

Seed formation in triploid loquat (*Eriobotrya japonica*) through cross-hybridization with pollen of diploid cultivars

Shinji Kikuchi*^{†1}, Miwako Iwasuna^{†2}, Aya Kobori^{†2}, Yasunori Tsutaki^{†3}, Akihiro Yoshida⁴, Yuri Murota^{5,6}, Eisho Nishino¹, Hidenori Sassa¹ and Takato Koba¹

¹) Graduate School of Horticulture, Chiba University, Matsudo 648, Matsudo, Chiba 271-8510, Japan

²) Faculty of Horticulture, Chiba University, Matsudo 648, Matsudo, Chiba 271-8510, Japan

³) Southern Prefectural Horticulture Institute, Chiba Prefectural Agriculture and Forestry Research Center, Yamamoto 1762, Tateyama, Chiba 294-0014, Japan

⁴) Awa Agriculture Office, Hojyo 402-1, Tateyama, Chiba 294-0045, Japan

⁵) Chiba Prefectural Agriculture and Forestry Research Center, Daizen-no 808, Midori, Chiba, Chiba 266-0006, Japan

⁶) Present address: Sanbu Agriculture Office, Higashishinjyuku 17-6, Togane, Chiba 283-0006, Japan

As the fruits of loquat (*Eriobotrya japonica*, $2n = 2x = 34$) carry large seeds, the breeding of seedless loquat has long been a goal. The recent creation of triploid cultivars ($2n = 3x = 51$) and the application of gibberellins allow commercial production of seedless loquat, but the possibility of seed formation in triploid loquats has not been carefully investigated. Through crossing experiments and cytological observations of meiosis and pollen tube growth, we found that the triploid line 3N-N28 was essentially self-sterile, but developed seeds on pollination with pollen from diploid cultivars at rates of up to 5.5%. Almost half of the seedlings survived to 5 months, and carried diploid ($2n = 34$), tetraploid ($2n = 68$), or aneuploid chromosome numbers. Our results suggest that triploid loquat cultivars might retain the risk of seed formation. Protection from pollination by diploid cultivars or the development of new triploid cultivars will be necessary to ensure the production of seedless loquat fruits.

Key Words: cross-hybridization, loquat, meiosis, pollen tube growth, seed formation, triploid.

Introduction

The loquat—*Eriobotrya japonica* (Thunb.) Lindl. ($2n = 2x = 34$)—is an evergreen fruit tree in the Amygaloideae sub-family of the Rosaceae. Native to central China, it grows well in subtropical to mid-temperate climates, and bears fruits in early spring. The loquat has been cultivated for more than a thousand years in Japan and China, and is now an export crop in Spain (Lin *et al.* 2007). In addition to its fruit, it is grown for medicinally valued materials in the treatment of lung-related diseases including cough, asthma, and chronic bronchitis (Lin *et al.* 2007). Breeding programs in loquat are being carried out (Badenes *et al.* 2013), for example, the development of loquat SSR markers (Watanabe *et al.* 2008), genetic analysis of resistance to loquat canker (Hiehata *et al.* 2012), and introduction of new loquat cultivars such as ‘Reigetsu’ and ‘Ryoho’ (Hiehata *et al.* 2008, Terai *et al.* 2007). Besides these endeavors, the production of seedless loquat has long been a goal in loquat breeding.

Loquat fruits usually hold 4–7 large seeds. Although the

flesh yield, at 65%–70%, is not much lower than that of other fruits, the heavy seeds, which constitute 15%–20% of fruit weight, create an impression that the edible proportion is limited. To overcome such negative aspect, breeders in Japan have bred seedless cultivars ($2n = 3x = 51$) by a combination of triploidy and the use of gibberellins (GA₃) and forchlorfenuron (Yahata *et al.* 2006). In China, Liang *et al.* (2011) identified 311 triploids from open-pollinated seeds of Chinese diploid cultivars, and selected six large-fruited seedless lines.

The use of triploids is a common technique used to grow seedless fruits (Kihara 1951). During meiosis in triploids, each trio of homologous chromosomes randomly segregates into the two daughter cells, producing sterile gametes with different chromosome numbers (aneuploidy). However, seeds can still form in some triploid fruits, including apple (Sato and Kanbe 2007, Sato *et al.* 2007), grape (Park *et al.* 1999), blueberry (Vorsa and Ballington 1991), *Hylocereus* (Tel-Zur *et al.* 2005), and Japanese butterbur (*Petasties japonicus*) (Koizumi *et al.* 2008). The possibility of seed formation in triploid loquats remains to be investigated.

Here, we investigated the mechanism that causes sterility in a triploid loquat line, 3N-N28. 3N-N28 showed sterility in most male and female gametes, but hand-pollination with pollen from diploid cultivars led to seed formation in 3N-N28

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*Corresponding author (e-mail: skikuchi@faculty.chiba-u.jp)

† These authors contributed equally to the work

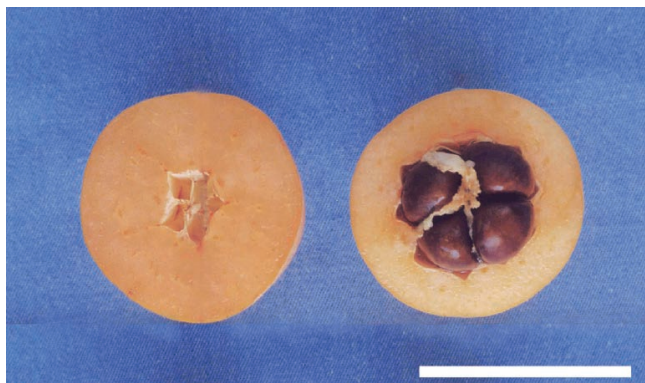


Fig. 1. Transverse sections of loquat fruits. Left: seedless fruit of triploid loquat 3N-N28. Right: diploid loquat fruit with seeds. Bar = 5 cm.

fruits at rates of up to 5.5%. Many of these seeds germinated, producing seedlings that were either euploids or aneuploids seedling(s).

Materials and Methods

Plant materials

The triploid loquat line 3N-N28 ($2n = 3x = 51$) (Fig. 1) was derived from a cross between a tetraploid of ‘Tanaka’ seedling and diploid ‘Nagasakiwase’. Trees were maintained at the Chiba Prefectural Agriculture and Forestry Research Center. Triploid nature of the hybrid was confirmed by flow-cytometric analysis.

Seeds collected from 3N-N28 in hand-pollination with pollen of diploid cultivars or in open-pollination were washed and immediately put in vermiculite to raise seedlings. The seedlings were maintained in a greenhouse controlled at $>12^{\circ}\text{C}$ without light supplement at Chiba University, in Chiba prefecture of Japan. The diploid cultivars ‘Fusahikari’, ‘Fusahime’, ‘Mizuho’, ‘Oobusa’, ‘Satomi’, ‘Tanaka’, and ‘Tomifusa’ were also used for observation of pollen tube growth, crossing experiments, and polymorphism analysis.

Cytological observations

For observation of meiotic chromosomes, anthers collected from immature floral buds of 3N-N28 at 12–16 days before flowering were fixed in 1 : 3 (v/v) acetic acid : ethanol at 4°C for 5 days. For observation of mitotic chromosomes, fresh root tips were pre-treated with 0.1% 8-hydroxyquinoline at 16°C for 4 h. Chromosome preparations were obtained as described in Kikuchi *et al.* (2008). Chromosomes were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured on a Leica DC350 F camera fitted to a Leica DM RXA2 microscope and processed using the Leica CW 4000 FISH software. Chromosome numbers were determined from at least 10 mitotic cells with well spread chromosomes.

Pollination and observation of pollen tube growth

Anthers were dehisced by incubation at 26°C for 12 h, and then fresh pollen grains of 3N-N28 or diploid cultivars were hand-applied to the stigmata of emasculated flowers of 3N-N28. The pollinated flowers were covered with paper bags. These cross-hybridization tests were performed in November–December of 2010, 2011, and 2012. The fruits started development in February–March of the subsequent year, and then matured fruits were harvested in April–June to observe seed formation. Open-pollinations were conducted by pollinators such as bees in a non-enclosed greenhouse.

Fresh pollen grains from more than ten anthers were collected to observe the morphology and placed in Milli-Q water on glass slides. After covering with a cover slip, the pollen grains were observed under a phase-contrast light microscope (Olympus BX41).

To observe pollen tube growth, the aniline blue staining method was followed as described by Kikuchi *et al.* (2007) with some modifications. In brief, a week after pollination, styles were fixed in 1 : 3 (v/v) acetic acid : ethanol at 4°C for a week, and then stored in 70% ethanol at 4°C until use. Fixed styles were rinsed in Milli-Q water for 30 min and placed in 70% lactic acid, which was held in boiling water for 2 min. After another rinse in Milli-Q water for 30 min, the styles were stained with 1% aniline blue in 0.1 M K_2HPO_4 for 60 min. Each stained style was torn into its two carpels with forceps under a stereomicroscope. The two carpels were placed on a glass slide with the transmitting tissue side up, mounted in 1% aniline blue in 0.1 M K_2HPO_4 , and then covered with a cover slip. Average rates of pollen tube growth were calculated as described in the footnote to Table 1.

Table 1. Rates of pollen tube growth in self-pollination of 3N-N28 and cross pollinations with diploid cultivars

Female	Male	No. of styles pollinated (No. of flowers pollinated) ^a	Average rate of pollen tube growth ^b
3N-N28	Oobusa	29 (9)	0.98 ± 0.03
	Satomi	37 (12)	0.95 ± 0.03
	Tanaka	41 (13)	0.91 ± 0.03
	Tomifusa	30 (10)	0.98 ± 0.02
	Mizuho	42 (13)	0.97 ± 0.02
	Fusahikari	16 (4)	0.72 ± 0.10
	3N-N28	11 (3)	0.32 ± 0.10

^a Pistil of loquat is compound pistil with 5 styles. The ovary consisted of 5 fused carpels, and each locules divided by the septums have 2 ovules. To observe the pollen tube growth, all fresh styles, except styles which died by 7 DAP, were used.

^b If >3 of pollen tubes elongated to ovary, the rate of pollen tube growth was defined as ‘1’. If 1–2 of pollen tubes arrived at ovary, the rate of pollen tube growth was defined as ‘0.5’. In case of no pollen tube growth, the rate of pollen tube growth was defined as ‘0’. The average rates with standard errors of pollen tube growth were calculated as average numbers of the rates of pollen tube growth.

Histological observation of ovules of pollinated flowers

At 7 and 20 days after pollination (DAP), ovules were fixed in 1% paraformaldehyde (pH 7.0) for 24 h, dehydrated through a graded ethanol series (80%, 90%, 95%, 99% for 1 h each), and stored in 100% ethanol. The dehydrated ovules were submerged in a 1 : 1 (v/v) mixture of Technovit 7100 resin (Heraeus Kulzer) and ethanol for 12 h, and then in 100% Technovit 7100 for 12 h. They were then embedded in a Technovit block according to the manufacturer's procedure, and histological sections were sliced at 5- μ m intervals with a microtome (Leica RM3165), floated on the distilled water and scooped on the slide glass. After drying on a hot plate (40°C), sections were stained with 1% safranin 'O' solution for 10 min and 0.5% fast green FCF in 95% ethanol solution for 10 s, and then dehydrated by 100% ethanol, and cleared by 100% Lemosol (Wako). Finally, sections were mounted in Entellan Neu medium (Merck) and observed through a bright-field microscope (Olympus BH2) equipped with a digital camera (Nikon DS-L1).

DNA extraction, PCR, and polyacrylamide gel electrophoresis

To verify cross-hybridization with the diploid cultivars, we performed PCR analysis with SSR markers and DNA of 3N-N28, diploid cultivars, and seedlings. Total DNA from young leaves of seedlings with veins and leaf hairs removed was purified using a standard CTAB method (Murray and Thompson 1980); polysaccharides were first removed by washing with HB buffer (10 mM Tris·HCl, 10 mM EDTA, 80 mM KCl, 0.5 M sucrose; Sassa and Hirano 1998) containing 2% polyvinylpyrrolidone (average mol. wt. 40 000; Sigma PVP-40). PCR was done in a 5- μ L mixture containing 1.5 μ L Milli-Q water, 2.5 μ L GoTaq master mix (Promega), 0.5 μ L 5 μ M primers (forward and reverse), and 0.5 μ L genomic DNA (10 ng). The program used 94°C for 5 min; 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min; and a final 72°C for 7 min. Three SSR primer sets (CH03d12: 5'-GCCCAGAAGCAATAAGTAAACC-3' and 5'-ATTGCTCCATGCATAAAGGG-3', CH05g03: 5'-GCTTTGAATGGATACAGGAACC-3' and 5'-CCTGTCTC ATGGCATTGTTG-3', NB105a: 5'-AAACAACCGACTGA GCAACATC-3' and 5'-AAAATCTTAGCCCCAAAATCTCC-3') (Watanabe *et al.* 2008) showing polymorphisms between 3N-N28 and any one of the seven diploid cultivars were used for PAGE. PCR products were run on a 13% polyacrylamide gel, and stained with 0.5 μ g/ml ethidium bromide.

Results

Meiosis and pollen formation in 3N-N28

As expected, meiocytes of 3N-N28 showed several abnormalities throughout all stages of meiosis (pachytene, diakinesis, metaphase I, anaphase I, tetrad). In pachytene, the chromosomes showed complex alignment patterns (Fig. 2A), and one chromosome group (pachytene chromosome) showed both association and local unpaired regions

(asterisks in Fig. 2A inset). Chromosomes of 3N-N28 ($2n = 3x = 51$) did not always align into 17 trivalents, and bivalents and multivalents with more than three chromosomes appeared frequently (Fig. 2B). Univalents, bivalents and multivalents were also observed in metaphase I (Fig. 2C). The chromosomes divided unequally between daughter cells, and several lagging chromosomes were also observed in anaphase I (Fig. 2D). After meiosis, tetrads reflecting unbalanced chromosome segregation were generated, and tetrads and spores carried some micronuclei (Fig. 2E, 2F). These observations show that the 51 chromosomes in 3N-N28 did not segregate reductionally into the gametes.

Observations of 550 pollen grains under a phase-contrast light microscope revealed 435 developed grains (79.1%) and 115 empty grains (20.9%) (Fig. 3). In addition, variations in the sizes of the developed grains suggest that the grains carried different DNA contents. As explained below, these pollen grains were essentially sterile.

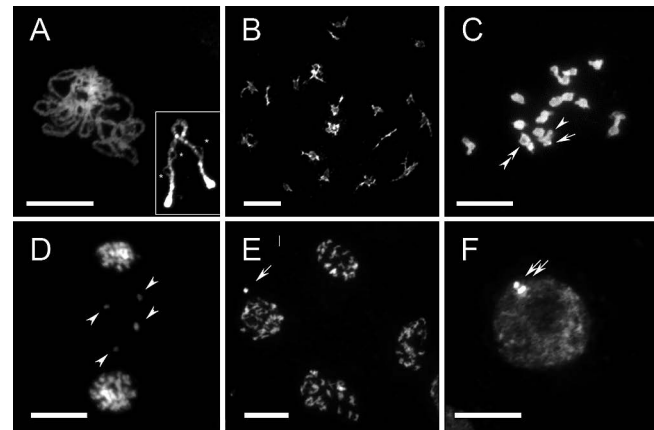


Fig. 2. Meiosis of triploid loquat 3N-N28 with DAPI staining. (A) Pachytene chromosomes. Inset shows a chromosome association with unpaired loops (asterisks). (B) Diakinesis, showing more than 17 chromosome groups (paired chromosomes). (C) Metaphase I, showing $x \approx 17$ groups of various chromosome sizes, including univalent (arrow), bivalent (arrowhead), and multivalent (double arrowhead). (D) Anaphase I. Arrowheads show lagging chromosomes on the metaphase plate. (E, F) Arrows show micronuclei formed in (E) tetrads and (F) microspore. Bars = 10 μ m.

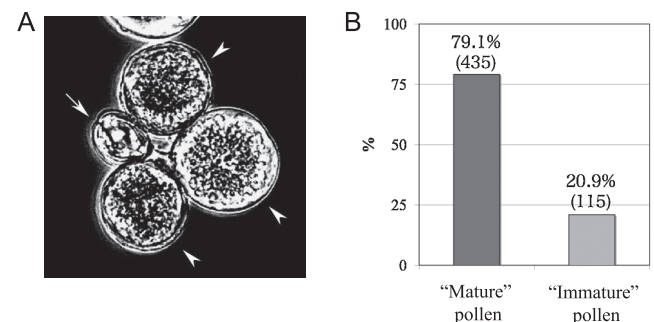


Fig. 3. Morphological characteristics of pollen grains of 3N-N28. Arrowheads show cytoplasm-packed grains ("mature pollen"). Arrow shows empty grain ("immature").

Pollen tube growth and seed formation efficiency in crosses between 3N-N28 and diploid cultivar pollen

Most pollen of self-pollinated 3N-N28 did not germinate on the stigma by 7 DAP (Fig. 4A). Several pollen tubes grew into the style but almost none were able to reach the ovary. On the other hand, pollen tubes of ‘Oobusa’, ‘Satomi’, ‘Tanaka’, ‘Tomifusa’, ‘Mizuho’, and ‘Fusahikari’ elongated straight and reached the ovary of 3N-N28 (Fig. 4B–4F; ‘Fusahikari’ not shown). The average rates of pollen tube growth were high when pollen of diploid cultivars was used and low when pollen of 3N-N28 was used (Table 1). Histological sections of the ovary at 20 DAP showed development of endosperm irrespective of the pol-

len donor (Fig. 5B, 5C), but not in ovaries of unpollinated flowers (Fig. 5A). About 12%–30% of ovaries developed endosperm, suggesting that most female gametes lost the ability for fertilization or embryogenesis might be due to an unbalanced chromosome segregation.

Crossing experiments during the subsequent 3 years revealed that pollination with pollen of diploid cultivars resulted in fruits with one or two fertile seeds at a rate of up to 5.5%, but self-pollination ($n = 220$) failed to generate any seed (Table 2).

Germination rate, survival rates, chromosome numbers, and confirmation of cross-hybridization using SSR markers in 3N-N28 seedlings

A total of 106 seeds were harvested from open pollination of 3N-N28 in 2011 and 2012, out of which 92 of the seeds (87%) germinated, and 47 (51.1% = 47/92) were still alive 5 months after sowing (Table 3). Seedlings from open pollination of diploid cultivars showed high survival rates (77%–93%). Nearly half of the 3N-N28 seedlings survived also. Most of those grew well (Fig. 6). We confirmed the chromosome number in 15 seedlings: seven (47%) had 68 chromosomes per somatic cell ($2n = 68$), three (20%) had $2n = 34$, and the other five (33%) had aneuploid numbers of $2n = 62$ – 77 (Fig. 7). Chromosome numbers of 68 and 34 equate to that of tetraploid and diploid, respectively, however, we could not identify the genome constitutions due to lack of chromosome markers.

Finally, we tried to confirm the pollen parentage of the seedlings obtained from open pollination. For this conformation,

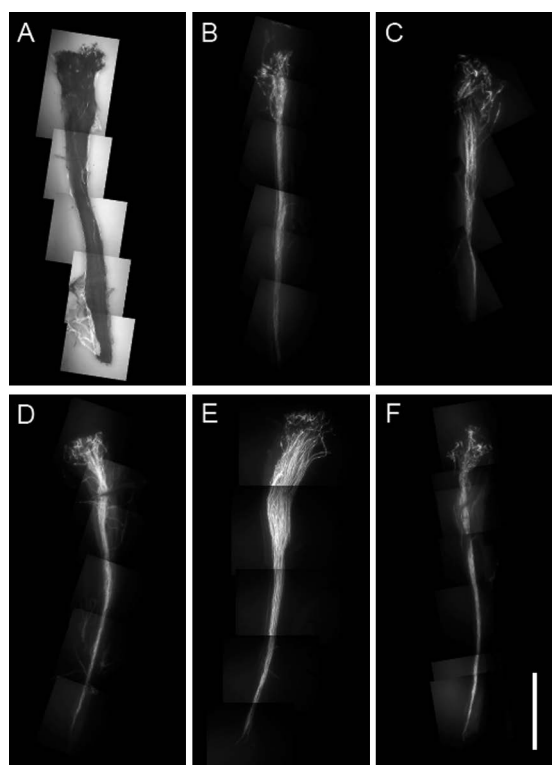


Fig. 4. Pollen tube growth from stigma to base of style of 3N-N28 in pollination by (A) 3N-N28, (B) ‘Oobusa’, (C) ‘Satomi’, (D) ‘Tanaka’, (E) ‘Tomifusa’, and (F) ‘Mizuho’, at 7 days after pollination. Pollen tubes were stained with aniline blue. Bar = 0.5 mm.

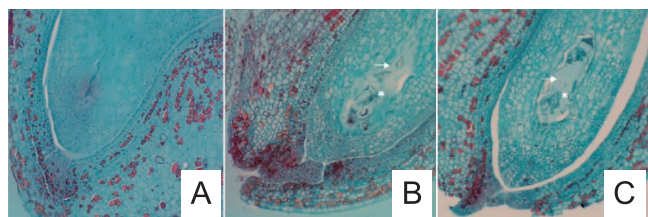


Fig. 5. Histological observations of 3N-N28 ovules at 20 days after pollination. Sections were stained with safranin O (red) and fast green FCF (green). (A) Ovule of an unpollinated flower shows undeveloped endosperm. (B) Ovule of self-pollinated flower. (C) Ovule of 3N-N28 flower pollinated by diploid ‘Tomifusa’. Arrow, endosperm; *, embryo-like structure.

Table 2. Rates of fruit formation with seeds in self-pollination of 3N-N28 and crosses with five diploid cultivars

Female	Male		Rate of fruit formation with seed (%) ^a			
	Cultivar	Ploidy	2010–2011	2011–2012	2012–2013	Mean
3N-N28	Tomifusa	2x	–	4.0	2.0	3.0
	Mizuho	2x	–	2.0	1.0	1.5
	Fusahime	2x	25.0	5.0	2.0	5.5
	Fusahikari	2x	–	0.0	0.0	0.0
	Satomi	2x	–	0.0	1.0	0.5
3N-N28	3x		0.0	0.0	0.0	0.0

^a Emasculated flowers ($n = 20$ in 2010–11, $n = 100$ in 2011–12 and 2012–13) of 3N-N28 were hand-pollinated.

Table 3. Rates of germination and survival of seedling of 3N-N28 and diploid cultivars derived in open pollination at 5 months after sowing

Line/Cultivar	No. of seeds produced	No. of seeds germinated (Germination rate, %) ^a	No. of seedlings survived (Survival rate, %) ^b
3N-N28	106	92 (87)	47 (51)
Mizuho	28	28 (100)	26 (93)
Fusahime	12	12 (100)	11 (92)
Tomifusa	13	13 (100)	10 (77)

^a Germination rate (%) = (No. of seeds germinated / No. of sowing seeds) × 100.

^b Survival rate (%) = (No. of seedlings survived / No. of seeds germinated) × 100.

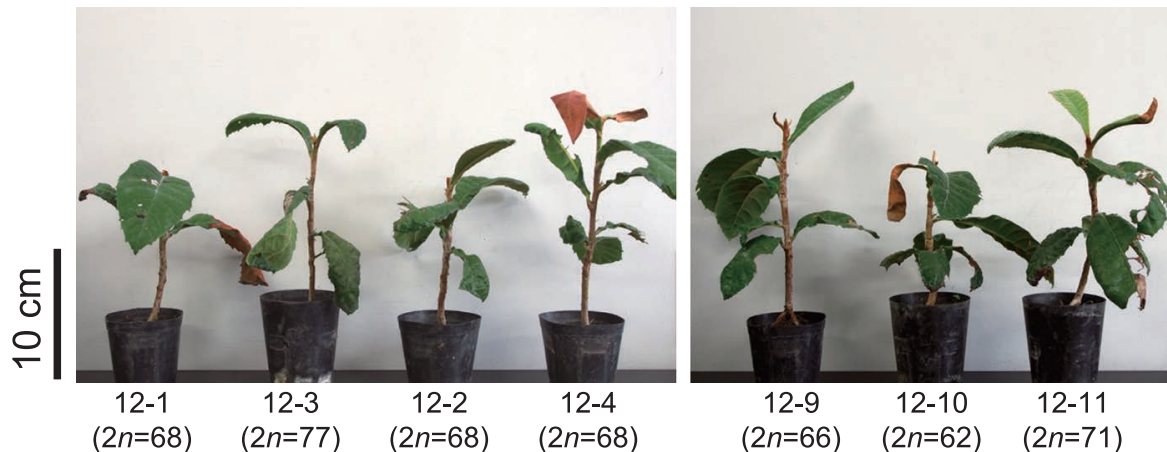


Fig. 6. One-year-old seedlings of 3N-N28 obtained in open-pollination. Seedling IDs and chromosome numbers coincide with those in Fig. 7.

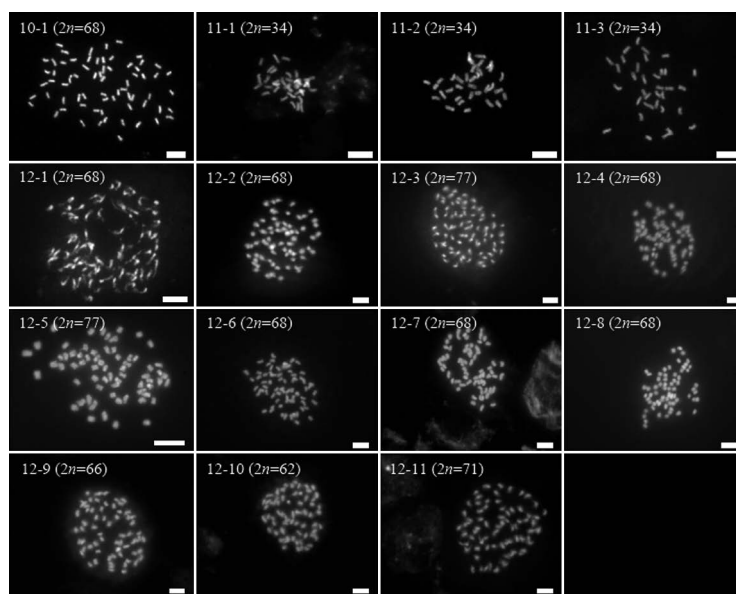


Fig. 7. Mitotic metaphase chromosomes of 15 3N-N28 seedlings stained with DAPI. Bars = 10 μ m.

3 primer sets (CH03d12, CH05g03, NB105a) were screened from 26 SSR markers (Table 4 in Watanabe *et al.* 2008) with polymorphisms between 3N-N28 and the any one of the seven diploid cultivars ('Tanaka', 'Oobusa', 'Mizuho', 'Satomi', 'Fusahikari', 'Tomifusa', 'Fusahime') that could indicate difference of DNA length on 13% PAGE. PCR amplification of the three SSR markers revealed that all 13 3N-N28 seedlings had PCR products different from those of 3N-N28 (Fig. 8), suggesting that these seedlings were hybrids between 3N-N28 and diploid cultivars.

Discussion

Functional errors throughout meiosis and pollen tube growth show that the triploid loquat line 3N-N28 is essentially self-sterile. However, seed formation is possible through cross-pollination by diploid cultivars in both hand pollination and open pollination. Seed formation by triploid loquats pro-

duced in Japan (Yahata *et al.* 2006) and China (Liang *et al.* 2011) has not previously been discussed. The risk of seed formation that our results reveal may compromise the commercial value of triploid loquat as seedless fruit.

Seed formation in triploid loquat 3N-N28

The results of cross-hybridization and polymorphism analyses suggest that seed formation by 3N-N28 may be caused by incomplete sterility of female gametes. Because every flower has 10 ovules, the frequency of seed formation per flower (0.0%–5.5%) equates to 0.0%–0.55% per ovule. These rates are high compared with the expected occurrence [$3 \times (1/2)^{17}$] of euploid gametes ($n = 17, 34, 51$) resulting from random disjunction of the chromosomes during meiosis. Interestingly, we found seedlings with tetraploid ($2n = 68$) and hyper-tetraploid ($2n = 71$ and 77) values, suggesting the occurrence of non-reduced gametes. Intensive counting of chromosomes in 45138 seeds of 21 diploid loquat

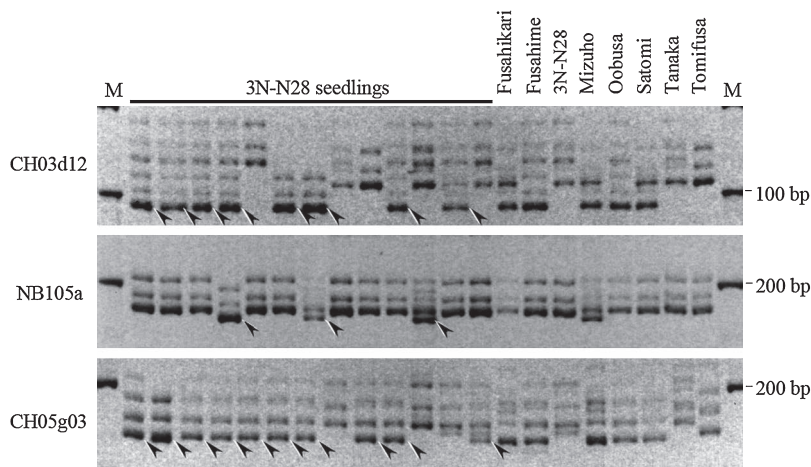


Fig. 8. Polymorphism analysis in 3N-N28 and 13 seedlings by SSR markers. Arrowheads indicate DNA fragments showing polymorphism with maternal 3N-N28.

cultivars derived from open pollination from 1997 to 2005 revealed 311 (0.68%) natural polyploids, including 225 triploids, which occurred through spontaneous chromosome doubling or the formation of non-reduced gametes (Guo *et al.* 2007). A line 77-1 had a frequency of 2.32% (Guo *et al.* 2007). In diploid *Malus* (also Maloideae), 0.07%–0.36% of 27542 F₁ seedlings were polyploids and aneuploids derived from non-reduced gametes (Considine *et al.* 2012).

Rates of seed formation in 3N-N28 varied, depending on the pollen parent, from 0% in 3N-N28 × ‘Fusahikari’ to 5.5%. Such varietal differences in seed formation by triploid × diploid (or diploid × triploid) crosses have been reported in apple (Sato and Kanbe 2007), grape (Park *et al.* 1999), blueberry (Vorsa and Ballington 1991), and Japanese butterbur (Koizumi *et al.* 2008), suggesting that genetically controlled crossability based on the diploid genotypes is a leading cause of the varietal differences in seed formation in triploids. Besides genetically unbalanced gametes, self-incompatibility could be a possibility for self-sterility of 3N-N28. Loquat has a gametophytic self-incompatibility (GSI) system based on S-RNase (Carrera *et al.* 2009), but most Japanese loquat cultivars are known as self-fertile (Ishimoto *et al.* 2014) due to a self-compatible *S*⁶ allele (Niska *et al.* 2010). Both parental cultivars of 3N-N28, ‘Tanaka’ (*S*²*S*⁶) and ‘Nagasakiwase’ (*S*⁶*S*¹⁰), are also known as self-compatible cultivars (Ishimoto *et al.* 2014). However, the tetraploid ‘Tanaka’ of female parent of 3N-N28 has been bred from chromosome doubling of a ‘Tanaka’ seedling in open pollination, thus, to clear up whether the 3N-N28 is self-incompatible, determination of the *S* genotype of 3N-N28 is necessary.

Protection against seed formation in triploid loquat

Cross-hybridization with diploid cultivars can lead to seed formation in 3N-N28, but pollination is not necessary for fruit formation by gibberellins and forchlorfenuron treatment (data not shown). As loquat growers often rely on pollinators such as bees, screening or bagging flowers against

insects is necessary to avoid the risk of seed formation in triploid loquat fruits. Since seed formation in 3x × 2x apple crosses depended on the triploid cultivar—e.g., ‘Jonagold’ (3x) × ‘Fuji’ (2x) gave 33.6% seed formation but ‘Mutsu’ (3x) × ‘Fuji’ (2x) gave 11.5% (Sato and Kanbe 2007)—production of new triploid loquat cultivars could also reduce the risk.

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