

Development of Molecular Markers Associated with Sexuality in *Diospyros lotus* L. and Their Application in *D. kaki* Thunb.

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Sexuality of crops affects both cultivation and breeding systems. Cultivated persimmon (*Diospyros kaki* Thunb) has a morphologically well-characterized polygamous or gynodioecious sexual system. However, the genetic basis of sexuality in *D. kaki* has yet to be characterized. Here, we used dioecious *D. lotus* L., a diploid wild relative species to hexaploid or nonaploid *D. kaki*, as a model to clarify the genetic basis of sexuality in *Diospyros* and to develop molecular markers associated with the sexuality of individuals. Using 62 F₁ offspring segregated into distinct male/female phenotypes, we found two amplified fragment-length polymorphism markers, DISx-AF4 and DISx-AF7, which cosegregated with maleness. This could suggest that the sexuality of *D. lotus* is controlled by a single gene/haploblock, and the male is dominant over the female. Thus, *D. lotus*'s sexuality can be described as the heterogametic male type, the XY-type, as reported for most other dioecious plant species. For unknown reasons, segregation of the phenotype of a sequence-characterized amplified region marker developed from DISx-AF4 (DISx-AF4S) and/or the male/female phenotype in two different crosses in *D. lotus* showed an apparent bias towards femaleness and better fitted 1:2 than 1:1, which is the theoretical segregation for a single genetic locus or haploblock in diploid *D. lotus*. DISx-AF4S could distinguish *D. kaki* cultivars with female and male flowers from cultivars with only female flowers, strongly indicating that the same genetic system controls *D. kaki*'s sexuality and that DISx-AF4S could be used as a genetic marker for sexuality in *D. kaki* breeding programs.

Key Words: AFLP, marker-assisted breeding, persimmon, polyploidy, sex expression, XY chromosome.

Introduction

Sex expression of crops affects cultivation systems as well as yields. Understanding the sexual system and its controlling mechanism would be useful to develop a stable and efficient production system for a given crop plant species. Sexuality in plants is more complicated than that in animals and can be defined at three different levels: (i) in the flower, (ii) in an individual, and (iii) in a population (Dellaporta and Calderon-Urrea, 1993). For flower sexuality, distinct unisexual types, male and female types, and a cosexual (hermaphrodite) type can be defined. The sexuality of an individual is revealed by combinations of flower sexualities. The sexuality in a population, generally called the “sexual system”, is defined by combinations of individual sexualities. A classic catalog of sexuality in 120,000 plant species showed that hermaphrodites are the predominant sex-

ual system, accounting for up to 75% of the species (Ming et al., 2007; Yampolsky and Yampolsky, 1922). Conversely, approximately 4% and 7% of the species are supposed to be strictly dioecious and monoecious, respectively. Nevertheless, research in the area of plant sexuality has mainly focused on dioecious systems or changing the mechanism between dioecious and co-sexual types (hermaphrodite or monoecious) (Dellaporta and Calderon-Urrea, 1993). Although environmental controls (Chailakhyan and Khryanin, 1978; Negri and Olmo, 1966) and genetic controls (Fechter et al., 2012; Fraser et al., 2009; Goldberg et al., 2010; Khattak et al., 2006; Onodera et al., 2011; Spigler et al., 2008; Wang et al., 2012) of dioecious sex determination have been clarified in some major crops to some extent, no general sexuality-controlling mechanism has been found in higher plants (Charlesworth, 2013; Dellaporta and Calderon-Urrea, 1993; Grant et al., 1994). Furthermore, genes or molecular mechanisms directly involved in sex determination have been identified only in a limited number of plant species, such as *Cucumis* (Boualem et al., 2009; Martin

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et al., 2009) and maize (*Tasselseed* genes, i.e., Chuck et al., 2007), although they have a monoecious system.

Diospyros kaki Thunb., often called simply “persimmon” or “kaki”, is one of the main fruit tree species in East Asia. Most *Diospyros* species are assumed to bear unisexual flowers and are classified as the dioecious type, with some exceptions, presumably because of the influences of minor genetic changes or environmental factors (Bawa, 1980; Duangjai et al., 2006), as reported in the other genera in the Ebenaceae, to which *Diospyros*, *Euclea*, *Royena*, and *Lissocarpa* genera belong (Duangjai et al., 2006; Salter, 1953). In terms of flower sexuality, *D. kaki* bears unisexual flowers, as do other *Diospyros* species. In addition to the unisexual flowers, some cultivars or genotypes bear hermaphrodite flowers. Although hermaphrodite flowers of most *D. kaki* cultivars or genotypes do not function fully as female flowers because they only develop seedless fruit, some cultivars, such as ‘Kubogata Obishi’, develop normal fruit with seeds (personal communication A. Sato). Sexuality of *D. kaki* at the individual level is different from that of most other *Diospyros* species and could be described as the gynodioecious type, based on the observation that monoecious individuals with both male and female flowers and individuals with only female flowers exist in major cultivars (Yonemori et al., 1993). However, some monoecious cultivars can bear hermaphrodite flowers as well as male and female flowers. Furthermore, individuals bearing only male flowers were recently found in China (Xu et al., 2008) and occasional male flower formation was reported in cultivars that usually bear only female flowers (Yakushiji et al., 1995). Thus, the sexuality of *D. kaki* appeared to be more strictly described by using the term polygamy. The change in the sexual system of *D. kaki* may be related to its polyploid nature ($x = 15$, $2n = 6x$ or $9x = 90$ or 135): most diploid *Diospyros* species that are closely related to *D. kaki*, such as *D. lotus*, *D. oleifera*, and *D. glandulosa* (Duangjai et al., 2006), show a distinct dioecious type. Changes in the sexual system according to ploidy changes are well documented in plants (Comai, 2005). The XY-type sexual system in some species, such as in *Rumex* and *Humulus*, is based on the dosage balance between X chromosomes and autosomes (Grant et al., 1994; Ming et al., 2007; Ono, 1935; Parker and Clark, 1991), which indicates that variation in ploidy or karyotype can result in changes in sex expression.

Most commercial *D. kaki* cultivars bear only female flowers; however, some cultivars, such as ‘Taishu’ and ‘Hanagosho’, bear male flowers as well. Furthermore, commercial orchards are interplanted with pollinizer trees with male flowers to ensure good fruit production. The ratio of male-to-female flowers in a tree varies, not only with the genotype or cultivar, but also with the environmental conditions and the age of the tree (Hume, 1913; Kajiura and Blumenfeld, 1989). For example, ‘Taishu’ produces excessive male flowers when the tree

becomes old or weakened, leading to an insufficient amount of crop load. Thus, some farmers are reluctant to plant ‘Taishu’, despite this cultivar bearing excellent fruit. From the viewpoint of breeding new cultivars, the fact that only limited numbers of cultivars bear male flowers limits crossing combinations. Elucidation of the genetic and molecular basis of sex expression in *D. kaki* may lead to the development of artificial control of sex expression in this important fruit species. To the best of our knowledge, however, there has been no report describing the genetic and molecular basis of the sexuality in *D. kaki*, although male and female floral differentiation and development have been described in monoecious cultivars (Nishida and Ikeda, 1961; Yonemori et al., 1993). The complicated sex expression and polyploidy in *D. kaki* make it difficult to identify genes involved in sex determination. In this study, we used one of the diploid dioecious species that is closely related to *D. kaki*, *D. lotus*, as a model to uncover the genetic and molecular basis of sexuality in *D. kaki*. We described (i) the mode of inheritance of sexuality in *D. lotus*, (ii) the development of molecular markers associated with sex expression in *D. lotus*, and (iii) genotyping of *D. kaki* using a sequence-characterized amplified region (SCAR) marker from DISx-AF4 (an amplified fragment length polymorphism (AFLP) marker) to determine if it could be applicable to determine the sexuality of *D. kaki*. Based on the results obtained, we discussed the genetic control of sex expression in *Diospyros* and marker-assisted breeding of sexuality in *D. kaki*.

Materials and Methods

Plant materials and DNA extraction

The KK F₁ population, consisting of 64 trees, was obtained from the cross *D. lotus* L. ‘Kunsenshi Female’ × *D. lotus* ‘Kunsenshi Male’ in the experimental orchard of Kyoto University in 2004. This F₁ population was used in experiments to test the segregation of sexuality and to develop markers associated with sex expression. The same cross was made in 2011, and the F₁ population obtained was named KK2. The VM F₁ population was obtained from the cross *D. lotus* ‘Budougaki’ × ‘Kunsenshi Male’ in 2009. Ninety-six and 119 trees of KK2 and VM, respectively, which were still in the juvenile phase and yet to flower, were used for the marker segregation test. We also used 21 *D. kaki* cultivars. The sexualities of the 21 *D. kaki* cultivars were classified into two types: type FM and type F, based on previous reports (Fruit Tree Experiment Station of Hiroshima Prefecture, 1979; Yamada et al., 1993; Yamane et al., 2001). The former are cultivars with female and male flowers and the latter are cultivars with only female flowers. Note that the frequency of male flowers on a tree considerably varies with the FM cultivar. The cultivars tested were: ‘Atago’ (F), ‘Amahyakume’ (FM), ‘Dojohachiya’ (FM), ‘Fujiwaragosho’ (FM), ‘Fuyu’ (F), ‘Hiratanenashi’

(F), 'Jiro' (F), 'Kakiyamagaki' (FM), 'Luo-Tian-Tian-Shi' (F), 'Meotogaki' (FM), 'Miyazakitanenashi' (F), 'Oyotsumizo' (F), 'Sangokuichi' (F), 'Shogatsu' (FM), 'Taishu' (FM), 'Ta-Mo-Pan' (F), 'Taiwanshoshi' (FM), 'Tohachi' (FM), 'Watarisawa' (F), 'Yokono' (F), and 'Zenjimarui' (FM). Three cultivars, 'Miyazakitanenashi', 'Hiratanenashi', and 'Watarisawa', are nonaploid, while all the other cultivars tested are hexaploid. Young leaves and/or dormant winter buds were used for DNA extraction. Total DNA was extracted by the CTAB method, according to Doyle and Doyle (1987). After purifying the DNA by phenol extraction and polyethylene glycol precipitation, DNA was used for AFLP and PCR analyses.

Bulked AFLP analysis and cloning sequences

DNA concentration was adjusted to 50 ng·μL⁻¹ for AFLP analysis. DNA digestion, adapter ligation, and preamplification were performed as described by Vos et al. (1995), using the AFLP analysis system I kit (Life Technologies, Carlsbad, CA, USA). The PCR steps and detection of AFLP signals were conducted according to Kanzaki et al. (2001), with some modifications. Briefly, total genomic DNA (0.25 μg) of bulked samples comprising DNA from five each of male or female offspring (0.05 μg DNA each from an individual plant), and DNA (0.25 μg) from the male and female parents, were digested with 2.5 U *Eco*RI and 2.5 U *Mse*I in a 25-μL reaction volume. Then, *Eco*RI and *Mse*I adapters were ligated to the digested DNA fragments. After a pre-amplification reaction with 5 μL template DNA (1:10, v/v solution diluted from the restriction-ligation mixture), the selective amplification reaction was performed with two primers based on the sequences of the *Eco*RI and *Mse*I adapters including three additional selective nucleotides at the 3' end of each primer. The amplified fragments were denatured and subjected to polyacrylamide gel electrophoresis (acrylamide-bisacrylamide 29:1). After electrophoresis, DNA in the gel was transferred to a Biotodyne B membrane (Pall Corporation, Port Washington, NY, USA). The membrane was hybridized with a 5' digoxigenin (DIG)-labeled oligonucleotide (Sigma-Aldrich Japan, Tokyo, Japan) designed to hybridize with the *Eco*RI adaptor sequence. After hybridization at 42°C for 2–8 h, the hybridization signal was detected using anti-DIG-alkaline phosphatase (Roche Applied Science, Upper Bavaria, Germany) and chemiluminescent substrate CDP star (Roche Applied Science). The image analyzer LAS3000 mini (Fujifilm, Tokyo, Japan) documented the chemiluminescence.

When AFLP bands showing distinct polymorphisms with regard to sexuality were found, the band was excised from the gel and cloned into a plasmid vector. In short, the AFLP reaction mixture was re-electrophoresed through a polyacrylamide gel and the gel was silver-stained using the Silver Sequence DNA sequencing system (Promega, Fitchburg, WI, USA). The

portion of polyacrylamide gel carrying the target AFLP band was excised from the gel. TE buffer (50 μL) was added to the excised gel fragment. DNA was extracted into the TE buffer by freeze-thaw cycles at –20°C and 4°C. After incubation at 95°C for 15 min, the DNA in the supernatant was purified by phenol/chloroform extraction and precipitated by ethanol. The recovered DNA was re-amplified by PCR under the same conditions as the selective amplification reaction. The PCR products were subcloned into the pGEM-T easy vector (Promega) and sequenced.

Evaluation of the SCAR marker

We designed a SCAR marker, DISx-AF4S, from the DNA sequence of an AFLP band that appeared to be associated with the sexuality of *D. lotus*. For amplification of the DISx-AF4S site, the PCR reaction mixture comprised a total volume of 25 μL containing 20 ng genomic DNA, 400 μM each of dNTPs, 1 U KOD FX Neo (Toyobo Life Science, Osaka, Japan), 400 nM each of forward (DISx-AF4-3F; 5'-ACA TCC AAA GTT CTG GAG AAT CA-3') and reverse (DISx-AF4-3R; 5'-ATT GGT GCT TGG TCA AAC ATA TC-3') primers, and 1× KOD FX PCR buffer (Toyobo Life Science). The PCR program consisted of pre-denaturation at 94°C for 2 min; 35 cycles at 98°C for 10–30 s, 56–60°C for 30 s, and 68–72°C for 1 min; followed by a final extension at 68–72°C for 7 min. PCR products were run on 1.5% agarose gels in 1× TAE buffer and visualized with ethidium bromide. The genomic fragment of *Diospyros kaki* *ANTHOCYANIDIN REDUCTASE* (*DkANR*; accession no. AB195284) was amplified as a reference to confirm the presence of genomic DNA, as reported by Akagi et al. (2010).

Results and Discussion

Segregation of sexuality in the KK F₁ population

Table 1 shows the segregation of sexuality in the KK F₁ population, comprising 64 trees investigated for 6 years from 2008 to 2013. Sixty-two trees were examined for their sexuality; however, two trees did not flower, even 8 years after planting in 2005. Except for the six trees that flowered for the first time in 2013, the other 56 trees that flowered were scored for their sexuality at least in two separate years. All of the segregated individuals showed distinct unisexual flowers and their sexuality was consistent throughout the tree. No sex change was observed, except for a single plant, tree no. 42. Tree no. 42 was first scored male in 2010, while it was scored female in 2012 and 2013. We did not investigate this tree in 2011. Considering that the other 55 trees that flowered and were scored in multiple years showed the same sexuality over the years, and the sexuality throughout the tree was consistent, the scoring of tree no. 42 in 2010 may have been an error, although it is still possible that sex conversion occurred in this tree.

In the KK F₁ population, trees with male flowers

Table 1. Sexuality, marker phenotype, and the first year of flowering of the *D. lotus* ‘Kunsenshi Female’ × ‘Kunsenshi Male’ (KK) F₁ population.

Tree no.	Male/Female	First flowering	DISx-AF4S ^z	DISx-AF7 ^z
No.1	♂	2008	p	p
No.2	♂	2008	p	p
No.3	♂	2008	p	p
No.4	♂	2008	p	p
No.5	♂	2008	p	p
No.6	♂	2009	p	p
No.7	♀	2009	a	a
No.8	♂	2009	p	p
No.9	♂	2008	p	p
No.10	♀	2008	a	a
No.11	♀	2009	a	a
No.12	♂	2008	p	p
No.13	♀	2008	a	a
No.14	♀	2008	a	a
No.15	♀	2012	a	a
No.16	♀	2008	a	a
No.17	♀	2009	a	a
No.18	♀	2009	a	a
No.19	♀	2012	a	a
No.20	♀	2009	a	a
No.21	♂	2008	p	p
No.22	♂	2008	p	p
No.23	♀	2009	a	a
No.24	♀	2008	a	a
No.25	♂	2009	p	p
No.26	ND ^y	ND ^y	a	a
No.27	♀	2009	a	a
No.28	♂	2008	p	p
No.29	♀	2009	a	p
No.30	♀	2012	a	a
No.31	♂	2009	p	p
No.32	♂	2008	p	p
No.33	♀	2010	a	a
No.34	♂	2008	p	p
No.35	♀	2009	a	a
No.36	♂	2008	p	p
No.37	♀	2012	a	a
No.38	♀	2010	a	a
No.39	♀	2009	a	a
No.40	♂	2008	p	p
No.41	♀	2009	a	a
No.42	♂/♀	2010	a	NC ^x
No.43	♂	2009	p	p
No.44	♀	2013	a	a
No.45	♀	2013	a	a
No.46	♀	2009	a	a
No.47	♀	2009	a	a
No.48	♂	2009	p	p
No.49	ND	ND	a	a
No.50	♀	2012	a	a
No.51	♀	2009	a	NC
No.52	♂	2008	p	p
No.53	♀	2013	a	a
No.54	♀	2010	a	a
No.55	♀	2013	a	NC
No.56	♀	2009	a	a
No.57	♂	2010	p	p
No.58	♀	2010	a	a
No.59	♀	2013	a	a
No.60	♀	2012	a	a
No.61	♂	2009	p	p
No.62	♀	2010	a	a
No.63	♂	2009	p	p
No.64	♀	2013	a	a

^z a = absent, p = present.^y Not determined.^x Not clearly determined.

appeared to flower earlier than female trees, with the female-to-male ratio increasing from 0.33 to 1.54 from 2008 to 2013 (Table 2). The observed ratio of female to male was not significantly different from 1:1 ($P > 0.1$), which is the theoretical ratio when hypothesizing the presence of a single pair of sex chromosomes. The observed ratio, however, was apparently biased towards females when almost all plants flowered in 2013, showing better fitness to the segregation of 1:2 in 2013 by chi-square test ($P = 0.319$, Table 2). Assuming that the sex expression of *D. lotus* is controlled by multiple genetic factors located on distinct chromosomes, this segregation ratio could be explained more appropriately. However, it is difficult to assume the involvement of multiple loci in the segregation of distinct male/female phenotypes in dioecious plants if we consider the evolution of sex expression in higher plants (Charlesworth, 2013; Ming et al., 2011). Supporting this hypothesis, no dioecious plant species in which sex expression is determined by multiple chromosomes has been described (Ming et al., 2007; Ono, 1935). It may be more appropriate to assume the involvement of lethal or semi-lethal genes to explain the distorted segregation ratio from the expected 1:1 (discussed below in more detail).

Identification and evaluation of male-associated markers

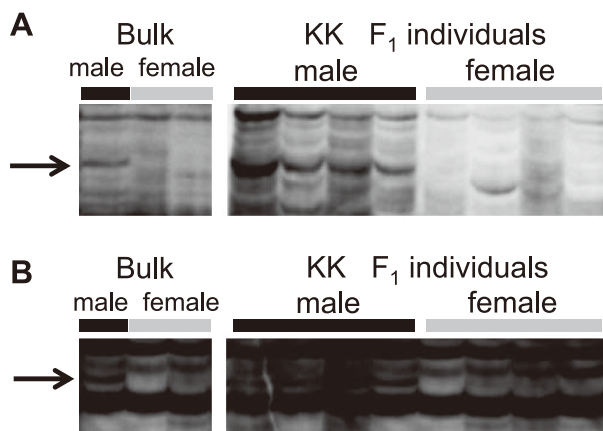
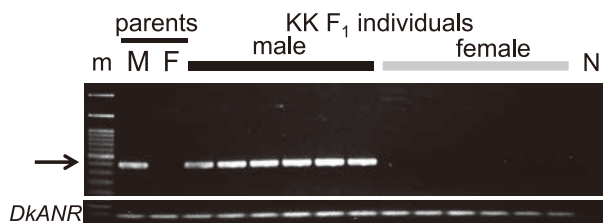
A total of 512 primer combinations of 16 *Eco*RI primers starting with “T” or “A” base and 16 *Mse*I primers starting with “A” base were used in bulked segregant analysis against subsets of the male and female bulks. Of the 512 primer sets tested, one primer combination (E-TGT/M-AAT) yielded a male-specific band, named DISx-AF4 (Fig. 1) and this marker could be converted to a SCAR marker, named DISx-AF4S (Tables 1 and 2). The DNA sequence of DISx-AF4 was deposited in the International Nucleotide Sequence Database Collaboration (INSDC) through DNA Data Bank of Japan (DDBJ) with the accession no. AB909127. Another primer combination (E-ATA/M-ATG) produced an AFLP band (DISx-AF7) co-segregating with the male phenotype, although DISx-AF7 could not be converted to a SCAR marker. As the presence or absence of the DISx-AF7 AFLP band was often difficult to determine unambiguously, we could not score three individuals (42, 50, and 54). Except for tree no. 29, the scoring of the DISx-AF7 marker phenotype was consistent with the scoring of the DISx-AF4S marker phenotype (Table 1). None of the primer combinations tested, however, yielded polymorphisms co-segregating with the female phenotype. If we excluded tree no. 42, which was scored both male and female depending on the year, DISx-AF4S showed perfectly male-specific amplification in all 61 individuals (Fig. 2). Considering that only male-specific dominant markers have been found and they are linked to each other, the sexuality in *D. lotus* was hypothesized to be controlled by a single locus or by a single haploblock containing gene clusters, and maleness is dominant

Table 2. Segregation for the sexuality of 63 trees of the *D. lotus* 'Kunsenshi Female' × 'Kunsenshi Male' (KK) F₁ population.^z

Year	Sexuality			Fitness to the expected ratio (<i>P</i> value) ^y	
	♂	♀	Unflowered	1:1	1:2
2008	15	5	43	0.025	< 0.00001
2009	23	20	20	0.647	0.005
2010	24	25	14	0.886	0.020
2011	24	25	14	0.886	0.020
2012	24	31	8	0.345	0.105
2013	24	37	2	0.096	0.319

^z Note that tree no. 42 (Table 1) was excluded from this table because it was scored male in 2010, while female in 2012 and 2013 (see text).

^y Chi-square test. The expected segregation ratio for male:female.

**Fig. 1.** AFLP fingerprinting of bulked male and female pools, and four each of male and female individuals in the *D. lotus* 'Kunsenshi Female' × 'Kunsenshi Male' (KK) F₁ population, using the (A) E-TGT/M-AAT and (B) E-ATA/M-ATG primer combinations, corresponding to DISx-AF4 and DISx-AF7, respectively. Arrows indicate the male-specific band.**Fig. 2.** DISx-AF4S PCR amplification of female/male segregated individuals in the *D. lotus* 'Kunsenshi Female' × 'Kunsenshi Male' (KK) F₁ population, and the parents. M, male parent 'Kunsenshi Male'; F, female parent 'Kunsenshi Female'; m, DNA ladder marker; and N, negative control. Six individuals each for male and female trees of the KK F₁ population were analyzed. Arrow indicates the DISx-AF4S signal co-segregated with the male phenotype. The genomic fragment of *DkANR* (Akagi et al., 2010) was amplified to confirm the presence of the genomic DNA.

over femaleness. Thus, the *D. lotus* sex determination is the heterogametic male type (XY type), as reported for most other dioecious plant species in angiosperms (Ming et al., 2011). To date, the sex determination of *Fragaria* species has been only described as the ZW type (Ahmadi

and Bringhurst, 1991; Spigler et al., 2008). Based on the chromosome observations of *D. lotus* and other *Diospyros* species (Choi et al., 2003a, b; Tamura et al., 1998; Zhuang et al., 1990), the X and Y chromosomes in *Diospyros* could be defined as homomorphic sex chromosomes.

DISx-AF4S completely co-segregated with the male phenotype in all 61 segregated individuals, implying that this marker locus would be very tightly linked to the sex determination locus. Genomic/genetic characterization of Y chromosomes in dioecious plant species, such as *Silene* species and papaya, indicated that the region including the sex determination gene(s) is supposed to be gradually degraded and have a wide Y-specific region (male-specific region of the Y-chromosome, MSY), depending on the time after the divergence of X and Y chromosomes (Liu et al., 2004; Ming et al., 2011; Wang et al., 2012). Recombinations are suppressed in the MSY and its surrounding regions (Charlesworth, 2013; Ming et al., 2011). As it appeared that DISx-AF4 had a male-specific sequence and showed no similarity to any transposable elements in the public databases (data not shown), we suggested that DISx-AF4S may be from MSY.

Inheritance mode of the sex determination locus

Using the DISx-AF4S marker, all 64 trees of KK F₁ population, 96 trees of the KK2 F₁ population, and 119 trees of the VM F₁ population were tested to estimate the behavior of X and Y chromosomes (Table 3). Chi-square test showed that segregations of DISx-AF4S in the KK, KK2, and VM F₁ populations are better fitted to the 1:2 ratio than the 1:1 ratio. The segregation in a single marker locus would theoretically reflect the behavior of the chromosomes where the marker sequence resides. Thus, the segregation of DISx-AF4S should have fitted the 1:1 ratio, if there was no preference for survival among the plants with homologous chromosomes. A possible explanation for this bias would be that the male might be weaker than the female, and female trees could survive better than male trees. Alternatively, we could explain this distorted segregation by assuming that (semi-)lethal effects of the Y chromosome in 'Kunsenshi

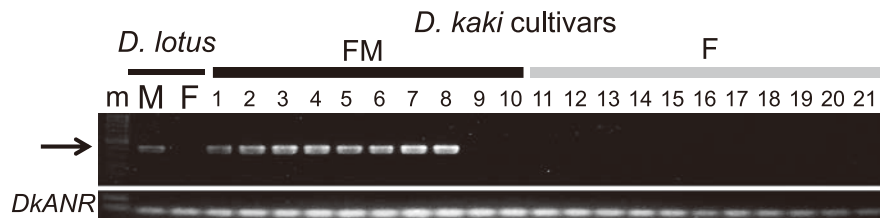
Table 3. Segregation of the DISx-AF4S marker phenotype in three *D. lotus* F₁ populations used in this study: ‘Kunsenshi Female’ × ‘Kunsenshi Male’ developed in 2004 (KK), ‘Kunsenshi Female’ × ‘Kunsenshi Male’ developed in 2011 (KK2) and ‘Budougaki’ × ‘Kunsenshi Male’ developed in 2009 (VM).^z

F ₁ Population	Sexuality	DISx-AF4S		Fitness to the expected ratio (<i>P</i> value) ^y	
		Present	Absent	1:1	1:2
KK	male	24	0	ND ^x	ND ^x
	female	0	37	ND	ND
	unflowered	0	2		
	combined	24	39	0.059	0.423
KK2	unflowered	37	59	0.025	0.279
KK + KK2	combined	61	98	0.003	0.178
VM	unflowered	40	79	< 0.001	0.948

^z Note that tree no. 42 tree of KK F₁ population (Table 1) was excluded from this table because it was scored male in 2010, while female in 2012 and 2013 (see text).

^y Chi-square test. The expected segregation ratio for present:absent.

^x Not determined.

**Fig. 3.** DISx-AF4S PCR amplification of 21 *D. kaki* cultivars and the male and female parents of the KK F₁ population. M: male parent ‘Kunsenshi Male’, F: female parent ‘Kunsenshi Female’, and m: DNA ladder marker. For hexaploid/nonaploid *D. kaki*, 10 cultivars with female and male flowers (FM, lanes 1–10), and 11 cultivars with only female flowers (F, lanes 11–21) were analyzed. 1, Taishu; 2, Tohachi; 3, Zenjimarui; 4, Meotogaki; 5, Shogatsu; 6, Taiwanshoshi; 7, Kakiyamagaki; 8, Fujiwaragoshio; 9, Dojohachiya; 10, Amahyakume; 11, Fuyu; 12, Jiro; 13, Luo-Tian-Tian-Shi; 14, Oyotsumizo; 15, Sangokuichi; 16, Yokono; 17, Atago; 18, Ta-Mo-Pan; 19, Watarisawa; 20, Hiratanenashi; and 21, Miyazakitanenashi. The genomic fragment of *DkANR* (Akagi et al., 2010) was amplified to confirm the presence of genomic DNA.

Male’ would appear when it is combined with one of the two X chromosomes from the female parent. However, considering that two independent crosses using different female parents showed the same tendency of segregation bias, the former reason may be more likely. Significant distortion of the sexuality in *Diospyros* species other than *D. lotus* has also been reported, although more male plants than female were observed in the previous report (Venkatasamy et al., 2007).

Sexuality determination of *D. kaki* cultivars by DISx-AF4S

DISx-AF4S was tested to see if it could be used as a molecular marker to determine the sexuality of *D. kaki* cultivars. DNA sequences of the amplified bands from *D. kaki* showed more than 95% sequence identity to that from *D. lotus* (data not shown). All FM cultivars tested, except for ‘Dojohachiya’ and ‘Amahyakume’, yielded DISx-AF4S bands (Fig. 3). This result suggested that sexuality, or the ability to develop androeciums, in *D. kaki* is controlled by the same genetic factor as *D. lotus*, if we assume recombination events between DISx-AF4S and sex determination loci in these cultivars. However, it is possible that the genetic control of sexuality in *D. kaki*

may be leakier than that in *D. lotus*. Although a previous report described that ‘Dojohachiya’ and ‘Amahyakume’ bear male flowers infrequently (Fruit Tree Experiment Station of Hiroshima Prefecture, 1979), we could not find any male flowers on the trees in our experimental orchard in Kyoto. Furthermore, ‘Dojohachiya’ and ‘Amahyakume’ trees in the National Agriculture and Food Research Organization (NARO) Institute of Fruit Tree Science (NIFTS) bore only female flowers in recent years (personal communication A. Sato). Considering occasional male flowerings in the type F cultivars ‘Jiro’ and ‘Fuyu’ (Yakushiji et al., 1995), and ‘Luo-Tian-Tian-Shi’ (personal communication A. Sato), ‘Amahyakume’, and ‘Dojohachiya’ may also be type F cultivars and occasionally bear male flowers under certain conditions. Sex expression in *D. kaki* could be more susceptible to environmental factors than that in *D. lotus*, presumably because of the gene dosage effects from the polyploid nature of *D. kaki* (Akagi et al., 2010; Bawa, 1980). We found significant association between DISx-AF4S and sexuality in *D. kaki*; therefore, one possible use of DISx-AF4S would be to diagnose sexuality in breeding programs.

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