. crassifolia* Swingle), the Round kumquat [*F. japonica* (Thunb.) Swingle] and the Changshou kumquat (*F. obovata* hort. ex Tanaka), showing tetraploid, which had been induced by treating the seeds with colchicine, were examined for origin and horticultural characteristics (e. g. morphology of leaves, flowers, pollen and fruits). Additionally, these tetraploid kumquats were crossed to produce triploid kumquats. All of the tetraploids were confirmed to be derived from the nucellar embryo of each original kumquat by examining their chromosomal composition with chromomycin $A_3$ staining. All of the tetraploid kumquats had the typical morphological characteristics of tetraploid *Citrus* plants, such as round and thick leaves, and large flowers and pollen grains. On the other hand, the fruits of these tetraploid plants showed desirable traits for kumquats such as thicker pericarp and higher soluble solids content. Furthermore, when these tetraploids were crossed with some diploid cultivars, triploid progenies were obtained from almost all of the cross combinations.

**Keywords:** chromomycin $A_3$ (CMA), chromosome, citrus, flow cytometry, polyploidy breeding

## 1. Introduction

The genus *Fortunella* (kumquat), a close relative of *Citrus* and *Poncirus* in the subfamily Aurantioideae (Citroideae) of the family Rutaceae (Swingle and Reece, 1967), consists of a few species and is primarily grown Japan, China and peninsular Malaysia. Kumquat fruit is used to produce preserves, candies, syrups, oriental medicines, as well as for fresh consumption because of its special flavor and thick pericarp. However, kumquats have seedy fruits, so the production of new seedless cultivars is desired.

The major method for producing seedless cultivars is the use of triploids (Soost and Cameron, 1980; 1985; Recupero, 2005). Generally, triploids have been efficiently obtained by reciprocally

crossing tetraploid and diploid plants (Esen and Soost, 1972; Kaneyoshi et al., 1997; Oiyama et al., 1991; Viloria and Grosser, 2005), and so tetraploids are vital to breeding triploids. In the genus *Citrus*, tetraploid plants have been produced by various techniques such as selection of nucellar seedlings (Frost, 1925), treating apical meristems, nucellar embryos and calluses with antimitotic agents (Gmitter and Ling, 1991; Oiyama and Okudai, 1986; Wu and Mooney, 2002; Zeng et al., 2006; Zhang et al., 2007). To obtain valuable information for the planned breeding, furthermore, the growth and morphological characteristics, fruit quality and reproductive potential of these tetraploids of *Citrus* were examined, and it was reported that they have rounder leaves and flowers, low pollen fertility and reduction of fruit quality compared to diploid plants (Lee, 1988; Tan et al., 2015; 2017).

Recently, triploid kumquat cultivars, 'Puchimaru' {diploid Oval kumquat [*F. margarita* (Lour.) Swingle] x tetraploid Meiwa kumquat} and 'Miyazaki-Yumemaru' (tetraploid Meiwa kumquat x diploid Meiwa kumquat), have been registered in Japan. These cultivars had desirable traits such as a small number of seeds and/or seedlessness, and high sugar content, so the usefulness of triploid breeding of kumquats is accepted. However, the production of tetraploid kumquat has been far behind that of *Citrus* species, and only two species, Hongkong kumquat [*F. hindsii* (Champ. ex Benth.) Swingle] and Meiwa kumquat has been produced (Longley, 1925; Kawase et al., 2005). Therefore, the basal information of the horticultural characteristics and the reproductive potential in tetraploid kumquats are poor (Nukaya et al., 2009). To breed new cultivars with unique characteristics such as seedless and attractive flavor, other tetraploid kumquat, production of tetraploid in other *Fortunella* species and their information of the horticultural characteristics and the reproductive potential are necessary.

Our research group have produced a lot of tetraploid plants using colchicine-treated seeds of polyembryonic kumquat species and/or cultivars (Yahata et al., 2004). When these tetraploids were grafted onto trifoliate oranges [*Poncirus trifoliata* (L.) Raf.], they showed vigorous growth.

3 - 5 years after top-graft onto trifoliate oranges, three species of tetraploid kumquats (the Meiwa kumquat, the Round kumquat and the Changshou kumquat) had many flowers and fruits.

To obtain the basal information of the tetraploid plant in the genus *Fortunella*, in the present study, we examined the origin and the morphological characteristics in three tetraploid kumquats that had been induced using colchicine-treated seeds of diploid polyembryonic kumquats. To produce the triploid progenies, furthermore, we performed reciprocal crosses between the tetraploid and diploid kumquats.

## 2. Materials and Methods

### *2.1. Plant materials*

Three tetraploid kumquats (the Meiwa kumquat, the Round kumquat and the Changshou kumquat that were induced by treating the seeds with colchicine) were used in the present study. Each of the original diploid kumquats were used as the control. These plant materials were grafted onto trifoliate oranges, and maintained for approximately 10 years in the greenhouse of the Faculty of Agriculture, Shizuoka University, Japan.

### *2.2. Confirmation of origin of tetraploid kumquats by CMA staining*

Young leaves (approximately 3-5 mm long) were excised from each plant, immersed in 2mM 8-hydroxyquinoline for 12 h at 10°C and fixed in a mixed solution of ethanol and acetic acid (3:1) for 12 h at 10°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 at 37°C for 40 min 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., Burlingame, CA, USA), 0.3% Pectolyase Y-23 (w/v) (Kyowa Chemical Products Co., Ltd., Osaka, Japan) and 200mM

ethylenediaminetetraacetic acid (EDTA). The chromosomes were stained with 2.0% Giemsa solution (Merck KGaA, Darmstadt, Germany) in a 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 BX51 (Olympus Co., Ltd., Tokyo, Japan).

After the chromosome numbers and each position were confirmed on a slide, the chromosomes were destained with both 70% and 100% ethanol. They were then stained with 0.1 mg $L^{-1}$ CMA and 0.1 mg $L^{-1}$ Distamycin A hydrochloride (Sigma-Aldrich Co., Steinheim, Germany) following the method described by Befu et al. (2000) with some modifications, and observed under a fluorescence microscope BX51 (Olympus Co., Ltd.). The Chromosomes were classified into the following seven types based on the number and position of CMA-positive bands according to Yamamoto et al. (2008) and Yamamoto and Tominaga (2003): type A: two telomeric bands and one proximal band, type B: one telomeric and one proximal band, type C: two telomeric bands, type D: one telomeric band, type E: no band, type F: one proximal band, type Dst: type D with a satellite chromosome.

### *2.3. Morphological characteristics of tetraploid kumquats*

The morphological characteristics of fully expanded leaves (e.g., leaf blade size, leaf weight per unit, guard cell size and guard cell density) and flowers just before bloom (e.g., sizes of flower bud, petal, pistil, ovary and pollen, and number of petal and stamens) were measured using 20 samples. Guard cells and pollen grains were observed using a scanning electron microscope (Miniscope® TM3030Plus, Hitachi High-Technologies, Tokyo, Japan). **The fruit weight**, size, pericarp weight, the number of locules and seeds, soluble solids content (SSC) and titratable acidity (TA) of each kind of fruit were measured used 20 samples, respectively.

Pollen fertility was evaluated by stainability and *in vitro* germination. Pollen stainability was estimated by staining the samples with 1% acetocarmine after squashing nearly mature anthers on

a glass slide. *In vitro* germination of the pollen grains was performed on microscope slides covered with a 2-mm layer of 1% (w/v) agar medium containing 10% sucrose. Five stamens, each from different flowers, 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. Each test was evaluated from 1,000 grains with five repetitions.

### *2.4. Crossing with diploid kumquats for production of triploid progenies*

Three tetraploid kumquats (the Meiwa kumquat, the Round kumquat and the Changshou kumquat), diploid Meiwa kumquat and monoembryonic Oval kumquat were used. The cross combinations are shown in Table 5.

The flowers were pollinated immediately after emasculation and covered with paraffin paper bags. Seeds were collected from each mature fruit of all the crosses and were classified into two groups, i.e., developed (normal growth embryo) and undeveloped (poor growth embryo) ones (Fig. 1). After being numbered and weighed, both developed and undeveloped seeds were cultured on Murashige and Skoog (MS) medium (1962) containing 500 mg $L^{-1}$ malt extract, 30 g $L^{-1}$ sucrose and 2 g $L^{-1}$ gellan gum at 25°C under continuous illumination (38 μmol $m^{-2}$ $s^{-1}$). After germination, the seedlings were transplanted into vermiculite in pots and were transferred to a greenhouse. Ploidy analysis of the seedlings was performed by flow cytometry (FCM, EPICS XL; Beckman Coulter, Inc., CA, USA) using young leaves and chromosome observation using root tips, according to the methods of Yahata et al. (2005a) and Yahata et al. (2015) with some modifications, respectively.

## 3. Results

### *3.1. Confirmation of origin of tetraploid kumquats by CMA staining*

The original diploid kumquats' chromosomal compositions (2n=2x=18) were analyzed by

CMA staining. The CMA banding patterns were 2A+2C+12D+1F+1Dst in the Meiwa kumquat (Fig. 2A), 2A+2C+12D+2Dst in the Round kumquat (Fig. 2C) and 1A+1B+2C+10D+3E+1F in the Changshou kumquat (Fig. 2E). On the other hand, each tetraploid kumquat (2n=4x=36) had twice the number of chromosomes of the original diploids and a chromosome composition of 4A+4C+24D+2F+2Dst in the tetraploid Meiwa kumquat (Fig. 2B), 4A+4C+24D+4Dst in the tetraploid Round kumquat (Fig. 2D) and 2A+2B+4C+20D+6E+2F in the tetraploid Changshou kumquat (Fig. 2F).

### *3.2. Morphological characteristics of tetraploid kumquats*

The morphological characteristics of tetraploid kumquats were compared with those of the diploid kumquats. All tetraploid kumquats had significantly rounder leaves as compared to those of the diploids (Table 1, Fig. 3A). Furthermore, the leaf weight per unit area of the tetraploids was significantly heavier than that of diploids, and the tetraploids had thicker leaves compared with the diploid kumquats. Although the guard cell size of all tetraploid kumquats was significantly larger than that of diploids, the number of guard cells per unit area in the tetraploids was less than that of the diploids (Fig. 4).

The flower organs of the tetraploids showed normal morphology. The flower buds and ovaries of the tetraploids were significantly larger than those of the diploids (Table 2, Fig. 3B). On the other hand, the tetraploids had a significantly reduced number of stamens compared with that of the diploids.

The average size of the pollen grains from the tetraploids was larger than that of the grains from diploids (Table 3, Fig. 5). The fertility of the pollen grains was evaluated by stainability with acetocarmine and *in vitro* germination. Pollen fertility in the tetraploids of the Meiwa kumquat and the Changshou kumquat was significantly lower than that of their diploids, whereas the tetraploid Round kumquat showed significantly higher pollen fertility compared with its

diploid.

The tetraploid had significantly larger fruits than the diploid in the Meiwa kumquat, whereas there was no significant difference between the tetraploids and the diploids in the fruit weight and fruit size of the Round kumquat and the Changshou kumquat (Table 4, Fig. 3C). The percentage of pericarp weight per fruit in all tetraploid kumquats increased significantly in comparison with that of diploids. In the Meiwa kumquat and the Changshou kumquat, the tetraploids had a reduced number of seeds per fruit compared with that of the diploids. SSC in the pericarp of the tetraploids was significantly higher than that of the diploids in all of the species.

### *3.3. Crossing with diploid kumquats for production of triploid progenies*

To produce the triploid progenies, crossing with diploid kumquats was carried out (Table 5). The frequency of developed seeds in the monoembryonic Oval kumquat was **9.9, 11.9 and 0%** when crossed with pollen from the tetraploid Meiwa kumquat, the Round kumquat and the Changshou kumquat, respectively, whereas that in open-pollinated fruit was 86.5%. When crosses between the diploid polyembryonic Meiwa kumquat and three tetraploid kumquats were carried out, the frequency of developed seeds (15.7-46.6%) was also lower than that of open-pollinated fruit (85.9%). Thus, a lot of undeveloped seeds were obtained when pollinated with tetraploids as the pollen parent. In the crosses with tetraploid kumquats as the seed parent, on the other hand, the frequency of developed seeds in each of the cross combinations was similar to that of open pollination. However, the crosses between tetraploid kumquats and diploid Meiwa kumquat produced all developed seeds with an average weight approximately 1/3 of that of the seeds obtained from each open pollination. Especially, their weight (38.0 mg) was approximately 1/6 of that of the seeds obtained from open pollination (227.3 mg) in the tetraploid Changshou kumquat (Table 5).

Although no seedlings were obtained from undeveloped seeds cultured on MS medium,

developed seeds germinated almost normally (Table 5). The ploidy level of these seedlings was confirmed by flow cytometry analysis and chromosome observation (Fig. 6). Consequently, when the Oval kumquat was used as the seed parent, the triploid seedlings (2n=3x=27) obtained from the crosses with the tetraploids of the Meiwa kumquat and the Round kumquat were five and one, respectively. In the crosses between the diploid Meiwa kumquat and the tetraploid kumquats, most of the seedlings were diploids, but three, six and one triploids were obtained from crossing with the tetraploids of the Meiwa kumquat, the Round kumquat and the Changshou kumquat, respectively. Moreover, one seedling obtained from a cross between the diploid Meiwa kumquat and the tetraploid Changshou kumquat showed a tetraploid DNA value. In the reverse crosses, on the other hand, triploid seedlings were also obtained from all cross combinations, i.e., the triploids were five in the tetraploid Meiwa kumquat, nine in the tetraploid Round kumquat and one in the tetraploid Changshou kumquat, respectively.

**4. Discussion**

The gametic seedlings may have been mixed with the nucellar seedlings because three tetraploid kumquats used in the present study were induced using colchicine-treated seeds. Therefore, we need to confirm their origins. In previous studies, isozyme, RAPD and microsatellite analysis were used for the identification of the origin of *Citrus* and its related genera (Germana et al., 1994; Germana and Chiancone, 2001; Rao et al., 2008; Tan et al., 2007). Recently, a combination of enzymatic maceration and fluorescent staining such as CMA and/or 4'-6-diamidino-2-phenyl-indole (DAPI) has been used for identifying *Citrus* and its related genera chromosomes and has been applied in biotechnological studies such as genome analysis, somatic hybridization, and ploidy manipulation (Guerra, 1993; Miranda et al., 1997; Yahata et al., 2005b; Yamamoto and Tominaga, 2004; Yasuda et al., 2016). By CMA staining the chromosomes, Yamamoto and Tominaga (2004) and Yahata et al. (2005b) easily demonstrated that diploid cells

arose from a haploid plant obtained from a Clementine (*C. clementina* hort. ex Tanaka) and a 'Banpeiyu' pummelo (*C. maxima* (Burm.) Merr.) were doubled haploid cells. In the present study, each of the three tetraploid kumquats had twice the number of chromosome composition with CMA staining compared with that of the Meiwa kumquat, the Round kumquat and the Changshou kumquat, respectively. This result showed that these tetraploid plants were confirmed to be derived from the nucellar embryo of each of the original diploids, and to be autotetraploids.

Generally, tetraploid plants increase each of their organs compared with those of diploid plants. As reported in genera *Citrus* (Lee, 1988), tetraploids showed round and thick leaves, large flowers and pollen grains, low pollen fertility, and poor fruit characteristics such as rough rind, thick pericarp and high acid as compared with diploids. The three tetraploid kumquats examined in the present study showed the typical morphological characteristics of tetraploid *Citrus* plants. But they had desirable traits for a kumquat with an edible pericarp such as a thicker pericarp with higher SSC, as compared with each original diploid kumquats. In the future, we need to carry out researches which not only utilizes triploid breeding but also investigates the commercial growing of these tetraploid kumquats.

Unlike the tetraploid Meiwa kumquat and the tetraploid Changshou kumquat, the tetraploid Round kumquat were shown to have significantly increased pollen fertility and number of seeds per fruit in comparison with those of the diploids, and were observed to have restoration of fertility. It was reported in many plant species that the plants of low fertility produced by the interspecific crosses recovered fertility by doubling their chromosomes (Ishizuka and Uematsu, 1994; Nimura et al., 2006; Yabuya, 1985). This is because a chromosome pairing was normally performed by carrying out chromosome doubling. It is supposed that the Round kumquat came across the sea from China before the 17th century (Iwamasa, 1976). However, the Round kumquat introduced into Japan shows polyembryony, while the Round kumquat grown wild in China shows monoembryony and differs in its morphological characteristics as compared with

that of the Round kumquat in Japan (Yin-Min, 1985). It is hypothesized that the cause of the low fertility in the Round kumquat of our country could based on an inbreeding depression by selfing or interspecific hybrid, and its hybrid was introduced into our country. Probably, the fertility restoration of the tetraploid Round kumquat was the result of the meiosis being performed normally because of the by doubling of its chromosomes. To account for these factors, detailed cytological observation, especially at meiosis, remain necessary in future research.

When the crossing with the tetraploid plants was carried out, the number of developed seeds and the size of the seeds varied from whether tetraploids were used as a seed parent or a pollen parent in genera *Citrus* (Esen and Soost, 1973; Esen et al., 1978; Oiyama et al., 1991; Viloria and Grosser, 2005). In the crosses between the diploids and the tetraploids, undeveloped seeds, early-degeneration of the embryos, occur frequently, and it is difficult to get developed seeds. Additionally, a lot of tetraploid progenies that resulted from the fertilization between diploid unreduced female gametes and diploid male gametes appeared from developed seeds. Conversely, the seeds obtained from the cross between the tetraploid and the diploid were well developed although their developed seeds were smaller than those of the cross among the tetraploid. These phenomenon are caused by an unbalance of ploidy ratio between the embryo and the endosperm (Esen and Soost, 1973; Johnston et al., 1980). In the present study, the results of the reciprocal crosses between the diploid kumquats and the tetraploid kumquats were similar to those of the genus *Citrus*. In genus *Fortunella*, it was also presumed that the number of developed seeds and the size of the seeds obtained from the polyploid crosses were determined by the ploidy ratio between the embryo and the endosperm. In the crosses between diploids and tetraploids, moreover, the frequency of developed seeds of the monoembryonic Oval kumquat and the polyembryonic Meiwa kumquat was different. This result is assumed to be due to the influence of nucellar embryony.

In the present study, a tetraploid seedlings was obtained in the cross between the

polyembryonic diploid Meiwa kumquat and the tetraploid Changshou kumquat. There are mainly two causes in occurrence of this tetraploid, i.e., spontaneous chromosome doubling of nucellar tissue and fertilization with unreduced gametes (Kaneyoshi et al., 1997; Viloria and Grosser, 2005). In the crosses between polyembyonic diploids and tetraploids of the genus *Citrus*, Kaneyoshi et al. (1997) reported that tetraploid plants obtained from the crosses between diploid Satsuma mandarin cultivars and tetraploid Ponkan mandarin (*C. reticulata* Blanco) were hybrids or spontaneous autotetraploids. It is also necessary to clarify the origin of this tetraploid obtained from the cross between the diploid Meiwa kumquat and the tetraploid Changshou kumquat. Furthermore, although many triploid seedlings were obtained from reciprocal crosses between diploid and tetraploid kumquats, we also plan to investigate these hybridity, the horticultural characteristics and the reproductive potential in a future study.

In conclusion, three autotetraploids of the Meiwa kumquat, the Round kumquat and the Changshou kumquat used in the present study had the typical morphological characteristics of tetraploid *Citrus* plants, such as large leaves, flowers and pollen grains with low pollen fertility. However, they had desirable traits for kumquats such as thick pericarp with high SSC, as compared with each original diploid kumquat. Furthermore, our results indicate that crossing the diploid kumquats with these autotetraploid kumquats can be useful as a parent for triploid breeding. In the future, we plan to carry out of research as which not only utilizes triploid breeding but also investigates the commercial growing of these tetraploid kumquats.

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**Legends for Figures**

Fig. 1. Comparision of developed seeds (left) and undeveloped ones (right) obtained from the reciprocal crosses between diploid and tetraploid kumquats. Bar = 1 cm.

430 Fig. 2. Photographs of the chromosomes with chromomycin $A_3$ (CMA) banding patterns of the diploid and the tetraploid in the Meiwa kumquat, the Round kumquat and the Changshou kumquat. Bars = 10 μm. A: Meiwa kumquat. B: Tetraploid Meiwa kumquat. C: Round kumquat. D: Tetraoloid Round kumquat. E: Changshou kumquat. F: Tetraploid Changshou kumquat.

435 Fig. 3. Comparison of the morphological characteristics of leaves (A), flowers (B) and fruits (C) in the diploid (left) and the tetraploid (right) of the Changshou kumquat (Bars = 3 cm). (The Changshou kumquat was represented on behalf of the three kumquats.)

Fig. 4. Scanning electron micrographs of guard cells in the diploid (A) and the tetraploid (B)
440 of the Changshou kumquat. Bars = 30 μm. (The Changshou kumquat was represented on behalf of the three kumquats.)

Fig. 5. Scanning electron micrographs of pollen grains in the diploid (A) and the tetraploid (B) of the Changshou kumquat. Bars = 30 μm. (The Changshou kumquat was represented on behalf
445 of the three kumquats.)

Fig. 6. Flow cytometric analysis and chromosome observation of the triploid seedling obtained from interploid crosses of the Meiwa kumquat. Bar = 10 μm. A: Flow cytometric analysis. B: the metaphase chromosomes in a root tip cell in one of the seedlings (2n = 3x = 27, Bar = 10 μm). [z]
450 the Meiwa kumquat was used as a control.

Table 1. Comparison of leaf characteristics in the diploid and the autotetraploid of the Meiwa kumquat, the Round kumquat and the Changshou kumquat.

| | Ploidy level | Leaf blade (mm) | | Shape index of leaf shape[y] | Leaf weight (mg / cm$^2$) | Guard cell (μm) | | Guard cell density (No. / mm$^2$) |
|---|---|---|---|---|---|---|---|---|
| | | Length | Width | | | Length | Width | |
| Meiwa kumquat | 2x | 84.8 | 31.5 | 2.7 | 25.2 | 24.6 | 21.1 | 415.4 |
| | 4x | 86.4 | 41.1 | 2.1 | 34.5 | 32.1 | 26.0 | 249.8 |
| | | NS[z] | * | * | * | * | * | * |
| Round kumquat | 2x | 63.1 | 24.3 | 2.6 | 22.2 | 21.2 | 18.9 | 460.4 |
| | 4x | 73.1 | 31.3 | 2.3 | 34.2 | 27.3 | 25.0 | 272.7 |
| | | * | * | * | * | * | * | * |
| Changshou kumquat | 2x | 93.7 | 49.8 | 1.9 | 21.9 | 20.2 | 18.8 | 537.8 |
| | 4x | 95.6 | 65.1 | 1.5 | 28.7 | 27.3 | 24.7 | 260.5 |
| | | NS | * | * | * | * | * | * |

[z] NS: No significantly different, *: mean significantly different at 1% levels by t-test.

[y] Length of leaf blade / Width of Leaf blade.

Table 2. Comparison of flower characteristics in the diploid and the autotetraploid of the Meiwa kumquat, the Round kumquat and the Changshou kumquat.

| | Ploidy level | Flower bud (mm) | | No. of petal | Petal (mm) | | Length of pistil (mm) | Ovary (mm) | | No. of stamen |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Length | Width | | Length | Width | | Diameter | Height | |
| Meiwa kumquat | 2x | 10.1 | 5.8 | 5.0 | 9.3 | 3.7 | 5.4 | 2.2 | 1.8 | 18.0 |
| | 4x | 9.2 | 7.4 | 5.0 | 9.2 | 5.7 | 5.3 | 2.8 | 2.3 | 16.6 |
| | | **[z] | ** | NS | NS | ** | NS | ** | ** | ** |
| Round kumquat | 2x | 9.0 | 4.4 | 5.0 | 7.8 | 3.1 | 4.1 | 1.4 | 1.4 | 18.3 |
| | 4x | 8.3 | 5.4 | 5.0 | 7.8 | 4.2 | 4.2 | 2.0 | 1.9 | 15.8 |
| | | ** | ** | NS | NS | ** | NS | ** | ** | ** |
| Changshou kumquat | 2x | 10.1 | 6.5 | 5.0 | 9.3 | 4.1 | 7.0 | 2.7 | 2.6 | 18.5 |
| | 4x | 11.4 | 8.7 | 5.0 | 10.9 | 5.7 | 7.0 | 3.1 | 2.9 | 17.3 |
| | | ** | ** | NS | ** | ** | NS | ** | ** | * |

[z] NS: No significantly different, *, **: mean significantly different at 5, 1% levels by t-test, respectively.

Table 3. Comparison of pollen characteristics and pollen fertility in the diploid and the autotetraploid of the Meiwa kumquat, the Round kumquat and the Changshou kumquat.

| | Ploidy level | Pollen grain (μm) | | Shape index of pollen grain[y] | Pollen fertility (%) | |
|---|---|---|---|---|---|---|
| | | Length | Width | | Stainability | *In vitro* germination |
| Meiwa kumquat | 2x | 30.2 | 16.9 | 1.8 | 95.9 | 32.5 |
| | 4x | 33.4 | 25.4 | 1.3 | 68.4 | 11.3 |
| | | *[z] | * | * | * | * |
| Round kumquat | 2x | 30.0 | 17.7 | 1.7 | 97.4 | 12.9 |
| | 4x | 35.8 | 25.5 | 1.4 | 93.6 | 22.0 |
| | | * | * | * | NS | * |
| Changshou kumquat | 2x | 33.8 | 18.7 | 1.8 | 90.6 | 15.5 |
| | 4x | 40.3 | 23.8 | 1.7 | 77.4 | 9.7 |
| | | * | * | * | * | * |

[z] NS: No significantly different, *: mean significantly different at 1% levels by t-test.

[y] Length of pollen grain / Width of pollen grain.

Table 4. Comparison of fruit characteristics in the diploid and the autotetraploid of the Meiwa kumquat, the Round kumquat and the Changshou kumquat.

| | Ploidy level | Fruit wt. (g) | Fruit (mm) | | Shape index of fruit[y] | Pericarp wt. (g) | Pericarp wt. / fruit wt. (%)[x] | No.of locule | No. of seed / fruit | Developed seeds / fruit (%) | SSC (°Brix) | TA (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Diameter | Height | | | | | | | | |
| Meiwa kumquat | 2x | 13.9 | 28.7 | 29.7 | 96.6 | 8.8 | 63.3 | 6.5 | 4.2 | 90.2 | 21.0 | 0.30 |
| | 4x | 16.9 | 30.9 | 32.3 | 95.7 | 11.9 | 70.4 | 6.5 | 2.6 | 74.2 | 27.1 | 0.16 |
| | | **[z] | ** | ** | NS | ** | ** | NS | ** | ** | ** | ** |
| Round kumquat | 2x | 5.1 | 20.9 | 19.9 | 105.1 | 2.5 | 48.9 | 5.4 | 2.6 | 92.1 | 22.7 | 0.89 |
| | 4x | 5.5 | 21.1 | 20.6 | 102.5 | 3.0 | 55.8 | 5.7 | 2.8 | 82.8 | 25.7 | 1.09 |
| | | NS | NS | NS | * | * | ** | NS | NS | NS | * | * |
| Changshou kumquat | 2x | 39.8 | 42.5 | 44.0 | 96.9 | 20.3 | 51.0 | 7.1 | 8.8 | 99.1 | 18.5 | 0.16 |
| | 4x | 38.1 | 43.2 | 42.4 | 102.2 | 23.1 | 60.7 | 7.2 | 6.0 | 89.9 | 26.9 | 0.23 |
| | | NS | NS | * | * | ** | ** | NS | ** | ** | ** | ** |

[z] NS: No significantly different, *, **: mean significantly different at 5, 1% levels by t-test, respectively.

[y] (Fruit width / Fruit height) ×100.

[x] (Pericarp wt. / Fruit wt.) ×100.

Table 5. Seed contents and ploidy level of the seedlings obtained from the reciprocal crosses between diploid kumquats and tetraploid kumquats.

| Cross combination | | No. of fruits set | No. of seed | | | No. of developed seed / fruit | Developed seed (%)[x] | Av. Developed seed wt. | No. of seedlings examined | Ploidy level | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Seed parent | Pollen parent | | Developed | Undeveloped | Total | | | | | 2x | 3x | 4x |
| Oval kumquat | Open pollinated | 10 | 64 | 10 | 74 | 6.4 a[z] | 86.5 a | 102.3 a | 30 | 30 | 0 | 0 |
| | Meiwa kumquat 4x | 23 | 9 | 82 | 91 | 0.4 b | 9.9 b | 91.0 a | 5 | 0 | 5 | 0 |
| | Round kumquat 4x | 30 | 8 | 59 | 67 | 0.3 b | 11.9 b | 59.3 b | 1 | 0 | 1 | 0 |
| | Changshou kumquat 4x | 23 | 0 | 27 | 27 | 0 b | 0 b | – | – | – | – | – |
| Meiwa kumquat | Open pollinated | 10 | 55 | 9 | 64 | 5.5 a | 85.9 a | 122.0 a | 30 | 30 | 0 | 0 |
| | Meiwa kumquat 4x | 18 | 41 | 47 | 88 | 2.3 b | 46.6 bc | 90.4 b | 47 | 44 | 3 | 0 |
| | Round kumquat 4x | 43 | 78 | 96 | 174 | 1.8 b | 44.8 b | 87.6 b | 81 | 75 | 6 | 0 |
| | Changshou kumquat 4x | 17 | 11 | 59 | 70 | 0.6 c | 15.7 c | 76.5 b | 12 | 10 | 1 | 1 |
| Meiwa kumquat 4x | Open pollinated | 10 | 38 | 14 | 52 | 3.8 | 73.1 | 173.1 | 30 | 0 | 0 | 30 |
| | Meiwa kumquat | 7 | 19 | 5 | 24 | 2.7 | 79.2 | 55.1 | 25 | 0 | 5 | 20 |
| | | | | | | NS[y] | NS | * | | | | |
| Round kumquat 4x | Open pollinated | 10 | 40 | 8 | 48 | 4.0 | 83.3 | 111.6 | 30 | 0 | 0 | 30 |
| | Meiwa kumquat | 8 | 29 | 3 | 32 | 3.6 | 90.6 | 41.0 | 24 | 0 | 9 | 15 |
| | | | | | | NS | NS | * | | | | |
| Changshou kumquat 4x | Open pollinated | 10 | 48 | 6 | 54 | 4.8 | 88.9 | 227.3 | 30 | 0 | 0 | 30 |
| | Meiwa kumquat | 14 | 47 | 6 | 53 | 3.4 | 88.7 | 38.0 | 63 | 0 | 1 | 62 |
| | | | | | | NS | NS | * | | | | |

[z] Different letters represent significant differences in Tukey's multiple test, 1%level.

[y] NS: No significantly different, *: mean significantly different at 1% levels by t-test.

[x] (No.of developed seed / No. of total seed) × 100.

**Fig. 1. Nukaya et al.**

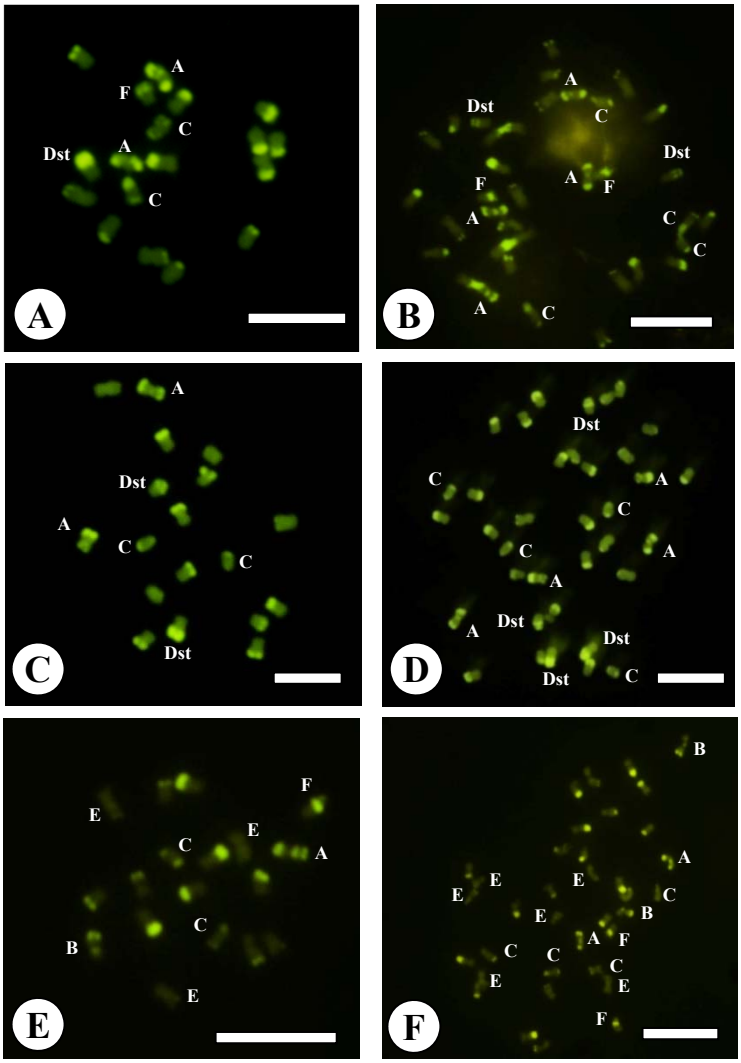


**Fig. 2. Nukaya et al.**

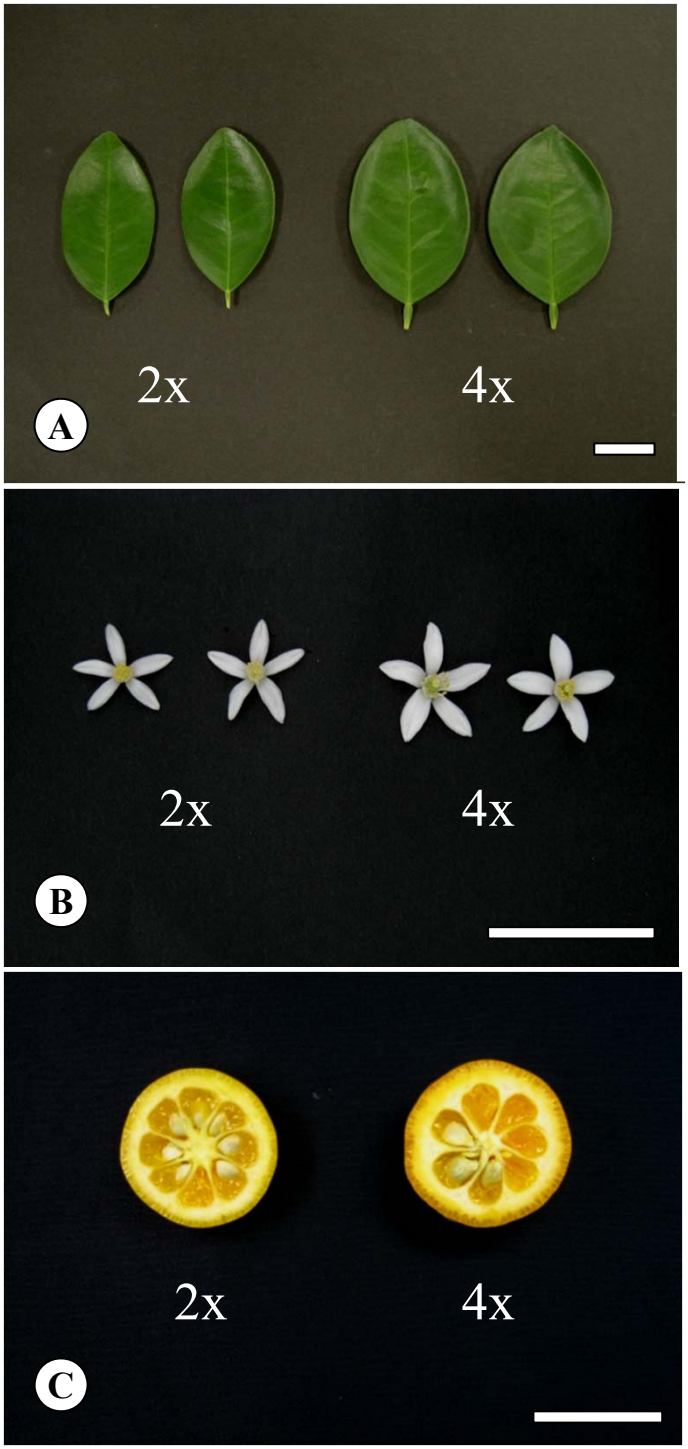


**Fig. 3. Nukaya et al.**

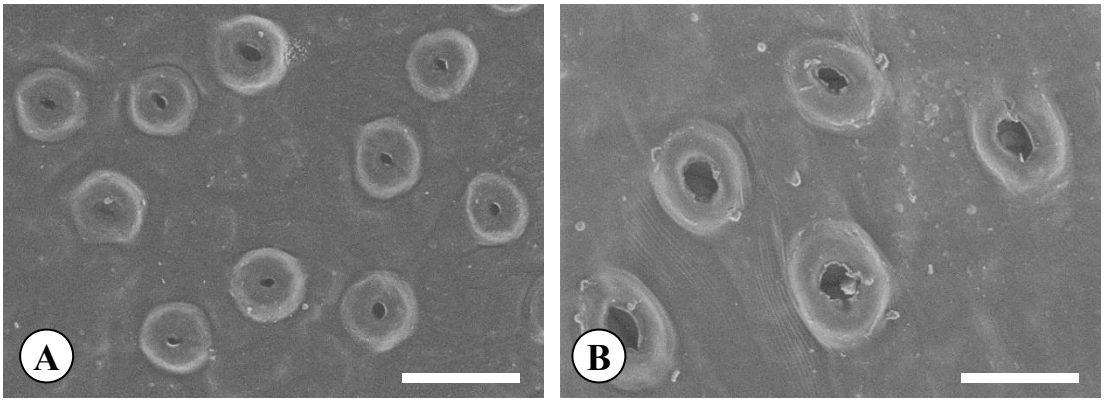


**Fig. 4. Nukaya et al.**

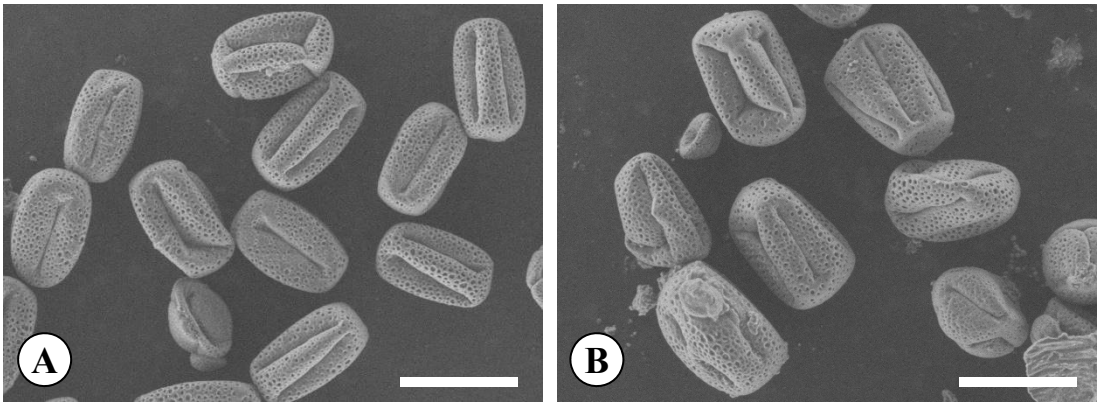


**Fig. 5. Nukaya et al.**

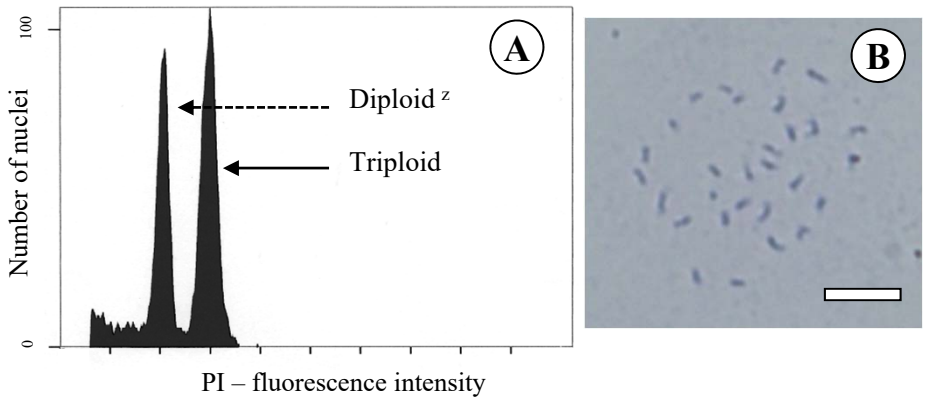


**Fig. 6. Nukaya et al.**