

Production of Nonaploid ($2n = 9x$) Japanese Persimmons (*Diospyros kaki*) by Pollination with Unreduced ($2n = 6x$) Pollen and Embryo Rescue Culture

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ABSTRACT. To produce nonaploid Japanese persimmon (*Diospyros kaki* L.f.) by artificial hybridization, we surveyed the natural occurrence of unreduced ($2n$) pollen among hexaploid cultivars and sorted them from normal reduced (n) pollen. The sorted $2n$ pollen was crossed with a hexaploid female cultivar and the resultant embryos were rescued by in vitro culture techniques to obtain plantlets. Three out of six male-flower-bearing cultivars ($2n = 6x = 90$) produced $2n$ pollen at rates of 4.8% to 15.5% varying with the cultivar, which was estimated by both pollen size and flow cytometry. After sorting giant ($2n$) from normal pollen grains by using nylon mesh, they were crossed with a hexaploid female cultivar. The seeds obtained from pollination with normal pollen were perfect, but those obtained from pollination with giant pollen were mostly imperfect, with embryo growth being suspended at the globular stage. Although the rate of survival was very low, some embryos at the globular stage were rescued successfully and grown in vitro. Both flow cytometric analysis and chromosome counting proved that the plantlets obtained were nonaploid.

Polyploid plants are very common in tree fruit species (Sanford, 1983). Such polyploids may have arisen either by sporadic chromosome doubling or by the union of unreduced gametes with other unreduced gametes or with normal reduced gametes. The latter case is considered more likely to occur in nature (Harlan and DeWet, 1975).

Japanese persimmon (*Diospyros kaki*) is a hexaploid tree fruit having a somatic chromosome number of 90 ($2n = 6x$, $x = 15$). The fruit is important commercially and has been cultured for centuries in such east Asian countries as China, Korea, and Japan. Japanese persimmon is now gaining popularity as a new fruit crop throughout the world. Until recently, information on the chromosome karyotype and ploidy of Japanese persimmon and its relatives has been limited. Namikawa and Higashi (1928) were the first to report that Japanese persimmon is a hexaploid plant. Namikawa (1930) also reported that date plum (*D. lotus* L.) and velvet apple, or mabolo (*D. discolor* Willd.) are diploid and American persimmon (*D. virginiana* L.) is hexaploid. Baldwin and Culp (1941) found two karyotypes in *D. virginiana* ($2n = 4x$, $6x$), but few reports on the cytogenetics of persimmon have since been published. Zhuang et al. (1990) reported that a few seedless Japanese persimmon cultivars have a nonaploid chromosome number ($2n = 9x = 135$) in contrast to most seeded cultivars. The origin of these nonaploid cultivars is unknown, but might have arisen from spontaneous crossing between reduced (n) and unreduced ($2n$) gametophytes in field culture, since no dodecaploid persimmons have been found in nature. There is substantial evidence in other fruit species that the natural occurrence of

unreduced gametophytes is common and contributes to their polyploidization (Sanford, 1983). In *D. kaki*, some pollinating cultivars seem to produce giant pollen which is supposedly unreduced (Zhuang, 1990). However, even if reduced and unreduced gametophytes were fertilized successfully, the possibility of producing a zygote with subsequent normal embryo development would be very low in nature probably due to endosperm genomic imbalance (Sanford, 1983). Such a zygote or an embryo, however, could be rescued successfully by means of in vitro culture.

To demonstrate the aforementioned possibility and to determine if this is a potential origin of nonaploid persimmon, we surveyed the occurrence of giant (unreduced) pollen in several cultivars of Japanese persimmon. We then attempted to produce artificially nonaploid persimmons by pollination of normal hexaploid cultivar with the sorted giant unreduced pollen followed by in vitro rescue culture of the resultant embryos. This method would be useful for breeding nonaploid seedless persimmons.

Materials and Methods

OBSERVATION OF GIANT POLLEN IN SELECTED JAPANESE PERSIMMON CULTIVARS. Staminate flowers were collected just before anthesis (middle to late May 1992–97) from mature trees of monoecious Japanese persimmon cultivars growing in an orchard at Kyoto University, Kyoto. After removing the corollas with forceps, male flowers were dried overnight at room temperature (≈ 20 °C) and the released pollen grains were placed in sealed glass vials. The vials were stored at 4 °C with silica gel desiccant until used. An aliquot of stored pollen was hydrated in 0.9 M mannitol in cell and protoplast washing (CPW) solution (Draper et al., 1988) for 2 h with agitation and then the diameter of ≈ 500 grains was measured under a light microscope. Measurements were made over two to three different seasons from 1992 to 1997, depending on the cultivar. The hexaploid cultivars used were

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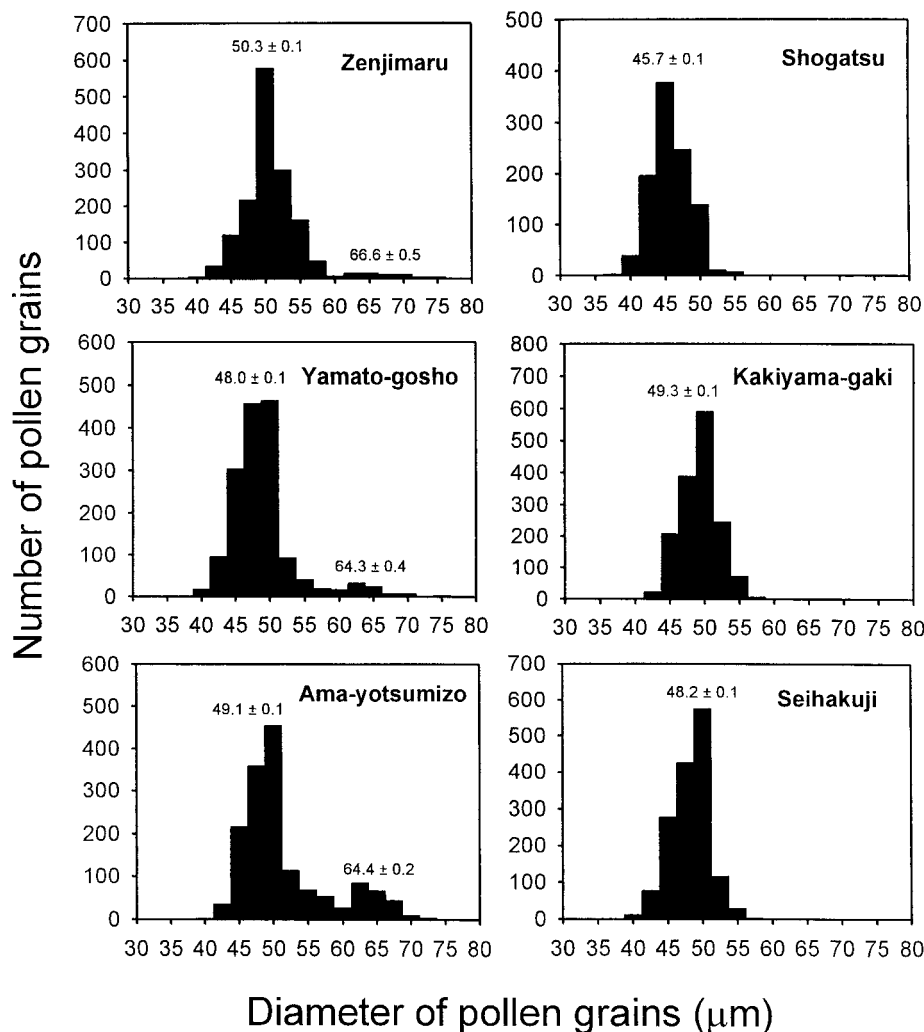


Fig. 1. Size distribution of pollen grains in selected persimmon cultivars. Data of 2 or 3 years were combined. Mean grain diameters (\pm SE) for major and minor peaks are indicated.

'Zenjimaruru', 'Yamato-gosho', 'Ama-yotsumizo', 'Shogatsu', 'Kakiyama-gaki', and 'Seihakuji'.

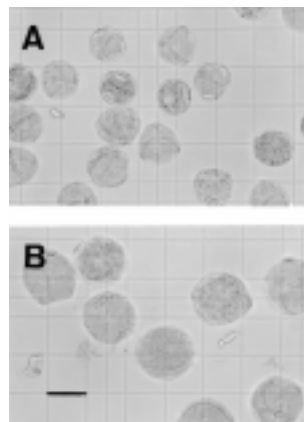
Data were subjected to two-way analysis of variance (ANOVA) with cultivar and year as main effects for giant pollen production. All percentage data were subjected to arcsin transformation before ANOVA. Since data for giant pollen production were balanced incompletely, ANOVA was conducted assuming no interactions were present.

DNA MEASUREMENT OF POLLEN NUCLEI BY FLOW CYTOMETRY. An aliquot of 'Zenjimaruru' pollen collected in 1996 and 1997 was hydrated in 0.9 M mannitol in CPW solution for 2 h and sorted with a 62 μ m nylon mesh (Kyoshin Riko Co., Tokyo) to separate giant and normal pollen grains. Persimmon pollen is not sticky and was sieved successfully through the nylon mesh without the pollen grains adhering to each other. The diameters of sorted pollen grains were measured under a light microscope and the nuclear DNA content was determined by flow cytometry as described previously (Sugiura et al., 1998). An aliquot of each sorted pollen was immersed in 2 mL of 10 mM Tris-HCl buffer (pH 7.0) containing 100 mM NaCl, 10 mM Na₂-EDTA, and 0.1% (w/v) Triton X-100 and chopped with a razor blade to release nuclei. The suspension was cooled on ice for 30 min and then passed through a 20- μ m nylon mesh to remove debris. A 1-mL aliquot of the filtrate was transferred to a test tube, and the nuclei in the

filtrate were stained with propidium iodide (PI) at 100 μ g·mL⁻¹. Relative nuclear DNA contents of the nuclei were determined with a flow cytometer using somatic 'Jiro' callus nuclei (2n = 6x = 90) as a reference standard. By comparing the fluorescence peak (channel number) of the somatic nuclei, we estimated the relative amount of DNA in normal and giant pollen nuclei.

POLLINATION WITH GIANT VS. NORMAL POLLEN AND RESCUE CULTURE OF THE RESULTANT EMBRYOS. During 28 to 31 May 1996, prebagged female flowers of monoecious 'Jiro' persimmon were pollinated with sorted giant and normal 'Zenjimaruru' pollen grains separately. For this purpose, a quantity of 'Zenjimaruru' pollen was immersed in 0.9 M mannitol in CPW solution for 2 h and sorted through a 62 μ m nylon mesh. This sorting procedure was repeated three times to reduce contamination as much as possible. Sorted pollen grains, giant and normal, were suspended in CPW solution containing 15% sucrose and used for pollination immediately. After pollination, flowers were bagged for \approx 2 weeks. Germination of sorted pollen was tested on a 1% agar medium containing 15% sucrose. Three days after pollination some of the pollinated pistils were sampled and fixed in 5 formalin : 5 acetic acid : 90 (70%) alcohol (by volume). Pollen tube growth on some stigmas and pistils was observed with a fluorescence microscope. This research was repeated during flowering of 'Jiro' persimmon in 18 to 21 May 1997.

Fruit were harvested 70 d after pollination in 1996 and 50, 60, 70, 80, 90 or 170 d (maturity) after pollination in 1997. After measuring fruit diameter and weight, the seeds were removed from the fruit and the embryos were excised aseptically. Both normal and rudimentary (globular) embryos were cultured on modified MS medium (Murashige and Skoog, 1962) supplemented with 3 μ M zeatin (Tao and Sugiura, 1992) under a 16-h light/8-h dark cycle at 28 °C. Light was provided from cool-white fluorescent lamps with a total light intensity of 60 μ mol·m⁻²·s⁻¹. From the plantlets thus obtained, callus was induced in the dark on MS (1/2 NO₃) medium supplemented with 10 μ M zeatin and 1 μ M indoleacetic acid, and the calli were subjected to flow cytometric analysis of nuclear DNA to determine ploidy levels. Chromosomes were also counted using root tips obtained from plantlets as described by Tamura et al. (1996).



Results

OCCURRENCE OF GIANT POLLEN. Size distribution (diameters)

Fig. 2. Giant and normal pollen grains of 'Zenjimaruru' persimmon sorted with a 62 μ m nylon mesh. (A) Normal pollen and (B) giant pollen. Scale bar = 50 μ m.

Table 1. Frequency of giant pollen production in selected cultivars of Japanese persimmon in different years. Giant and normal pollen grains were separated at a diameter of 60 μm .

Cultivar	Year	No. of pollen grains			Giant (%)
		Normal	Giant	Total	
Zenjimaruru	1995	478	25	503	5.0
	1996	473	35	508	6.9
	1997	516	14	530	2.6
	Total	1467	74	1541	4.8
Yamato-gosho	1995	444	48	492	9.8
	1996	551	13	564	2.3
	1997	485	38	523	7.3
	Total	1480	99	1579	6.3
Ama-yotsumizo	1992	459	41	500	8.2
	1995	418	101	519	19.5
	1996	428	97	525	18.5
	Total	1305	239	1544	15.5
Shogatsu	1995	511	1	512	0.2
	1996	514	0	514	0.0
	Total	1025	1	1026	0.1
Kakiyama-gaki	1992	500	2	502	0.4
	1995	523	2	525	0.4
	1996	517	1	518	0.2
	Total	1540	5	1545	0.3
Seihakuji	1992	499	1	500	0.2
	1995	506	0	506	0.0
	1996	516	0	516	0.0
	Total	1521	1	1522	0.1

of hydrated pollen grains is illustrated in Fig 1. The cultivars, Zenjimaruru, Yamato-gosho, and Ama-yotsumizo, showed a major peak ≈ 45 to $50 \mu\text{m}$ and a minor peak at ≈ 64 to $67 \mu\text{m}$. The two distribution curves with these peaks seem to be separated at $\approx 60 \mu\text{m}$. The curve with a major peak is assumed to be normal pollen while that with a minor peak, giant pollen, which is ≈ 1.3 -fold larger on average than the normal pollen. Normal and giant pollen grains sorted with a $62 \mu\text{m}$ nylon mesh are illustrated in Fig. 2, and Table 1 presents data on the number of normal and giant pollen grains, and percentage of giant pollen in each cultivar. ANOVA showed that there was a significant ($P < 0.01$) cultivar effect for giant pollen production but the year effect was nonsignificant. Highest producer of giant pollen was ‘Ama-yotsumizo’ persimmon, followed by ‘Yamato-gosho’ and ‘Zenjimaruru’. Only a few giant pollen grains were observed in the other three cultivars.

RELATIVE NUCLEAR DNA CONTENT OF SORTED POLLEN GRAINS FROM ‘ZENJIMARURU’ PERSIMMON. Size distribution of nonsorted, sorted giant and sorted normal ‘Zenjimaruru’ pollen grains is presented in Fig. 3 and they were subjected to flow cytometric analysis (Fig. 4). Nonsorted pollen (Fig. 4A) and sorted normal pollen (Fig. 4C) showed a major peak at ≈ 260 channels, while sorted giant pollen (Fig. 4B) a peak at ≈ 500 channels. Fluorescent intensity of a minor peak of nonsorted pollen was the same as that of a major peak channels of giant pollen. Although there was some overlapping in grain size between normal and giant pollen, giant pollen had twice as much nuclear DNA as normal pollen, indicating that the giant pollen is unreduced and hexaploid (Fig. 4).

POLLINATION WITH SORTED POLLEN. Although the frequency of pollen tube growth was much lower than that in normal pollen,

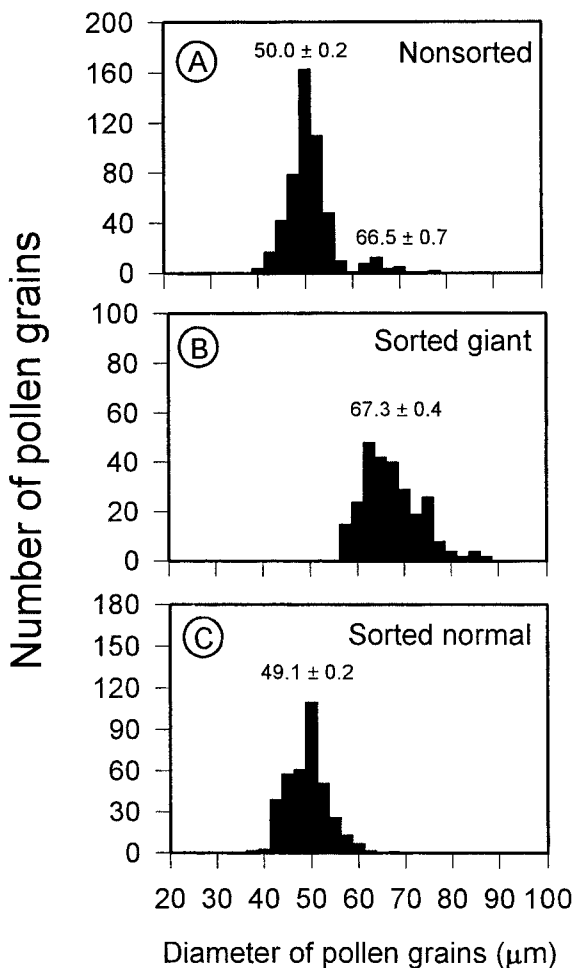


Fig. 3. Size distribution of ‘Zenjimaruru’ persimmon pollen grains sorted with a $62 \mu\text{m}$ nylon mesh. (A) Nonsorted pollen, (B) giant pollen, and (C) normal pollen. Mean grain diameters ($\pm \text{SE}$) for major and minor peaks are indicated.

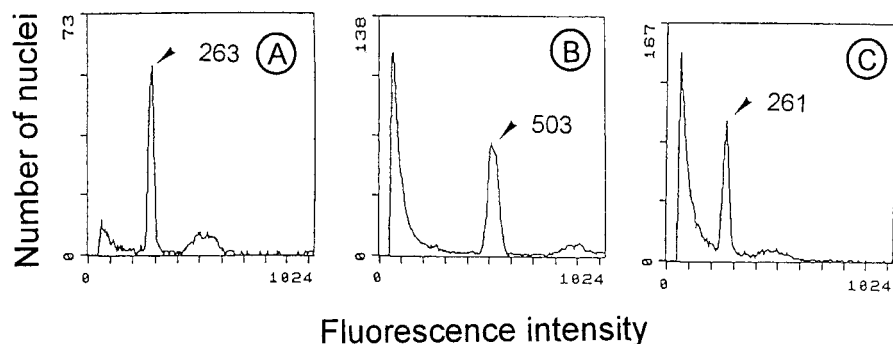


Fig. 4. Flow cytometry of pollen nuclear DNA of 'Zenjimaru' persimmon. (A) Nonsorted pollen, (B) giant pollen, and (C) normal pollen. Arrows and numbers indicate the peak channels of relative nuclear DNA content.

probably due to fewer numbers of pollen loaded, some giant pollen tubes emerged and penetrated into the style. The percentage germination of normal pollen on an agar medium was 69%, while that of giant pollen was 67% in 1996. Germination of normal versus giant pollen in 1997 was 60% and 57%, respectively. Chi-square tests revealed that the germination percentage between normal and giant pollen was not significantly different, but was significantly different between 1996 and 1997 ($P < 0.01$).

Fruit set, fruit diameter and weight, and seed number per fruit of 'Jiro' persimmon harvested 70 d after pollination in 1996 are presented in Table 2. Average seed number in fruit from pollination with giant and normal pollen was 1.8 and 5.2 seeds per fruit, respectively, and no seeds occurred in nonpollinated fruit. The longitudinal length of seeds from normal pollen pollination ranged almost from 14 to 17 mm ($x = 15.5 \pm 0.1$ mm SE), but that from giant pollen pollination produced two peaks separated by ≈ 12 mm. Most ($n = 529$) were < 10 mm, with a mean of 7.3 ± 0.1 mm (Fig. 5). About 20% of seeds ($n = 130$) from giant pollen were similar in size as those from normal pollen (15.0 ± 0.1 mm), which were probably derived from contaminating normal pollen. We determined from Fig. 5 that those seeds > 12 mm were perfect, while those < 12 mm were imperfect. The normal seeds produced by normal pollen had cotyledons visible to the naked eye. In contrast, the small seeds produced by the giant pollen were imperfect with a globular embryo visible only under a stereomicroscope.

IN VITRO EMBRYO CULTURE. All embryos from perfect seeds germinated quickly and developed into normal appearing plantlets during culture, but only a few embryos (2%) from imperfect seeds germinated (Table 3), producing abnormal shoots such as those lacking one of the cotyledons. In addition, embryos from imperfect seeds took 3 to 6 months to germinate in culture. These

abnormal plantlets, however, grew normally after the second subculture. Shoots from embryos of both perfect and imperfect seeds were proliferated, rooted, acclimatized, and transferred to the pots.

In an experiment repeated in 1997, the number of seeds per fruit at each inoculation date was 1.6 to 2.6 and 4.9 to 6.0 for giant pollen and normal pollen, respectively. Only seven of 245 embryos excised from imperfect seeds germinated with abnormal growth, whereas most embryos from perfect seeds grew normally (Table 4). Embryos from

perfect seeds were torpedo-like in shape 60 d after pollination. They developed cotyledons by day 70, while those from imperfect seeds remained at the globular stage even at maturity (170 d) when globular embryos embedded in degenerating endosperm were observed. Optimum timing for embryo rescue could not be determined (Table 4), because survival did not appear to be affected by fruit maturity.

PLOIDY LEVELS OF EMBRYOS AND REGENERATED PLANTLETS. Ploidy levels of calli induced from plantlets derived from embryos of imperfect and perfect seeds were determined by flow cytometry. The nuclei from somatic calli of hexaploid 'Jiro' and nonaploid 'Hiratanenashi' were used as reference standards (Fig. 6). The fluorescence peak of calli from imperfect seeds coincided with that of nonaploid 'Hiratanenashi' while the fluorescence peak of calli from perfect seeds coincided with that of the female hexaploid parent 'Jiro'. It was also determined that the chromosome number of root tip cells from the plantlets regenerated from imperfect seeds was $2n = 9x = 135$ (Fig. 7).

Discussion

In Japanese persimmon, unreduced hexaploid pollen (giant pollen) was produced naturally at various frequencies (0.1% to 15.5%) depending on the cultivar. Growth of embryos produced by the giant pollen was suspended at the globular stage and showed abnormal growth at an early stage in vitro but became normal after subculture, resulting in plantlets that had a nonaploid chromosome number ($2n = 9x = 135$).

Morphologically, the hydrated pollen grains could be classified into two groups with diameters $>$ or $<$ $60 \mu\text{m}$. This enabled giant pollen to be separated mechanically from normal pollen and most pollen retained by a $62 \mu\text{m}$ mesh screen was verified by flow cytometric analysis to be unreduced. The presence of unreduced pollen has been reported in *Citrus* L. (Esen and Soost, 1971), *Malus* Mill. (Zhang et al., 1988a), *Prunus* L. (Dermen, 1937), *Vaccinium* L. (Cockerham and Galletta, 1976; Ortiz et al., 1992a,

Table 2. Effects of pollination with giant and normal pollen grains of 'Zenjimaru' persimmon on fruit set, growth, and number of seeds per fruit in 'Jiro' persimmon measured 70 d after pollination in 1996.

	Pollinated with		
	Giant pollen	Normal pollen	None
No. flowers pollinated	481	58	54
No. fruit set	363	50	25
Fruit set (%)	75.5	86.2	44.4
Fruit diameter (mm) ^z	56.8 ± 0.2	59.6 ± 0.4	55.1 ± 0.5
Fruit wt (g) ^z	76.4 ± 0.6	88.0 ± 1.7	71.0 ± 0.5
No. seed/fruit ^z	1.8 ± 0.1	5.2 ± 0.2	0.0 ± 0.0

^zMean \pm SE.

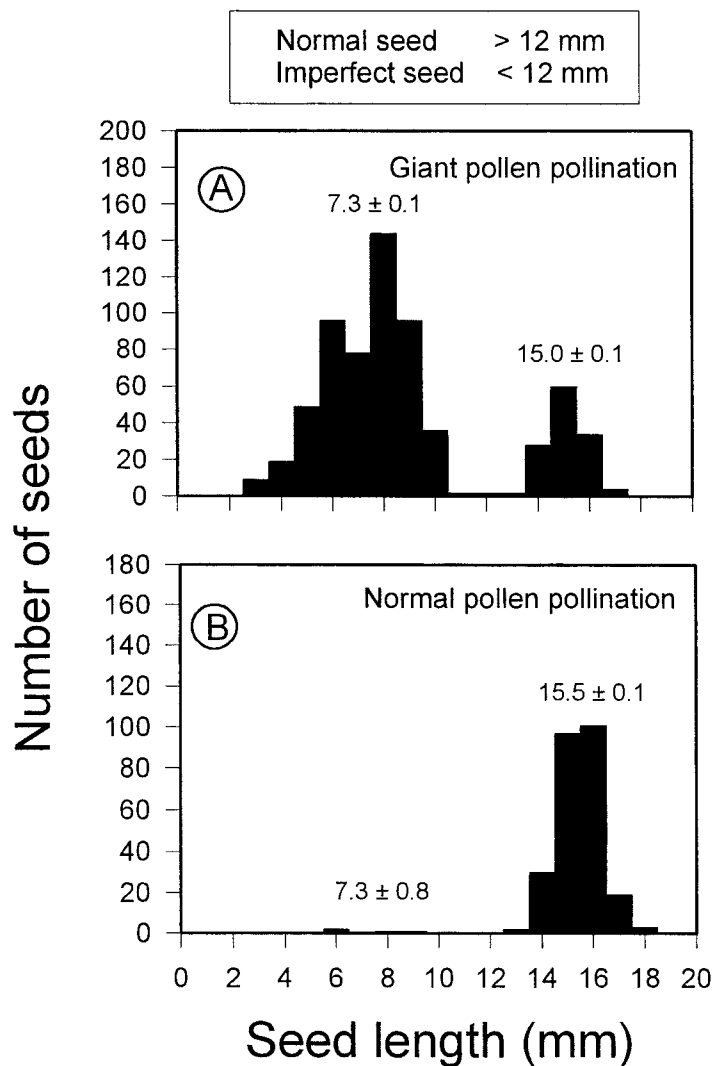


Fig. 5. Size distribution of seeds obtained from the fruit pollinated with (A) giant pollen and (B) normal pollen. Mean lengths (\pm SE) of imperfect (left) and perfect (right) seeds are indicated.

Table 3. Number of embryos developing into plantlets during embryo culture (1996). Data in the table show number of developed embryos per number of cultured embryos. Embryos were excised from seeds 70 d after pollination with normal or giant pollen.

Seed type	Pollination with	
	Giant pollen	Normal pollen
Perfect seed	92/92	132/132
Imperfect seed	6/294	0/2

Table 4. Number of embryos developing into plantlets after inoculation at different dates (1997). Data in the table represent number of developed embryos/number of cultured embryos.

Pollination with	Days from pollination to inoculation						Total
	50	60	70	80	90	170	
Normal pollen							
Perfect seed	17/34	47/49	25/25	26/26	22/23	---	137/157
Giant pollen							
Perfect seed	10/16	4/4	32/32	22/22	26/26	---	94/100
Imperfect seed	2/46	1/44	0/39	1/50	2/49	1/17	7/245

1992b; Shoemaker-Megalos and Ballington, 1988), and *Vitis* L. (Martens et al., 1989; Zhang et al., 1998b). Unreduced gametes are generally considered to be the source mechanism for natural polyploids (Harlan and DeWet, 1975). The frequency of unreduced pollen in persimmon varied with the cultivar examined. This means that the production of unreduced pollen is determined by genetic factors, although the mechanisms remain unknown. More cultivars should be examined to determine whether formation of unreduced pollen is common in Japanese persimmon. Environmental factors such as low or high temperatures often seem to impair meiosis, resulting in formation of unreduced gametophytes (Bretagnolle and Thompson, 1995; Iwamasa and Iwasaki, 1963). Although only unreduced pollen was investigated in this study, unreduced eggs may also be produced and become a source for polyploid formation.

In an interploid crossing, an endosperm genomic balance is important for successful hybridization. A 2:1 maternal and paternal ratio in the endosperm is generally necessary for endosperm viability but if the ratio is violated, the seed usually tends to abort or be underdeveloped, though many exceptions exist (Bretagnolle and Thompson, 1995; Sanford, 1983). In this case of persimmon with reduced egg crossed with unreduced pollen, the ratio would be 6:6 (1:1), violating the normal ratio. This may have caused poor development of embryos. A closer examination will be needed to determine the relevance of endosperm balance in the formation of nonaploid persimmon in nature.

In the present experiment, 'Zenjimaru' was used as the pollen source for pollination because it is an abundant pollen producer. Although a large quantity of pollen was hydrated and sorted carefully with a 62 μ m nylon mesh, mixture of some giant and normal pollen grains was inevitable. This caused \approx 20% formation of normal seeds from pollination with giant pollen. If we had used a mesh screen $>$ 62 μ m for sorting, such contamination of normal pollen may have been minimized further.

Plantlets recovered from immature embryos had a nonaploid chromosome number, as indicated by flow cytometric analysis and chromosome counting. Though only a small number of sustained embryos germinated in this study, the number might be increased by modification of the culture medium.

Three nonaploid cultivars have been found thus far in the persimmon germplasm collection at Kyoto University, namely 'Hiratanenashi', 'Miyazaki-tanenashi', and 'Watarizawa', all seedless astringent cultivars (Zhuang, 1990). The origins of these nonaploid cultivars derived from open pollination is difficult to determine, but the novel method of using sorted giant pollen in pollination combined with in vitro culture techniques is a useful technique for breeding seedless nonaploid persimmons.

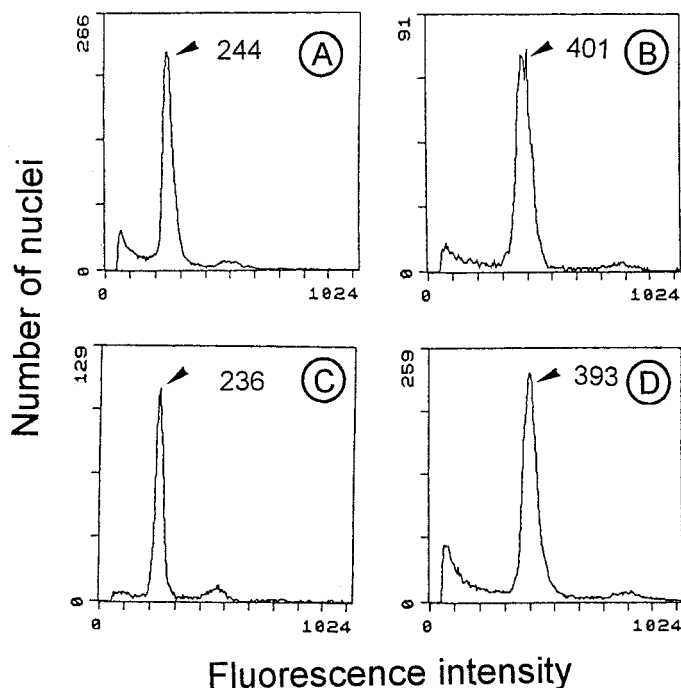


Fig. 6. Flow cytometry of nuclear DNA from cultured embryos. (A) Somatic callus cells of 'Jiro' ($2n = 6x = 90$), (B) somatic callus cells of 'Hiratanenashi' ($2n = 9x = 135$), (C) embryo callus obtained from a perfect seed, and (D) embryo callus obtained from an imperfect seed. Arrows and numbers indicate the peak channels of relative nuclear DNA content.

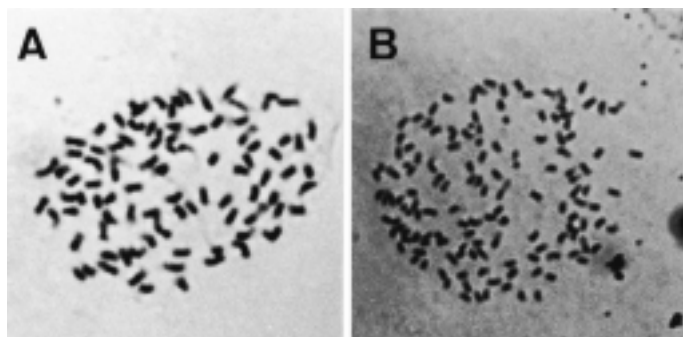


Fig. 7. Root tip chromosomes of (A) hexaploid 'Jiro' persimmon and (B) nonaploid plant derived from fertilization of 'Jiro' persimmon with giant pollen from hexaploid 'Zenjimarū' persimmon.

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