Title of the article:

A male determinant gene in diploid dioecious *Diospyros*, *OGI*, is required for male flower production in monoecious individuals of Oriental persimmon (*D. kaki*)

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Abstract

The sexuality of crops is an important trait affecting production and breeding efficiencies. Recently, a Y-chromosome-encoded gene, designated *Oppressor of MeGI (OGI)*, has been shown to play a central role in dioecious sex determination in diploid persimmons (*Diospyros* spp.). However, little is known about the relationship between *OGI* and the irregular sexuality in polyploid persimmon (*D. kaki*). *D. kaki*, which is mostly hexaploid with some nonaploid individuals, is mainly constituted of monoecious and female individuals. In this study, we attempted to investigate the association of sexuality with *OGI* allele diversities/dosages in a wide range of *D. kaki* cultivars. The results indicated that *OGI* is indispensable for the monoecious phenotype producing male flowers, which suggested a conserved role for *OGI* as a key to expressing maleness in *D. kaki*, as in dioecious diploid *Diospyros*. However, the existence of *OGI* alleles did not always lead to production of male flowers in *D. kaki*. The dosage or composition of *OGI* alleles had no substantial effect on the sexuality of the cultivars tested. We discuss the potential use of the obtained information on *OGI* allele diversities/dosages for breeding of *D. kaki* cultivars.

Highlights

- *OGI* is basically indispensable for producing male flowers in Oriental persimmon.
- Allelic status of *OGI* has no substantial effect on the sexuality.
- *OGI* will be applicable for marker assisted selection for sexuality in Oriental persimmon.

Keywords: dioecy, *Diospyros*, marker-assisted selection, monoecy, *OGI*, sex chromosome

1. Introduction

Separate sexes in plants, as represented by dioecy (the presence of separated male and female individuals), monoecy (formation of separate male and female flowers in an individual) or their variants, is one of the main strategies to maintain genetic diversity in a species (Charlesworth, 2013). More than 75% of flowering plant species are supposed to show hermaphrodite systems, while a minority of plant species have evolved separate sexes (Ming et al., 2011; Yampolsky and Yampolsky, 1922). In crops with separate sexes, artificial control of sexuality is an important issue for both production and breeding (Rowlands, 1964). In general, fruit production depends on the amount of female flowers/trees, while male flowers/trees are indispensable for fertilization and seed formation. Thus, the balances of male and female are critical for production efficiency. Cross breeding for new superior cultivars depends on potential fruit characters of both parents. In cross-breeding of dioecious fruit trees, breeders usually have to select a male parent whose genetic effects on fruit characteristics are unknown.

In contrast to the sex determination system in animals, the genetic factors controlling plant sexualities are less known (Bachtrog, 2013). Genes involved in the expression of monoecy or its associated sexualities have been isolated in maize (*Zea mays*) and *Cucumis* species (Chuck et al., 2007; Martin et al., 2009). Conversely, the genetic determinants for dioecy, which are defined as located on the “sex chromosome”, have remained unidentified for a long time, although plant sex chromosomes have been studied for over a century (Charlesworth, 2015; Ming et al., 2011). This is partially because of the heteromorphism of old plant sex chromosomes, which is generally associated with a long suppressed recombination region. In the XY heterogametic male system, this region is called the “male specific region of the Y-chromosome (MSY)” (Charlesworth, 2013; Ming et al., 2011). Recently, progress was made on the homomorphic sex chromosomes in some dioecious plant species, such as Caucasian persimmon (*D. lotus*), kiwifruit (*Actinidia* spp.) and grape (*Vitis* spp.) (Akagi et al., 2014a, b; Picq et al., 2014; Zhang et al., 2015). In *D. lotus*, a genome-wide cataloging of male-specific subsequences with high coverage revealed the sex determinant, *OGI*. *OGI* is a Y-chromosome-encoded gene that produces small-RNAs targeting an autosomal homologous gene, *Male Growth Inhibitor (MeGI)*, acting as a repressor of male organ development (Akagi et al., 2014a). *OGI* is conserved specifically in male individuals in a wide variety of *Diospyros* species, which corresponds well with the notion that the establishment of this dioecious sex determination system predates the divergence of the *Diospyros* genus,

and the species nested in this genus share the same sex determination mechanisms (Akagi et al., 2014a). However, hexaploid persimmon (*D. kaki*) shows flexible sexuality generally not observed in other diploid *Diospyros* species; it is mainly constituted of female and monoecious trees (Yonemori et al., 1993), with male trees occurring rarely (Xu et al., 2008; Yakushiji et al., 1995). Importantly, preliminary data
65 suggested that the monoecious trait was associated with the existence of the Y-chromosome in *D. kaki*, although the details remain to be elucidated (Akagi et al., 2014a, b; Kajita et al., 2015).

Dioecious plant species that have experienced polyploidization often show a change in sexuality to “intersexuality”, such as a hermaphroditic system. Although the molecular mechanisms behind the change in sexuality with polyploidization are still unknown, the allele balances of X-chromosomes and
70 autosomes are supposed to be an important factor for the expression of intersexuality in some XY-type dioecious species, such as *Rumex* and *Humulus* (Ming et al., 2007, 2011; Ono, 1935; Parker and Clark, 1991). Conversely, papaya (*Carica papaya*), *Silene latifolia*, and kiwifruit (*Actinidia* spp.) show no distinct changes in sex expression with polyploidization (Ming et al., 2011). The mechanisms underlying these differences in sexuality have not been elucidated, presumably because the sex determinants remain
75 to be identified. In hexaploid persimmon, six homologous chromosomes are suggested to have autohexaploid or autoallohexaploid inheritance, at least in the locus regulating the astringency of the fruit (Akagi et al., 2009, 2012). However, the relationship between the polyploid nature or allele balances of the sex chromosomes in *D. kaki* and its flexible sexuality remains to be clarified. Here, we aimed to examine the association of sexuality with the allele diversities/dosages of the Y-encoded *OGI* gene in
80 various *D. kaki* cultivars.

2. Materials and Methods

2.1. Definition of sexuality in *D. kaki* cultivars

One-hundred and seventy-two *D. kaki* cultivars, which are maintained in the orchard of Kyoto
85 University, Kyoto, Japan, were assessed for their sex expression patterns in 2007–2016. Most of the 172 cultivars were hexaploid ($2n = 90$, $x = 15$) but two were nonaploid (Table 1). Here, we define “monoecious (FM)” and “female (F)” as cultivars bearing both male and female flowers, and bearing only female flowers, respectively. FM-type cultivars occasionally bear hermaphrodite-like flowers with a developed ovary and fully functional stamen. The sexualities of the 172 cultivars were also annotated

90 according to a previous report (Fruit Tree Experiment Station of Hiroshima Prefecture, 1979) and the
NIAS Genebank database (https://www.gene.affrc.go.jp/databases-plant_search_char.php) (Table 1). Note
that there were some differences in sexuality among our observations, the report and the database, as
shown in Table 1. In this study, cultivars were annotated as monoecious when the existence of male
flowers was confirmed in at least our observations or the latest database of the NIAS Genebank.

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2.2. PCR detection of *OGI* in *D. kaki* cultivars and F_1 progeny

Genomic DNAs were extracted from all the cultivars described above using the CTAB method
(Doyle and Doyle, 1987), and then *OGI* was amplified by PCR using two primer pairs, *OGI*-candF1 and
OGI-spR (Akagi et al., 2014a), and *OGI*-F1 (5'-AATGCAAGCGGATACACACA-3', T_m : 60.1) and
100 *OGI*-R2 (5'-CCGTCAATTTTGAGGGAGAG-3', T_m : 59.7), with a program of 95°C for 3 min followed
by 32 cycles of 95°C for 20 s, 56°C for 15 s, and 72°C for 1 min. The genomic fragment of *D. kaki*
ANTHOCYANIDIN REDUCTASE (*DkANR*; accession no. AB195284) was amplified as a reference to
confirm the presence of genomic DNA, as described by Akagi et al. (2010). Fifty-nine of the 172 cultivars
gave *OGI* amplification and were subjected to quantitative genotyping and sequencing analysis of *OGI*, as
105 described below.

To estimate the manner of inheritance and the dosage of *OGI* alleles, PCR analyses, using the
OGI-candF1 and *OGI*-spR primers and the PCR program described above, were conducted to detect *OGI*
in *D. kaki* F_1 populations derived from four crosses; the 310-line ('Luo-tian-tian-shi' $\times$ 'Taishu'), the
RI-line ('Luo-tian-tian-shi' $\times$ 'Iwasedo'), the RO-line ('Luo-tian-tian-shi' $\times$ 'Okugosho'), and the RY-line
110 ('Luo-tian-tian-shi' $\times$ 'Yotsumizo'), as previously reported (Ikegami et al., 2005, 2006). The expected
ratio of *OGI*-positive individuals under the autohexasomic and autoallohexasomic inheritance models in
the F_1 progeny was calculated according to Akagi et al. (2010), as suggested for the
ASTRINGENCY-linked loci. The fit of the *OGI*-positive ratio in each F_1 progeny to the expected ratio was
assessed by the Chi-square test.

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2.3. Quantitative genotyping of *OGI*

The 59 cultivars showing putative *OGI* amplification were subjected to quantitative genotyping
of *OGI* by qPCR using a LightCycler 480 (Roche Diagnostics, Mannheim, Germany), according to a

previous report on the quantitative genotyping of *ASTRINGENCY*-linked loci (Akagi et al., 2010) with small modifications. This corresponded to detection of the dosage of the Y-chromosomes. The primer set OGI-F2 (5'-AACCCCATCGCATTTGATAA-3', T_m : 60.2) and OGI-R2 was used to detect the *OGI* genomic region. The primer sets DkANR-RTF1 and DkANR-RTR1, and Consv-5F and Consv-5R were used to detect the reference regions *DkANR* and *L5R*, which encode an anthocyanidin reductase and putative ORF with no annotation, respectively (Akagi et al. 2010). Various *Diospyros* species show high sequence similarity for these reference regions (T. Akagi unpublished), and six alleles in each site are conserved among the *D. kaki* cultivars as reported by Akagi et al. (2010, 2012). qPCR analyses were conducted under the following conditions: 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s, using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). Three technical replicates were performed for each cultivar.

2.4. Allele sequencing of *OGI* in *D. kaki* cultivars

The PCR products amplified from the 59 cultivars in the PCR detection of *OGI*, using two primer pairs described above, OGI-candF1 and OGI-spR, and OGI-F1 and OGI-R2, with the PCR conditions described above, were used for sequencing of almost the full length of *OGI*. After alkaline phosphatase and exonuclease treatment using ExoSAP-IT (Affymetrix, Tokyo, Japan), the treated PCR products were directly subjected to sequencing analysis. Furthermore, the amplified *OGI* sequences from three of the 59 cultivars ('Nagara', 'Zenjimar', 'Hanagosho') were purified using a FastGeneTM Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and then cloned into pGEM-T easy vectors to be sequenced. Unreliable residues of the sequences were cut off using BioEdit. For direct sequencing analysis, the SNPs in the nucleotide sequences were visually characterized from the overlapped waves. For TA-cloning sequence analysis, about 10 clones were sequenced and the sequences obtained were aligned by using MAFFT v. 7 (Katoh and Standley, 2013) to detect informative SNPs among the alleles. Haplotypes among the SNPs observed across the 59 cultivars were examined, and the association between the *OGI* allele compositions and individual sexualities was assessed by Fisher's exact test.

3. Results and Discussion

3.1. Association in *OGI* gene and the sexuality in *D. kaki* cultivars

PCR amplification for *OGI* was positive in 59 of the 172 cultivars assessed in this study (Fig. 1,

Table 1). The PCR results using two different primer pairs were consistent (data not shown). All 35
monoecious cultivars (or cultivars bearing both male and female flowers) had *OGI* alleles in their
genomes (Fig. 1). Among 137 female cultivars tested, 24 cultivars showed *OGI* PCR amplification, while
other 113 cultivars had no *OGI* alleles. Thus, these results suggested that the presence of *OGI* allele is
essential for expressing maleness in polyploid *D. kaki*, as in other dioecious *Diospyros* species (Akagi et
al., 2014a). The results also suggested that the presence of an *OGI* allele is not always genetically
sufficient for expression of maleness. Considering that monoecious *D. kaki* cultivars that are genetically
male trees carrying *OGI* alleles have the ability to bear female flowers in addition to male flowers, it is
possible that expression of *OGI* is mostly silenced or under the control of environmental factors in *D.*
kaki.

Compared with diploid *Diospyros* species, polyploid persimmons potentially have much more
complicated allele compositions for their sex chromosomes (Table 2). If we consider the heterozygotic
composition of hexaploid persimmons, the sex chromosome composition can be from simplex (XXXXXY,
single dose for Y) to quintuplex (XYYYYYY, pentavalent dose for Y), as with other loci involved in fruit
astringency (Akagi et al., 2010). Quantitative genotyping of *OGI* locus by qPCR analysis showed *OGI*
allele dosage variations from a single to triple doses in the 59 cultivars with *OGI* (Table 3), although we
could not rule out the possibility that *OGI* was duplicated on the same Y chromosome or in the autosomal
region. Our preliminary qPCR genotyping results obtained using a marker linked to *OGI*, D1SxAF4S
(Akagi et al., 2014b), for some duplex cultivars (data not shown) were consistent with the *OGI* qPCR
genotyping results, indicating that our analyses could precisely detect allele dosage of *OGI* (or
Y-chromosomes). Quantitative genotyping indicated a weak association between the *OGI* allele dosage
and the ability to produce male flowers (Table 3, $P = 0.21$). The cultivar showing triplex *OGI*, ‘Tohachi’,
is a representative male-rich cultivar, at least in the experimental orchard of Kyoto University. Some of
the cultivars showing duplex *OGI*, such as ‘Iwasedo’, ‘Hanagoshō’, and ‘Taishū’, are also representative
monoecious cultivars. However, even cultivars with duplex *OGI*, such as ‘Kunitomi’, bear only female
flowers. Thus, we concluded that the dosage of *OGI* is not a crucial determinant of monoecy. The results
from the quantitative genotyping were supported by the *OGI* allele segregation ratio in the four
segregating populations. In these populations, the ratio of *OGI* positive in the crosses of ‘Luo-tian-tian-shi’

x ‘Taishu’ or ‘Iwasedo’ (see Table 3 for their estimated *OGI* dosages by qPCR) gave better fit in nulliplex x duplex, than in nulliplex x simplex (Table 4). On the other hand, ‘Luo-tian-tian-shi’ x ‘Okugosho’ or ‘Yotsumizo’ showed better fit in nulliplex x simplex. The segregation ratio of them mostly fitted to the expected ratio under autohexasomic or autoallohexasomic inheritance modes, as is the case with the *ASTRINGENCY* locus (Akagi et al., 2012) (Table 4).

Some monoecious persimmon cultivars bear morphologically hermaphrodite flowers in addition to male flowers, although the genetic and environmental factors controlling hermaphrodite flower formation are unknown. Some dioecious plant species, such as *Rumex* or *Humulus*, occasionally show intersexuality (hermaphrodite and male/female flowers) upon polyploidization, for which the dosage balance between the X-chromosomes and autosomes is critical (Grant et al., 1994; Ming et al., 2007; Ono, 1935; Parker and Clark, 1991). Although the frequency of hermaphroditic flowers in monoecious persimmon cultivars remains to be characterized fully, the cultivars used in this study showed no clear association between hermaphrodite-like (intersexual) flower formation and the balance of X- or Y-chromosomes to autosomes (Table 3). This situation fits the so-called “active-Y” system found in kiwifruit or *Silene* spp., in which sexuality is determined by the presence of Y-chromosome but not affected by the ratio in sex chromosomes and autosomes (Ming et al., 2011; Seal et al., 2012). The sex determination system in persimmons can be also described as “active-Y” because the dosage of *OGI* seems to be unimportant for expression of maleness and intersexuality.

3.2. Allele sequencing of *OGI* in *D. kaki* cultivars

Although the sizes of PCR amplicons of *OGI* were identical, two kinds of SNPs were found in the directly sequenced PCR products of the *OGI* alleles from 59 cultivars. Alignments of about 10 each sequences obtained from separately cloned *OGI* alleles in the 12 cultivars gave the consistent results to the results obtained from direct sequencing analysis and the phases (or haplotypes) of the clones were likely conserved among the cultivars (Fig. 2A and Table 3). Furthermore, the allele types of these two SNPs were consistent with the estimated *OGI* allele dosage that was estimated by qPCR. In the cultivars with duplex *OGI*, for instance, the two SNPs haplotypes were expected to exist in 1:1, except ‘Taishu’ which seemed to have two identical *OGI* sequences (accession LC120366, Table 3). On the other hand, in the cultivars with simplex *OGI*, only one of the two SNPs haplotypes were detected. No association or

co-segregation was detected between the *OGI* allele composition and individual sexuality in the tested persimmon cultivars (Fig. 2B, Table 3). One of the two SNPs was located inside the inverted repeat region which may produce small-RNAs by hybridizing with the forward repeat region (Fig. 2A). The other one was in an intron which is not critical for the production of small-RNAs in *OGI*. Sequence variations in the target of the *OGI* gene, *MeGI*, might be associated with the ability to bear male flowers, because the *OGI* smRNAs act only when transitive RNAi is stimulated for the *MeGI* mRNA (Akagi et al., 2014a). Comprehensive sequencing of the putative polysomic alleles of *MeGI* and investigation of the sequence affinity to *OGI* alleles may further clarify the molecular basis of the complicated and unstable sexuality in *D. kaki*.

3. Conclusion

This study suggested that the presence of *OGI* alleles is indispensable for male flower formation in *D. kaki*, but not sufficient to express a monoecious phenotype. Future physiological analysis, especially focusing on the smRNA regulatory network involving *OGI* and *MeGI*, would give new insights into this unique sex expression mechanism in *D. kaki*. Although the allele dosage might have a small effect on male flower formation ability, variations in the *OGI* sequence seemed to have little effect on sex determination in the cultivars tested in this study. Information on the dosage of *OGI* would be important to determine parentages in breeding. In a hexasomic inheritance mode, crossing a female parent with nulliplex *OGI* and a monoecious parent with multiplex *OGI* would produce at least 80% offspring with *OGI* alleles (Table 2), which could potentially become monoecious trees. The production of male flowers is not desirable for persimmon cultivation (Hayashi et al., 2001) and thus marker-assisted selection of *OGI*-null female offspring could be a good strategy for future persimmon breeding. Furthermore, selection of male parents with less *OGI* alleles would reduce the ratio of potentially monoecious offspring, resulting in efficient breeding of new female cultivars. A drawback of MAS using the *OGI* sequence is that female individuals are present among the individuals with *OGI* gene, which would be judged as individuals potentially producing male flowers and removed during selection.

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Figure legends

Figure 1. (A) PCR detection of *OGI* in 35 monoecious cultivars (FM; 1–35) and 44 female cultivars (F; 36–79). Each number corresponds to the cultivar shown in Table 1. Arrows (ca 970-bp) indicate the expected size of amplification products. m, DNA size marker; M, *D. lotus* ‘Kunsenshi’ male tree (positive control); F, ‘Kunsenshi’ female tree (negative control); N, distilled water (negative control). *D. kaki* *ANTHOCYANIDIN REDUCTASE* (*DkANR*) was amplified as a reference to confirm the presence of genomic DNA. (B) Number and ratio of cultivars that produced *OGI* amplification among the 172 cultivars used in this study. The results of monoecious (FM) and female (F) cultivars are shown separately. OGI+ and OGI- represent *OGI*-positive and -negative, respectively.

Figure 2. (A) Positions of the two SNPs (SNP-1 and SNP-2) detected by sequencing analyses of *OGI*. The positions of SNP-1 (C or T) and SNP-2 (T or C), which were observed across the 59 cultivars carrying *OGI*, are shown with grey triangles on the schematic structure of *OGI*. Boxes and lines represent the putative exons and introns of the *OGI* gene, respectively. The phases of these two SNPs were conserved among the cultivars as shown with dashed line arrows, making two kinds of haplotypes (haplotype-1 and -2) for *OGI*. (B) *OGI* allele compositions and individual sexuality in the tested persimmon cultivars. H1 and H2 indicate cultivars with haplotype-1 and -2 for *OGI*, respectively, and “H1 + H2” represents those with both. FM, monoecious cultivars; F, female cultivars; n.s., not significant for the association of *OGI* allele compositions and individual sexuality.

Table 1

Definition of sexuality and detection of *OGI* in various persimmon cultivars.

No.	Cultivar	Reference sexuality ^z			Sexuality in this study	<i>OGI</i> ^v
		KT ^y	HS ^x	NIAS ^w		
1	Akazu	FM	F	-	FM	p
2	Amayotsumizo	FM	FM	-	FM	p
3	Cal. Fuyu	FM	-	-	FM	p
4	Egoshō	FM	FM	FM	FM	p
5	Emon	FM	FM	F	FM	p
6	Fudegaki	FM	FM	FM	FM	p
7	Fujiwaragoshō	FM	FM	F	FM	p
8	Hanagoshō	FM	FM	FM	FM	p
9	Hazegoshō	FM	FM	FM	FM	p
10	Ibogaki	FM	F	F	FM	p
11	Issaigaki	FM	-	-	FM	p
12	Iwasedo	FM	FM	FM	FM	p
13	Kakiyamagaki	FM	FM	FM	FM	p
14	Kanshu	FM	-	-	FM	p
15	Kikumanju	FM	-	-	FM	p
16	Kyara	FM	FM	F	FM	p
17	Meotogaki	FM	FM	FM	FM	p
18	Muraya	FM	FM	FM	FM	p
19	Mushirodagoshō	FM	FM	-	FM	p
20	Nishimurawase	FM	FM	FM	FM	p
21	Okugoshō	FM	FM	FM	FM	p
22	Saburouza	FM	FM	F	FM	p
23	Seihakujō	FM	FM	FM	FM	p
24	Shiroutodamashi	FM	FM	FM	FM	p
25	Shougatsu	FM	FM	FM	FM	p
26	Shoujō	FM	FM	FM	FM	p
27	Shouzaemon	FM	F	-	FM	p
28	Taishu	FM	-	-	FM	p
29	Taiwan-shoushi	FM	FM	FM	FM	p
30	Tohachi	FM	FM	-	FM	p
31	Toyoka	F	FM	FM	FM	p
32	Yamagaki	FM	-	-	FM	p
33	Yamatogoshō	FM	F	F	FM	p
34	Yotsumizo	FM	FM	FM	FM	p
35	Zenijamaru	FM	FM	FM	FM	p
36	Atagobou	F	F	-	F	p
37	Beniemon	F	F	F	F	p
38	Beniwase	F	F	F	F	p
39	Chagone	F	-	-	F	p
40	Chichibu-issaigaki	F	-	-	F	p
41	Deshimaru	F	F	-	F	p
42	Kawagone	F	-	-	F	p
43	Koudagoshō	F	F	-	F	p
44	Kunitomi	F	F	F	F	p
45	Mikado	F	F	F	F	p
46	Monpei	F	F	F	F	p
47	Nagara	F	F	F	F	p
48	Nanshi	F	-	-	F	p
49	Nitari	F	F	-	F	p
50	Oogoshō	F	F	F	F	p
51	Ooniwa	F	F	F	F	p
52	Saishō	F	F	F	F	p
53	Sanenashi	F	F	F	F	p
54	Shimokitahagakushi	F	F	-	F	p
55	Shouro	F	F	-	F	p
56	Suruga	F	F	F	F	p
57	Tenjingoshō	F	FM	F	F	p
58	Yashima	F	F	-	F	p
59	Yoshino	F	F	-	F	p
60	Aburatsubo	F	F	F	F	n
61	Aizumishirazu	F	F	F	F	n
62	Amahyakume	F	FM	F	F	n
63	Anzai	F	F	F	F	n
64	Aoso	F	F	-	F	n
65	Atago	F	F	F	F	n
66	Benigoshō	F	F	F	F	n
67	Benisakigake	F	-	-	F	n
68	Butsushigano	F	F	F	F	n
69	Chousengaki	F	F	-	F	n
70	Dennai	F	F	F	F	n
71	Doujohachiya	F	FM	F	F	n
72	Eboshi	F	F	F	F	n
73	Fuyu	F	F	F	F	n
74	Gionbou	F	F	F	F	n
75	Gobangaki	F	F	F	F	n
76	Gofu	F	F	F	F	n
77	Hacchiuri	F	F	F	F	n
78	Hagakushi	F	F	F	F	n
79	Hiragaki	F	F	F	F	n
80	Hiratanenashi ^h	F	F	F	F	n
81	Hiratanenashi-edagawari	F	-	-	F	n
82	Hiroshima-shimofuri	F	F	-	F	n
83	Houkikoushi	F	F	F	F	n
84	Houkisuishi	F	F	-	F	n
85	Ichidagaki	F	F	F	F	n
86	Ichiryō	F	F	F	F	n
87	Inayama	F	F	F	F	n
88	Izaemon	F	F	F	F	n
89	Jiro	F	F	F	F	n
90	Jou-hon-shi	F	F	F	F	n
91	Jouren	F	F	F	F	n
92	Kaibuchi	F	F	F	F	n
93	Kanro	F	F	-	F	n
94	Karasumi	F	F	-	F	n
95	Kawabata	F	F	F	F	n
96	Keizanbanshi	F	F	F	F	n
97	Kikuhira	F	F	F	F	n
98	Kiyosu-tanenashi	F	F	F	F	n
99	Koedagaki	F	-	F	F	n
100	Kojimawase	F	-	-	F	n
101	Komino	F	F	-	F	n
102	Koushuhyakume	F	F	F	F	n
103	Kouyouwase	F	-	-	F	n
104	Kuramitsu	F	F	F	F	n
105	Kurogaki	F	F	F	F	n
106	Kurokuma	F	-	F	F	n
107	Luo-tian-tian-shi	F	-	-	F	n
108	Maekawajiro	F	F	F	F	n
109	Matsumotowasefuyu	F	F	F	F	n
110	Midai	F	F	F	F	n
111	Mie	F	F	-	F	n
112	Mikatanigoshō	F	F	F	F	n
113	Mino	F	F	F	F	n
114	Miyazaki-tanenashi	F	F	F	F	n
115	Mizushima	F	F	F	F	n
116	Nanyousuishi	F	-	-	F	n
117	Niikura	F	F	F	F	n
118	Nijugaki	F	-	-	F	n
119	Nikorokonashiba	F	F	-	F	n
120	Obishi	F	F	F	F	n
121	Okujisha	F	F	-	F	n
122	Okumyoutan	F	F	F	F	n
123	Okusaijyo	F	-	-	F	n
124	Onihira	F	F	-	F	n
125	Oohachiya	F	-	-	F	n
126	Ookouzu	F	F	F	F	n
127	Oonaga	F	F	-	F	n
128	Ootenenashi	F	-	-	F	n
129	Ooyotsumizo	F	F	-	F	n
130	Oshoro	F	F	F	F	n
131	Oumidansu	F	F	F	F	n
132	Pansu	F	F	F	F	n
133	Pa-shi	F	F	F	F	n
134	Rashoutomoe	F	-	-	F	n
135	Rokuzaemon	F	F	-	F	n
136	Saijyo	F	F	F	F	n
137	Sakata	F	F	-	F	n
138	Sakushu-mishirazu	F	F	F	F	n
139	Sangokuichi	F	F	F	F	n
140	Sanja	F	F	F	F	n
141	Shakokushi	F	F	F	F	n
142	Shimamyoutan	F	F	-	F	n
143	Shinpei	F	F	-	F	n
144	Shinshu	F	-	-	F	n
145	Soushu	F	-	-	F	n
146	Spur Hiratanenashi	F	F	-	F	n
147	Sugitawase	F	-	F	F	n
148	Suimyou	F	-	-	F	n
149	Suishi	F	F	F	F	n
150	Sunami	F	-	-	F	n
151	Taiwan-koushi	F	F	-	F	n
152	Takase	F	F	F	F	n
153	Takeda	F	F	-	F	n
154	Takura	F	F	-	F	n
155	Ta-mo-pan	F	F	F	F	n
156	Tanenashi-jiro	F	-	-	F	n
157	Tenpougai	F	-	-	F	n
158	Tenryubou	F	FM	-	F	n
159	Tokudagoshō	F	F	-	F	n
160	Tonewase ^a	F	-	-	F	n
161	Totsutanenashi	F	-	-	F	n
162	Toushi	F	F	-	F	n
163	Wakamiya	F	F	F	F	n
164	Wasamatsuo	F	F	F	F	n
165	Wasejsha	F	F	F	F	n
166	Watarisawa	F	F	-	F	n
167	Yamagata-benigaki	F	FM	F	F	n
168	Yamato	F	F	F	F	n
169	Yatsudera	F	F	F	F	n
170	Yokono	F	F	F	F	n
171	Yomizo	F	-	-	F	n
172	Yotsuyasaijyo	F	F	F	F	n

^zFM; monoecious cultivar, F; female cultivar, -; data unknown.

^yExperimental Orchard of Kyoto University.

^xFruit Tree Experiment Station of Hiroshima Prefecture, 1979.

^wDatabase of the NIAS Genebank (https://www.gene.affrc.go.jp/databases-plant_search_char.php).

^vp; positive, n; negative.

^uNonaploid cultivar.

Table 2

Ratio of *OGI*-positive individuals in the F₁ progeny of crosses between cultivars with multiplex *OGI* and without *OGI*.

Hybridization (cultivar with <i>OGI</i>) x (cultivar without <i>OGI</i>)	<i>OGI</i> allele dosage ^z	Expected ratio of <i>OGI</i> positive in F ₁ (%) ^y	
		Autohexaploid	Autoallohexaploid
YXXXXX x XXXXXX	Single	50	50
YYXXXX x XXXXXX	Double	80	83.3 (YYXX/XX), 75 (YXXX/YX)
YYYXXX x XXXXXX	Triple	95	100 (YYYX/XX or YXXX/YY), 91.7 (YYXX/YX)
YYYYXX x XXXXXX	Quandrivalent	100	100
YYYYYX x XXXXXX	Pentavalent	100	100
YYYYYY x XXXXXX	Hexavalent	100	100

^zCorresponding to the dosage of Y-chromosomes.
^yThe expected ratio of *OGI*-positive individuals in the F₁ progeny was calculated according to Akagi et al. (2010), focusing on the inheritance mode and the allele dosage of *ASTRINGENCY*-linked loci in hexaploid persimmon cultivars.

Table 3

Estimation of *OGI* allele dosage and constitution of sex chromosomes by qPCR analysis.

No. ²	Cultivar	Sexuality	Calculated <i>OGI</i> allele dosage (no. $\pm$ SE) ³		SNP alleletype ⁴		Estimated constitution of the sex chromosomes ⁵	
			<i>Dk</i> ANR reference	L5R reference	SNP-1	SNP-2	<i>Dk</i> ANR reference	L5R reference
1	Akazu	FM	0.95 $\pm$ 0.05	0.86 $\pm$ 0.04	C	T	Y ₁ XXXXX	Y ₁ XXXXX
2	Amayotsumizo	FM	1.38 $\pm$ 0.03	1.13 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
3	CalFuyu	FM	1.24 $\pm$ 0.02	1.09 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
4	Egoshō	FM	0.93 $\pm$ 0.03	0.79 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
5	Emon	FM	0.92 $\pm$ 0.01	0.86 $\pm$ 0.01	C	T	Y ₁ XXXXX	Y ₁ XXXXX
6	Fudegaki	FM	1.05 $\pm$ 0.00	0.84 $\pm$ 0.00	T	C	Y ₂ XXXXX	Y ₂ XXXXX
7	Fujiwaragoshō	FM	1.02 $\pm$ 0.02	0.87 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
9	Hazegoshō	FM	1.15 $\pm$ 0.03	0.94 $\pm$ 0.03	C	T	Y ₁ XXXXX	Y ₁ XXXXX
10	Ibogaki	FM	1.11 $\pm$ 0.07	0.94 $\pm$ 0.06	C	T	Y ₁ XXXXX	Y ₁ XXXXX
11	Issaigaki ^v	FM	0.96 $\pm$ 0.01	0.91 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
13	Kakiyamagaki ^v	FM	1.10 $\pm$ 0.03	0.91 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
14	Kanshu	FM	1.10 $\pm$ 0.03	0.88 $\pm$ 0.03	C	T	Y ₁ XXXXX	Y ₁ XXXXX
15	Kikumanju	FM	0.85 $\pm$ 0.04	0.79 $\pm$ 0.03	T	C	Y ₂ XXXXX	Y ₂ XXXXX
16	Kyara	FM	1.14 $\pm$ 0.10	0.91 $\pm$ 0.08	T	C	Y ₂ XXXXX	Y ₂ XXXXX
17	Meotogaki ^v	FM	0.82 $\pm$ 0.03	0.67 $\pm$ 0.03	T	C	Y ₂ XXXXX	Y ₂ XXXXX
18	Muraya	FM	1.08 $\pm$ 0.02	0.89 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
19	Mushirodagoshō	FM	0.92 $\pm$ 0.00	0.77 $\pm$ 0.00	C	T	Y ₁ XXXXX	Y ₁ XXXXX
20	Nishimurawase	FM	0.98 $\pm$ 0.03	0.81 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
21	Okugoshō	FM	1.12 $\pm$ 0.07	0.94 $\pm$ 0.06	C	T	Y ₁ XXXXX	Y ₁ XXXXX
23	Seihakuji	FM	0.92 $\pm$ 0.02	0.85 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
24	Shiroutodamashi	FM	1.15 $\pm$ 0.01	1.07 $\pm$ 0.01	C	T	Y ₁ XXXXX	Y ₁ XXXXX
25	Shougatsu ^v	FM	1.03 $\pm$ 0.10	0.91 $\pm$ 0.09	T	C	Y ₂ XXXXX	Y ₂ XXXXX
26	Shoujou	FM	1.26 $\pm$ 0.03	1.09 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
27	Shouzaemon	FM	0.66 $\pm$ 0.02	0.61 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
29	Taiwan-shoushi ^v	FM	1.27 $\pm$ 0.03	1.12 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
31	Toyoka	FM	0.90 $\pm$ 0.02	0.88 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
32	Yamagaki	FM	1.09 $\pm$ 0.06	0.83 $\pm$ 0.04	T	C	Y ₂ XXXXX	Y ₂ XXXXX
34	Yotsumizo	FM	1.05 $\pm$ 0.02	0.89 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
35	Zenjimarū	FM	1.11 $\pm$ 0.02	0.88 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
36	Atagobou	F	1.21 $\pm$ 0.02	1.02 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
37	Beniemon	F	0.91 $\pm$ 0.00	0.71 $\pm$ 0.00	T	C	Y ₂ XXXXX	Y ₂ XXXXX
38	Beniwase	F	0.97 $\pm$ 0.05	0.68 $\pm$ 0.03	T	C	Y ₂ XXXXX	Y ₂ XXXXX
39	Chagone	F	1.22 $\pm$ 0.01	0.99 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
40	Chichibu-issaigaki	F	0.77 $\pm$ 0.01	0.75 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
41	Deshimaru	F	1.10 $\pm$ 0.03	0.92 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
42	Kawagone	F	1.44 $\pm$ 0.07	1.16 $\pm$ 0.06	T	C	Y ₂ XXXXX or Y ₂ Y ₂ XXXX	Y ₂ XXXXX
43	Koudagoshō	F	1.15 $\pm$ 0.02	0.95 $\pm$ 0.01	C	T	Y ₁ XXXXX	Y ₁ XXXXX
45	Mikado	F	0.96 $\pm$ 0.03	0.83 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
46	Monpei	F	1.19 $\pm$ 0.01	1.14 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
47	Nagara	F	1.25 $\pm$ 0.02	1.08 $\pm$ 0.02	C	T	Y ₁ XXXXX	Y ₁ XXXXX
48	Nanshi	F	1.01 $\pm$ 0.01	0.88 $\pm$ 0.01	C	T	Y ₁ XXXXX	Y ₁ XXXXX
49	Nitari	F	0.86 $\pm$ 0.04	0.66 $\pm$ 0.03	C	T	Y ₁ XXXXX	Y ₁ XXXXX
50	Oogoshō	F	1.11 $\pm$ 0.02	1.01 $\pm$ 0.02	T	C	Y ₂ XXXXX	Y ₂ XXXXX
51	Ooniwa	F	0.99 $\pm$ 0.01	0.96 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
52	Saishō	F	0.91 $\pm$ 0.21	0.81 $\pm$ 0.19	C	T	Y ₁ XXXXX	Y ₁ XXXXX
53	Sanenashi	F	1.23 $\pm$ 0.04	0.97 $\pm$ 0.03	C	T	Y ₁ XXXXX	Y ₁ XXXXX
54	Shimokitahagakushi	F	0.97 $\pm$ 0.01	0.80 $\pm$ 0.01	C	T	Y ₁ XXXXX	Y ₁ XXXXX
55	Shouro	F	1.14 $\pm$ 0.07	0.87 $\pm$ 0.05	T	C	Y ₂ XXXXX	Y ₂ XXXXX
56	Suruga	F	0.89 $\pm$ 0.01	0.75 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
57	Tenjingoshō	F	1.15 $\pm$ 0.03	1.03 $\pm$ 0.03	C	T	Y ₁ XXXXX	Y ₁ XXXXX
58	Yashima	F	1.10 $\pm$ 0.02	0.94 $\pm$ 0.01	T	C	Y ₂ XXXXX	Y ₂ XXXXX
59	Yoshino	F	1.11 $\pm$ 0.03	0.98 $\pm$ 0.03	C	T	Y ₁ XXXXX	Y ₁ XXXXX
8	Hanagoshō	FM	2.11 $\pm$ 0.03	1.73 $\pm$ 0.02	C : T = 1 : 1	T : C = 1 : 1	Y ₁ Y ₂ XXXX	Y ₁ Y ₂ XXXX
12	Iwasedo	FM	2.32 $\pm$ 0.05	2.02 $\pm$ 0.05	C : T = 1 : 1	T : C = 1 : 1	Y ₁ Y ₂ XXXX	Y ₁ Y ₂ XXXX
22	Saburouza	FM	2.08 $\pm$ 0.04	1.93 $\pm$ 0.04	C : T = 1 : 1	T : C = 1 : 1	Y ₁ Y ₂ XXXX	Y ₁ Y ₂ XXXX
28	Taishu ^v	FM	2.10 $\pm$ 0.04	1.87 $\pm$ 0.03	T	C	Y ₂ Y ₂ XXXX	Y ₂ Y ₂ XXXX
34	Yamatogoshō	FM	1.77 $\pm$ 0.02	1.60 $\pm$ 0.02	C : T = 1 : 1	T : C = 1 : 1	Y ₁ Y ₂ XXXX	Y ₁ Y ₂ XXXX
45	Kunitomi	F	1.79 $\pm$ 0.01	1.52 $\pm$ 0.01	C : T = 1 : 1	T : C = 1 : 1	Y ₁ Y ₂ XXXX	Y ₁ Y ₂ XXXX
31	Tohachi ^v	FM	3.28 $\pm$ 0.03	2.87 $\pm$ 0.03	C : T = 1 : 2	T : C = 1 : 2	Y ₁ Y ₂ Y ₂ XXX	Y ₁ Y ₂ Y ₂ XXX
(negative control)								
73	Fuyu	F	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	-	-	XXXXXX	XXXXXX
136	Saijo	F	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	-	-	XXXXXX	XXXXXX

^zCorresponding to the cultivar No. in Table 1.

^y*OGI* allele dosage was calculated as [(relative DNA dosage of *OGI*)/(relative DNA dosage of the reference gene)] × 2, based on the possibility that ‘Taishu’ has two *OGI* alleles in its genome (see Table 4). The relative DNA dosage was calculated from each Ct using the standard curves of the genomic DNA of ‘Taishu’.

^xAllelotypes at SNP-1 and SNP-2 are shown in Fig. 2. Approximate ratios of the signal strength derived from each allele in direct sequencing are shown in some cultivars carrying multiple *OGI* alleles.

^wConstitution of the sex chromosomes was estimated from the calculated *OGI* allele dosage and SNP allelotypes; Y₁, C/T and Y₂, T/C at SNP-1/SNP-2, respectively.

^vCultivars often giving hermaphrodite-like flowers in Kyoto University Experimental Orchard.

Table 4

Segregation of *OGI* in four *D. kaki* F₁ populations; ‘Luo-tian-tian-shi’ × ‘Taishu’ (310), ‘Luo-tian-tian-shi’ × ‘Iwasedo’ (RI), ‘Luo-tian-tian-shi’ × ‘Okugosho’ (RO) and ‘Luo-tian-tian-shi’ × ‘Yotsumizo’ (RY).

F ₁ population ^z	PCR signal of <i>OGI</i>		Fitness to the expected ratio of <i>OGI</i> positive (<i>P</i> value) ^y					
			Autohexaploid			Autoallohexaploid		
	Positive	Negative	Single (50%) ^x	Double (80%)	Triple (95%)	Single (50%)	Double (83.3% / 75%)	Triple (100% / 91.7%)
310 (‘Taishu’)	18	8	0.037	0.137	< 0.001	0.037	0.041 / 0.414	ND ^w / < 0.001
RI (‘Iwasedo’)	78	17	< 0.001	0.608	< 0.001	< 0.001	0.755 / 0.110	ND / < 0.001
RO (‘Okugosho’)	39	61	0.028	< 0.001	< 0.001	0.028	< 0.001 / < 0.001	ND / < 0.001
RY (‘Yotsumizo’)	63	51	0.261	< 0.001	< 0.001	0.261	< 0.001 / < 0.001	ND / < 0.001

^zThe cultivar crossed with ‘Luo-tian-tian-shi’ in each cross is shown in parentheses. Note that ‘Luo-tian-tian-shi’ has no *OGI* alleles, while the other cultivars have at least one *OGI* allele (see Table 1 and Fig. 1). **At least for the 310 population, the sexuality was completely co-segregated with the *OGI* genotype. For the other three populations, we have not fully examined the sexuality yet.**

^yChi-squared test for expected ratio of *OGI*-positive (: negative) individuals shown in Table 2.

^xPutative allele dosage of *OGI* in the cultivars crossed with ‘Luo-tian-tian-shi’. The expected *OGI*-positive ratio is shown in parentheses. Note that the results for quadrivalent, pentavalent and hexavalent are not shown because *P* values could not be determined by the Chi-square test.

^wNot determined.

Figure 1

5

10

15

Figure 2