

Genomics-based discrimination of 2n gamete formation mechanisms in polyploids: a case study in nonaploid *Diospyros kaki* 'Akiou'

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Abstract

Unreduced gametes (2n gametes), possessing double the haploid genome, whatever ploidy that happens to be, are a common source of ploidy variation in plant populations. First and second division restitution (FDR and SDR) are the dominant mechanisms of 2n gamete production; all else being equal, FDR gametes have a higher degree of heterozygosity, thus they are advantageous in breeding. The discrimination of these mechanisms from the consequence of hybridization is challenging, especially in higher polyploids, and usually requires information on centromere location. In this study, we propose a genotyping-based strategy to uncover the mechanisms of 2n gamete formation in progeny that has a higher ploidy than its parents. Simulation of 2n gamete production revealed that FDR and SDR pathways can be discriminated based on allele transmission patterns alone without information on centromere location. We applied this strategy to study the formation mechanism of a nonaploid *Diospyros kaki* 'Akiou', which was bred via hybridization between *D. kaki* hexaploid cultivars. The result demonstrated that 'Akiou' was derived from the fertilization of a normal female gamete by a 2n male gamete and that this 2n gamete was produced through FDR. Consequently, the distinct duplex transmission pattern in the FDR gamete enabled us to infer the genomic characteristics of polyploid persimmon. The method could be tested only for the plant being polyploid, which allows for the ability to discriminate causes of 2n gamete formation using allele dosage in progeny, and will be useful in future studies of polyploid genomics.

Keywords: persimmon; unreduced gamete; recombination; centromere; genetic simulation

Introduction

2n gamete production in plants

Unreduced (2n) gametes are an important source of polyploid formation and interploidy gene flow in mixed ploidy populations. Production of 2n gametes is mainly attributed to aberrations during meiosis I (first division restitution, FDR) or II (second division restitution, SDR) (Dewitte et al. 2012). In both cases, gametes with somatic (2n) rather than haploid (n) chromosome numbers are formed (Lieseback et al. 2015). In a strict sense, the FDR gametes are formed with the full omission of meiosis I, and thus fully retain parental heterozygosity. However, FDR gametes may also be formed after partial completion of meiosis I; in that case, recombination may occur and some of the parental heterozygosity is lost; this is referred to as broad sense FDR (Ramanna and Jacobsen 2003; De Storme and Geelen 2013). Most plants typically produce only a few 2n gametes averaging 0.1–2.0%, whereas some individuals, such as interspecific hybrids, produce substantially more (>10%)

(Alexander and Beckett 1963; Hahn et al. 1990; Ramsey and Schemske 1998; Ramsey 2007; Mai et al. 2019).

Value of 2n gametes in plant breeding

Sexual polyploidization using 2n gametes typically results in greater variability, fitness, and heterozygosity than somatic doubling (Peloquin et al. 1999; Carputo et al. 2003), and thus is beneficial in breeding. Additionally, hybridizations involving 2n gametes often produce a progeny with an odd number of genomes and result in seedlessness, which is valuable especially in fruit crop production. Thus, sexual polyploidization using 2n gametes has been a topical idea for plant breeding (De Storme and Geelen 2013; Younis et al. 2014). For example, several ploidy series of potato (Carputo and Barone 2005), and seedless triploid citrus hybrids, have been developed by sexual polyploidization techniques using 2n gametes (Aleza et al. 2010).

FDR gametes are more effective in transmitting parental heterozygosity and thus are more advantageous than those obtained

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by SDR for breeding purposes (Zhang *et al.* 2009; Ferrante *et al.* 2010; Dewitte *et al.* 2012). In potato, FDR is generally more than twice as effective as SDR in transmitting heterozygosity (Barone *et al.* 1995; Peloquin *et al.* 2008). Progenies bred by FDR 2n gametes tend to be more vigorous due to higher allelic diversity (Yao *et al.* 2013).

Reported methods for assessing the mechanism generating 2n gametes

The identification of the mechanism that resulted in 2n gametes is complex (Cuenca *et al.* 2015). Cytological methods have been widely used for determining the mechanisms; however, these methods are only practically applicable to characterize paternal meiosis. Genotyping-based analysis is another powerful approach that has been widely used (Ramanna and Jacobsen 2003; De Storme and Geelen 2013). In this method, the formation mechanism of 2n gametes is distinguished based on the genotype of centromeric markers: if a diploid individual has a centromeric marker genotype of AB, then gametes with the A and B alleles are produced with equal probability in normal meiosis. In this case, the FDR mechanism produces only gametes with AB genotype, whereas SDR produces AA and BB gametes with equal probability. In addition, the probabilities of a 2n gamete being heterozygous or homozygous as a consequence of FDR or SDR mechanisms can be considered as direct functions of the marker–centromere distance, taking recombination into account (Cuenca *et al.* 2015). This method has been used in populus (Dong *et al.* 2015) and citrus (Cuenca *et al.* 2015; Rouiss *et al.* 2017). However, although centromeric markers, or centromeric region, are essential for distinguishing the mechanisms, the centromeric region is often unknown in most plant species. Therefore, in order to study the biology of the 2n gamete formation in a broad range of species, including polyploids, we need a method to differentiate between mechanisms based on the genotype of markers distributed over the genome, rather than markers known *a priori* to be located in the centromeric regions.

At higher ploidy levels, 2n gametes are often produced at high frequencies. For example, Sugiura (2000) identified three hexaploid persimmon (*Diospyros kaki* Thunb.) cultivars that produced 2n pollen at rates of 4.8–15.5%, varying by cultivar. Yamada and Tao (2006) found that ‘Fujiwaragoshō’, a hexaploid *D. kaki*, frequently produced 2n embryo sacs. In polyploids, markers have several states of heterozygosity differing in allele dosage. Recent technical advances have enabled to precisely call polyploid genotypes with the allele dosage information. However, to our knowledge, the genotyping-based method for distinguishing the FDR or SDR origin of 2n gametes has not been intensively applied to any polyploid species.

Recent breeding trends in polyploid persimmon

Persimmon is a hexaploid species and is an important temperate fruit tree species, which originated in East Asia. Persimmon cultivars were classified into two types: pollination-constant nonastringent (PCNA) and non-PCNA (Akagi *et al.* 2011; Xu *et al.* 2016). The PCNA trait is valuable for commercial production because PCNA-type trees naturally produce nonastringent fruit without artificial treatment (Akagi *et al.* 2011). The PCNA type is a commercial standard in Asian countries, with associated genetic traits in modern commercial cultivars being conferred by a recessive allele at a single locus, AST (Ikeda *et al.* 1985; Yamada and Sato 2002; Nishiyama *et al.* 2018). In PCNA breeding, crosses among PCNA cultivars have been extensively performed, because crossing between PCNA and non-PCNA types is highly inefficient

without applying recently developed marker-assisted selection (Kanzaki *et al.* 2010; Sato and Yamada 2016). Due to the high genetic similarity among the PCNA cultivars, inbreeding depression has become an issue in PCNA breeding programs (Yamada *et al.* 1994; Pei *et al.* 2015).

In order to alleviate this problem, polyploid breeding has become an increasingly attractive strategy in PCNA persimmon breeding over the last two decades. The most remarkable achievement using this strategy is the nonaploid seedless PCNA persimmon ‘Akiou’ (also known as ‘Fukuoka K1 gou’). This was achieved through a controlled cross using the elite PCNA ‘Fuyu’ and ‘Taishuu’, as (hexaploid) maternal and paternal parents, respectively, followed by the embryo rescue of imperfect progeny seeds; however, the genomic constitution of ‘Akiou’ is largely unknown (Chijiwa *et al.* 2008).

In the present study, simulation of 2n gamete genotypes from a polyploid individual was conducted to visualize the allele transmission pattern at different allele dosages. This allowed us to develop an inheritance model as a potential means to discriminate the mechanisms, particularly focusing on double allele transmission. The proposed strategy was used to uncover the origin and formation mechanism of the 2n gamete that bred ‘Akiou’.

Materials and methods

Simulation of 2n gamete formation in polyploids

The 2n gametes resulting from the FDR broad sense and SDR mechanisms were simulated using PedigreeSim (Voorrips and Maliepaard 2012). PedigreeSim is a Java program that can simulate n gametes from one founder polyploid individual with a variety of meiotic models, including both bivalent and quadrivalent formation, different degrees of preferential pairing of homo(eo)logous chromosomes, and different quadrivalent configurations. Here, we assumed that 2n gametes result from modifications in the meiotic process that occur after the initial chromosome pairing (i.e. recombination in the first meiotic division is completed normally) and that the recombination configuration does not affect chromosome segregation. Under these assumptions, the fusion of pairs of two n gametes from the normal meiotic process allows the simulation of 2n gametes (Figure 1). Broad sense FDR (with recombination) gametes were simulated by the fusion of two n gametes, randomly selected from each pole. SDR gametes were simulated by the fusion of two gametes from the same pole (Figure 1). These processes were applied using different parameters for the quadrivalent formation fraction. This gametic data generation method was implemented in PedigreeSim version 2.1, which is available on github (<https://github.com/PBR/pedigreeSim>).

Throughout the simulation, we assumed a hexaploid founder individual, which is our target ploidy level, and simplex markers with known linkage phases distributed over the six homologous chromosomes. A population of 10,000 of 2n gametes for both mechanisms was simulated for different fractions of quadrivalent formation. In the present study, we assumed that the multivalent formation in hexaploid meiosis consists of one quadrivalent plus one bivalent, as explained in Bourke *et al.* (2019), and thus the quadrivalent parameter in this study controls the frequency of the meioses with a quadrivalent. A single meiosis produces two 2n gametes. Here, one of the two 2n gametes was randomly selected in each meiosis and allele transmission was recorded for each dosage separately. The average allele transmission to progeny was summarized over genetic distance based on the Haldane’s map function.

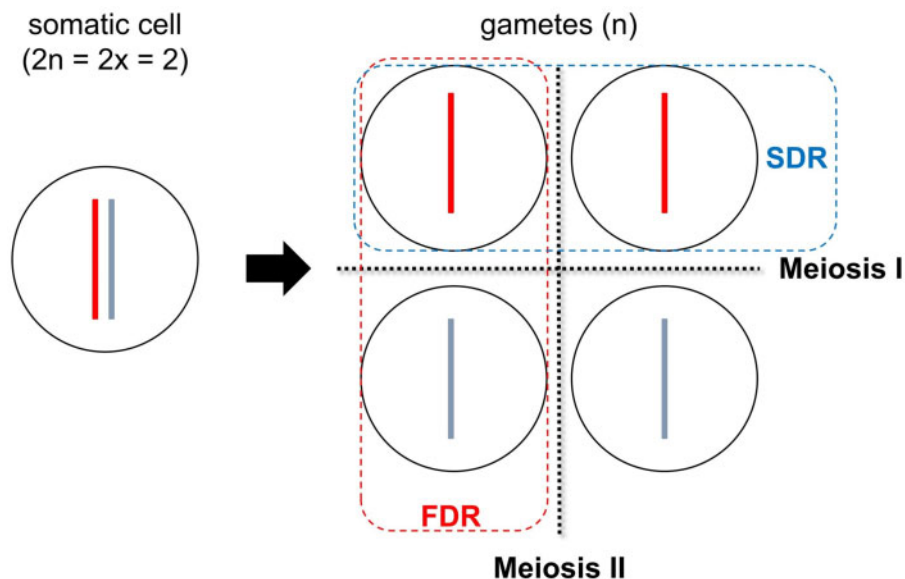


Figure 1 Graphical representation of the simulation method for 2n gamete production. In this example, a diploid organism with one chromosome pair was assumed; FDR and SDR were simulated by omitting the chromosome separation of Meiosis I and II, respectively.

For each simulated gamete, we also recorded possible ranges of the allele transmission by a single 2n gamete against recombination counts at meiosis. In this simulation, we assumed chromosomes with only one arm for easy interpretation. First, we extracted six chromosomes in one of the two 2n gametes produced in a single meiosis: these chromosomes are a mosaic of parental chromosomes by recombination. Positions of recombination breaks among the six chromosomes were ordered from the centromeric to the telomeric end, and the gametic genotypes created by each recombination were recorded and displayed by allele transmission dosage against the counts of recombination breaks from the centromeric end. For this analysis, at least 5 million 2n gametes for each setting were generated and analyzed. It should be noted that the method did not reflect all the recombination breaks produced during the meiosis; only the recombinations in the recorded 2n gamete were counted. This is because what we can actually observe in a progeny is one of the two 2n gametes from a single meiosis, not both.

Genomic DNA extraction, library construction, and sequencing

Total genomic DNA was extracted from fresh leaves of ‘Akiou’, ‘Fuyu’, and ‘Taishuu’ using the cetyl trimethyl ammonium bromide method and purified by phenol/chloroform extraction. Then, 1.5 µg of DNA from each sample was used to prepare short-read genomic libraries according to the following steps. First, DNA molecules were enzymatically fragmented and size-selected to 400–800 bp using AMPure (0.4:1 v/v AMPure: reaction). The resulting fragments were subjected to genomic library construction using the GenNext NGS Library Prep kit (Toyobo, Japan) and Truseq CD indexes (Illumina, USA) according to the manufacturers’ instructions and pooling guide. After amplification, libraries were further refined to remove fragments longer than 800 bp and shorter than 300 bp by AMPure. Finally, three genomic sequence libraries, with a gradient insert size of 300–800 bp, were mixed in equal proportions and sequenced using two lanes of the Illumina HiSeqX PE 150 platform (Illumina, San Diego, CA, USA). A total of 127.67, 93.47, and 68.73 Gb of raw DNA sequencing data (approximately 20-fold coverage of the polyploid genomes) of

‘Akiou’, ‘Fuyu’, and ‘Taishuu’, respectively, were used for further analysis.

Single nucleotide polymorphism selection

Sequences with low quality, adaptor sequences, and sequences shorter than 35 bp were removed by fastp (Chen et al. 2018). Subsequently, clean paired-end reads were mapped to an optimized version of the *Diospyros oleifera* genome (unpublished data), a close diploid relative of *D. kaki*, using the Burrows–Wheeler Aligner mem option and the paired-end model (Li and Durbin 2010). The optimization of the reference genome was performed on a published version of the *D. oleifera* reference genome (Suo et al. 2020) using a BioNano optical mapping-assisted assembly (unpublished data). The initial vcf file was created using the VarScan mpileup2snp mode (Koboldt et al. 2012), and only biallelic single nucleotide polymorphisms (SNPs) were retained using vcftools (Danecek et al. 2011). Based on the distribution of alternative allele read frequencies (Supplementary Figure S1), 3% of the alternative allele frequencies were used as the threshold for the heterozygous genotype call (Supplementary Figure S2). Only SNP genotypes with a read depth between half and twice the genome-wide average for each cultivar were retained. Furthermore, SNPs with missing data in any one or more of the three cultivars were filtered out using vcftools.

SNPs found in the parents ‘Fuyu’ and ‘Taishuu’ were analyzed to select informative sites. An SNP allele heterozygous in ‘Fuyu’ and not present (homozygous for the reference allele) in ‘Taishuu’ was defined as maternal informative allele (MIA), while the opposite definition was used for paternal informative allele (PIA). The inheritance ratio of MIAs and PIAs to ‘Akiou’ was calculated, and the SNP density per one megabase (Mb) along the genome was calculated using vcftools.

Characterization of allele transmission in the ‘Akiou’ pedigree

SNPs with simplex genotypes in one of the parents and nulliplex in the other were selected for further analysis. SNPs with a read frequency of the alternative allele below 1% and between 13% and 18% were considered nulliplex and simplex, respectively, in these

hexaploid cultivars. Then, the transmission of the simplex allele was measured in the nonaploid progeny based on the following ranges for the read frequency of the alternative allele: nulliplex 0–3%, simplex 4–17%, and duplex 18–31% (Supplementary Figure S3). These thresholds were determined based on the alternative allele frequency of each library (Supplementary Figure S1).

Alleles in the centromeric region show distinct transmission patterns in the FDR and SDR mechanisms at any ploidy level. Figure 2 shows the potential genotypes of gametes and progenies at centromeric markers. The nonaploid progeny of a hexaploid × hexaploid cross is produced by hybridization of an *n* gamete of one parent and a *2n* gamete from the other. If the parent producing the *n* gamete is simplex at a centromeric marker and the other parent is nulliplex, the nonaploid progeny has equal probability to be nulliplex or simplex (cross-combination II and III in Figure 2). If the parent producing the *2n* gamete is simplex and the other parent is nulliplex, there are two possibilities: if the *2n* gamete arose through FDR the nonaploid progeny is always simplex, but if SDR was involved, the progeny has equal chances to be nulliplex or duplex (cross-combination I and IV in Figure 2).

In *2n* gametes, even if the centromeric region is not known, the presumed pericentromeric region can be defined based on the ratio of allele transmission of simplex markers (see Results). As mentioned above, simplex MIA/PIAs at centromere always show simplex transmission through FDR gametes, whereas they show equal chances of nulliplex and duplex transmission through SDR gamete. The residual sum of squares (RSS) was used to evaluate fitting to the transmission rate at centromere and was calculated as follow:

$$RSS = (y_0 - \hat{y}_0)^2 - (y_1 - \hat{y}_1)^2 - (y_2 - \hat{y}_2)^2,$$

where y_i represents actual counts of loci with transmissions of MIA/PIA in a given region, \hat{y}_i represents expected counts of loci in a given region, and i (0–2) represents allele dosage. The presumed centromeric regions were defined by comparing RSSs in a given region against expected transmission ratio at centromere under

the FDR or SDR mechanism and at regions with a possible combination of parental chromosomes generated by recombination.

We applied this method to the ‘Akiou’ pedigree. Here, we first defined the candidate centromeric regions under both mechanisms. The regions with more than 95% transmission of the simplex MIA/PIAs were defined as the presumed pericentromeric regions under the FDR mechanism. For the case of SDR, the regions with approximately 50% duplex transmission of the simplex MIA/PIAs were defined as presumed pericentromeric regions. Then, the goodness-of-fit of the candidate regions with centromeric region was evaluated based on the transmission rate.

Data availability

Sequence data generated in this study are available in the DDBJ Sequenced Read Archive under the accession number DRA011719. Simulation method developed in this study was implemented in PedigreeSim version 2.1, which is available on github (<https://github.com/PBR/pedigreeSim>).

Supplementary material is available at G3 online.

Results

Simulation of *2n* gametes formation

In the simulation of the *2n* gametes population, we first assumed an individual and simplex markers with known linkage phase across the genome, and simulated transmission in *2n* gametes. The result was summarized in Supplementary Figure S4 and Table S1. As expected, the transmission of the alternative allele across the genome showed different patterns between the broad sense FDR and SDR mechanisms. Changes in simplex transmissions along the genome resulted from recombination. The simplex transmission patterns represented the heterozygosity restitution rate reported in diploid species (Cuenca *et al.* 2011). Markers in the centromeric region showed only simplex transmission in FDR gametes and equal fractions of duplex and nulliplex transmissions in SDR gametes. Due to recombinations

	Genotypes of Parents	Alternative allele Frequency of Parents	Ploidy and Genotypes of Gametes	Genotypes of Progeny	Alternative allele Frequency of Progeny
I	'Fuyu' ♀ aaaaaa	0 (0-1%)	<i>n</i> → aaa		1/9 (4-17%) 2/9 (18-31%) 0 (0-3%)
	'Taishuu' ♂ Aaaaaa	1/6 (13-18%)	FDR → Aaaaa SDR { AAaaaa aaaaa		
II	'Fuyu' ♀ Aaaaaa	1/6 (13-18%)	<i>n</i> → { Aaa aaa		1/9 (4-17%) 0 (0-3%)
	'Taishuu' ♂ aaaaaa	0 (0-1%)	<i>2n</i> → aaaaaa		
III	'Fuyu' ♀ aaaaaa	0 (0-1%)	<i>2n</i> → aaaaaa		1/9 (4-17%) 0 (0-3%)
	'Taishuu' ♂ Aaaaaa	1/6 (13-18%)	<i>n</i> → { Aaa aaa		
IV	'Fuyu' ♀ Aaaaaa	1/6 (13-18%)	FDR → Aaaaa SDR { AAaaaa aaaaa		1/9 (4-17%) 2/9 (18-31%) 0 (0-3%)
	'Taishuu' ♂ aaaaaa	0 (0-1%)	<i>n</i> → aaa		

Figure 2 The theoretical genotypes in nonaploid progeny when inheriting simplex genotype of centromere markers from one of its hexaploid parents.

between the centromere and the markers, the duplex transmission increased toward the telomeres in FDR gametes but decreased in SDR gametes. Multivalent formation decreased the net allele transmission (i.e. simplex plus duplex) under both mechanisms, but to a larger extent in SDR gametes. Increased quadrivalent formation resulted in an increase in the duplex inheritance rate, and a decrease in the simplex inheritance rate, in both gamete types. This change was more obvious in telomere side in FDR gametes, and in centromere side in SDR gametes (Supplementary Figure S5). In order to discriminate the mechanisms, we focused on the pattern of the duplex to simplex transmission ratio across the genome (Supplementary Figure S6). In the FDR gametes, this ratio reaches its minimum value in the centromere and increases toward the telomere. In contrast, in SDR gametes, the duplex: simplex ratio is at its maximum value in the centromeric region and decreases toward the telomere. Simulations on very long chromosomes (>1k cM) showed that the ratio does not exceed 0.4 in the FDR gametes and does not fall below 0.2 in the SDR gamete in any quadrivalent formation fraction. In order to apply this ratio for the discrimination, it is required to measure an allele frequency of genome-wide markers in a 2n gamete population or in hybrids resulted from 2n gametes.

When the mechanism for an individual gamete or hybrid needs to be discriminated, allele transmission pattern through a single 2n gamete across the genome can be a primal focus (Figure 3). With both 0% and 100% quadrivalent formation, the ranges of simplex or nulliplex/duplex transmission overlapped between FDR and SDR mechanisms with more than 2 recombinations from the centromere; with 0 or 1 recombinations, the possible ranges of both mechanisms were distinctly different (Figure 3, A–D). In other words, beyond the 2nd recombination point, there could be a mixture of markers that are simplex and that are nulliplex or duplex depending on which homologous chromosome carried the simplex allele and which homologous chromosomes were involved in the recombinations, and thus those regions cannot be used for discriminating the mechanisms.

Based on the results, we propose a genome-wide scan of both transmission ratio at the SDR-centromere (TR-SDRc; i.e. nulliplex: simplex: duplex = 1:0:1) and transmission ratio at the FDR-centromere (TR-FDRc; i.e. nulliplex: simplex: duplex = 0:1:0) as a means to discriminate between the two mechanisms. This type of scan is achieved by fitting the nulliplex, simplex, and duplex transmission ratio in a given region to the possible transmission model at the centromere. At the centromere, all simplex SNPs in the parent remain simplex in FDR gametes, whereas they are either duplex or nulliplex in SDR gametes. When the SNPs are far from the centromere and recombination among homologous chromosomes occurs, both mechanisms produce simplex, nulliplex, or duplex status in the gametes (Figure 4). Specifically, allelic dosage doubling can only occur by recombination between marker and centromere in the FDR case but occurs over the entire chromosome including the centromere in the SDR case, leading to different distribution patterns.

There are limitations to this method. According to the genetic model and simulation, TR-SDRc in FDR gametes, and TR-FDRc in SDR gametes could be formed with ploidy/2 recombinations (three in hexaploid) from the centromere (Figure 3, E–J). Therefore, this method can only be applied to polyploid species and cannot discriminate the mechanisms in diploid species where a single recombination results in the centromeric transmission pattern of the alternative mechanism. Besides, the discrimination power differs between the formation mechanisms. According to the simulation, in a hexaploid, this method can

identify the correct mechanism in more than 85% of the homologous groups in FDR gametes with five recombination breaks in an arm (corresponding to approximately 100 cM in average; Supplementary Figure S7) and in more than 45% of the homologous groups for SDR gametes in hexaploid species (Figure 3, G and H).

Inheritance of MIA and PIA observed in the progeny

Here, we attempted to analyze a hybrid persimmon, ‘Akiou’, which was bred through a controlled cross using the elite PCNAs ‘Fuyu’ and ‘Taishuu’, as (hexaploid) maternal and paternal parents, respectively, by applying the method presented. Since the subject is a single hybrid, in the following, we focused on the allele transmission pattern through a single 2n gamete across the genome.

In total, 25,099,250 SNPs, which are heterozygous in at least one of the parents, were identified using our SNP selection criteria. Among them, 18,630,483 SNPs were heterozygous in both ‘Fuyu’ and ‘Taishuu’. Thus, the ratio of heterozygous SNPs shared between ‘Fuyu’ and ‘Taishuu’ was 74.23%. This high sharing ratio between ‘Fuyu’ and ‘Taishuu’ is in accordance with the fact that ‘Fuyu’ was the maternal parent of ‘Taishuu’.

To apply the discrimination strategy, SNPs in progeny ‘Akiou’ should be distinguished by their transmission origin. Following the SNP selection criteria, 3,658,671 MIA and 2,809,961 PIA were found (Table 1). Among these SNPs, 2,208,808 MIA and 2,577,264 PIA were found in ‘Akiou’ in heterozygous state (Table 1). The average density of MIA and PIA inherited to ‘Akiou’ was 3.08/kilobase (kb) (Figure 5A) and 3.58/kb (Figure 5B), respectively. The inheritance ratio was 60.37% (Figure 5C) for MIA and 91.72% (Figure 5D) for PIA, which suggested that the 2n gamete that bred nonaploid ‘Akiou’ was derived from the paternal parent, namely, ‘Taishuu’.

In order to discern the origin of the 2n gamete and the formation mechanism, we focused on simplex SNPs in the parents and analyzed the allele transmission in the progeny. The simplex SNPs in the parents should transmit to the progeny with a specific ratio depending on the 2n gamete formation mechanism, genetic distance from the centromere, and pairing pattern (Figures 2 and 3). Therefore, the RSS value was used to report on the goodness-of-fit for the nulliplex: simplex transmission pattern in the progeny against expected ratio for n gamete (1:1) to identify the 2n gamete origin and possible formation mechanism. The simplex MIA showed nulliplex (386,300 loci), simplex (445,397 loci), and duplex (17,215 loci) transmissions in the progeny (Figure 6A). Similarly, the dosages of the simplex PIA in ‘Akiou’ were nulliplex (41,113 loci), simplex (489,869 loci), and duplex (34,983 loci) (Figure 6B). The nulliplex: simplex transmission pattern of simplex MIA in the progeny showed a much better fit of a 1:1 model ($RSS = 1.7 \times 10^9$) than the simplex PIA ($RSS = 1.0 \times 10^{11}$), which indicated that the simplex MIA followed the inheritance ratio of n gamete as described in the hypothetical cross-combination II in Figure 2. Thus, the maternal parent ‘Fuyu’ supplied an n gamete and the paternal parent ‘Taishuu’ supplied a 2n gamete to originate nonaploid ‘Akiou’.

Distribution of allele transmission

Genome-wide distribution of duplex (Figure 7A) and simplex (Figure 7B) inheritance of the PIA was plotted. It was observed that most of the PIA occurred in the simplex state in progeny (Figure 7B), which fits the transmission of FDR gamete. Additionally, it appeared that regions with a high ratio of duplex

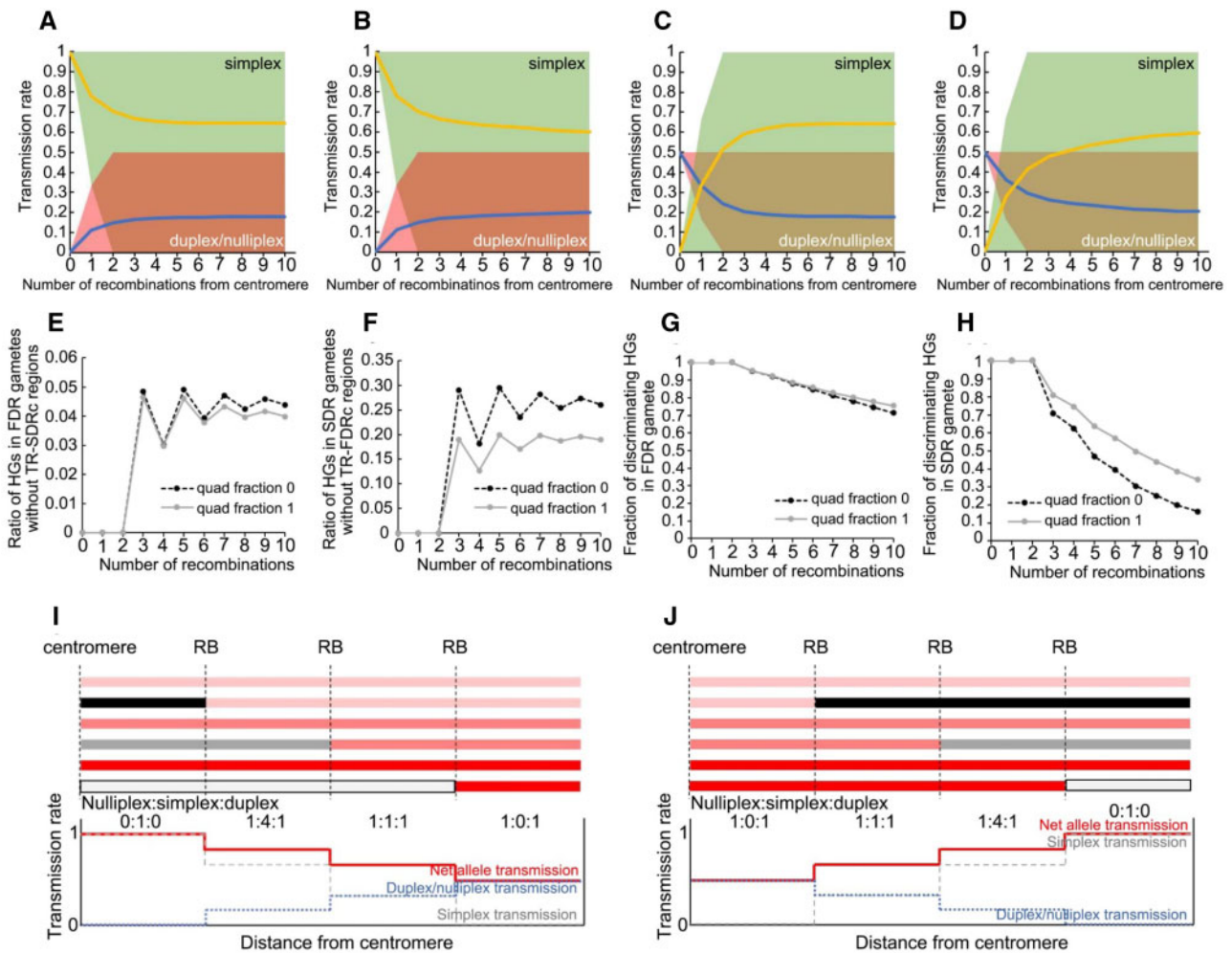


Figure 3 Simulation of the range of allele transmission in $2n$ gametes. (A–D) Simulated $2n$ gametes produced by a hexaploid individual. More than 5 million $2n$ gametes were generated for each experiment. Green and red areas represent the possible range of simplex and duplex/nullplex transmission rates, respectively. Yellow and blue lines represent the mean values of simplex and duplex/nullplex transmission rates, respectively. (A, B) Simulated $2n$ gametes generated by the broad sense FDR (with recombination) mechanism. (C, D) Simulated $2n$ gametes generated by the SDR mechanism. Individual $2n$ gamete was generated with 0% (A, C) and 100% (B, D) quadrivalent fraction, respectively. (E) Occurrence of homologous groups (HG) in FDR gametes with regions in which the allele transmission pattern accidentally coincides with the transmission ratio at the SDR-centromere (TR-SDRC). It should be noted that we assumed chromosomes with only one arm during this simulation. (F) Occurrence of HGs in SDR gametes with regions in which the allele transmission pattern accidentally coincides with the TR-FDRc. (G) Fraction of HGs without the TR-SDRC regions in FDR gametes. These types of HGs cannot be observed in SDR gametes, and thus were referred to as discriminating HGs. (H) Ratio of HGs in SDR gametes without the TR-FDRc regions. (I, J) Example of the recombination process for the $2n$ gamete produced via (I) the FDR broad sense mechanism with the TR-SDRC regions and (J) the SDR mechanism with the TR-FDRc regions. Each color bar represents different homologous chromosomes in a $2n$ gamete produced by a hexaploid individual. Recombination events at the recombination breakpoint (RB in the figure) were assumed. It should be noted that the reported counts of recombination in this figure took place in one of the two $2n$ gametes generated by a single meiosis but the other was not considered.

inheritance matched regions with a low ratio of simplex inheritance (Figure 7, A and B).

In order to verify the conclusion that ‘Taishuu’ supplied a $2n$ gamete that was the result of FDR, we investigated the alternative scenario whereby the $2n$ gamete was the result of SDR. In this SDR mechanism, the simplex SNPs in the pericentromeric region of the parent, which offered the $2n$ gamete, should be duplex or nullplex with equal probability in the progeny, whereas they should be entirely simplex in the FDR mechanism (Figure 4). Therefore, no simplex inheritance of the PIA to the progeny in the pericentromeric region would occur, and the nullplex:simplex:duplex ratio should be 1:0:1 (Figures 3J and 4). The presumed pericentromeric regions identified in the SDR scenario were defined based on the high fraction of duplex inheritance, and the regions of Chr2: 50.5–51.5 Mb, Chr3: 3.3–4.1 Mb, Chr 3: 33.9–34.5 Mb, Chr4: 0.1–2.9 Mb, Chr7: 0–2 Mb, Chr7: 41.9–42.6 Mb,

Chr10: 32.1–33.7 Mb, Chr12: 0.5–1.3 Mb, and Chr12: 50.7–52 Mb were selected (Figure 7A). Subsequently, the major concern is that whether the simplex dosage inherited from simplex PIA in these regions is negligible. If so, these regions are supported to be authentic pericentromeric regions in the SDR scenario. If not, the SDR scenario will be rejected. Here, we validated this by comparing RSSs against expected transmission ratio at SDR centromere (TR-SDRC) and at a region with a chromosome combination formed by single recombination from the SDR centromere combination (nullplex:simplex:duplex = 1:1:1; Figure 3, I and J). The PIA was found in nullplex (350 loci), simplex (847 loci), and duplex (694 loci) SNPs in ‘Akiou’ within the region of Chr2: 50.5–51.5 Mb, and the nullplex:simplex:duplex ratio fitted more to 1:1:1 (RSS = 1.3×10^5) than 1:0:1 (RSS = 1.1×10^6) (Figure 8A). Additionally, the nullplex:simplex:duplex ratio was fit best with the 1:1:1 model, in comparison to the 1:0:1 model, for the

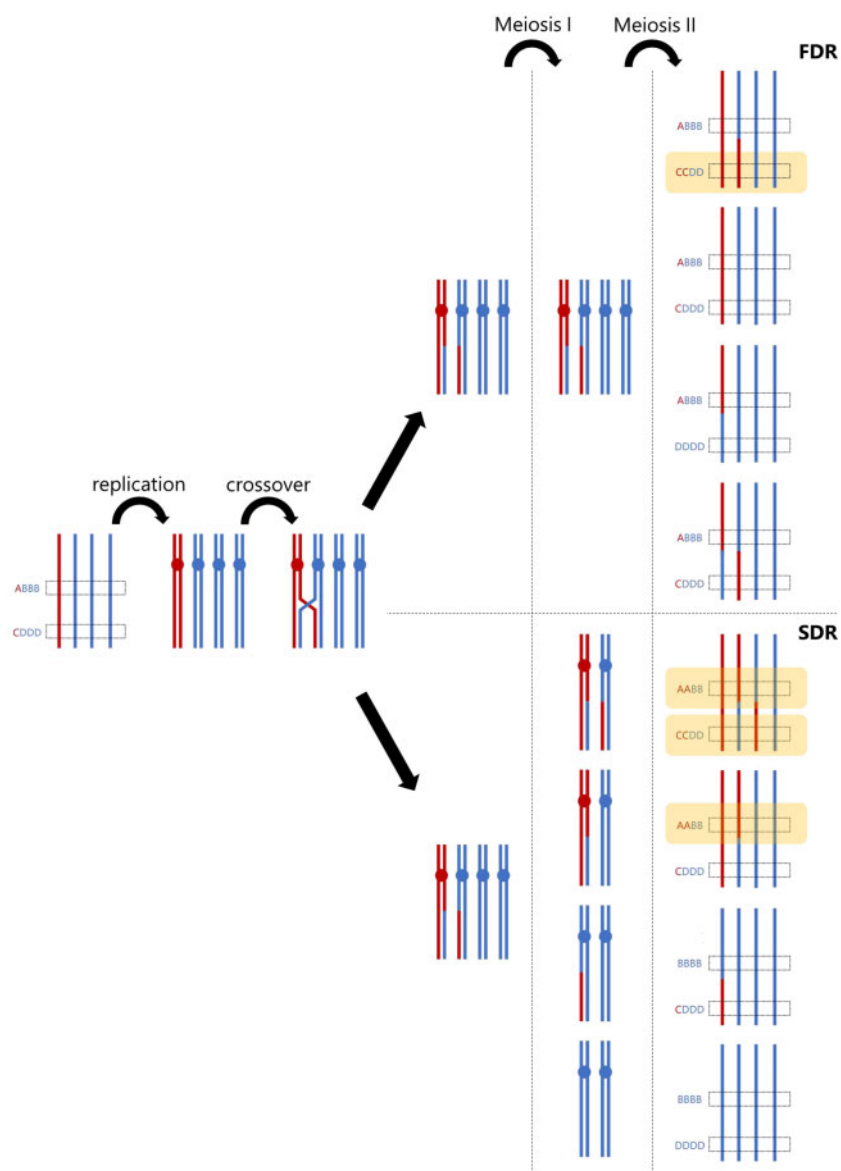


Figure 4 A model of the allelic dosage doubling mechanism in $2n$ gamete formation. A tetraploid case was used for simplicity, and the broad sense FDR (with recombination) and SDR mechanisms are shown. The SNPs with doubled alleles are highlighted in yellow.

Table 1 The number of maternal/paternal specific SNPs inherited to the progeny

	Total heterozygous loci	Heterozygous loci with an allele inherited to 'Akiou'	Inheritance ratio (Heterozygous loci with an allele inherited to 'Akiou'/Total heterozygous loci) (%)
'Fuyu'—specific MIA	3,658,671	2,208,808	60.37
'Taishuu'—specific PIA	2,809,961	2,577,264	91.72

remaining eight regions (Figure 8, B–I). Thus, the putative centromere regions, defined based on the duplex inheritance pattern in SDR, did not show TR-SDRC, but fit more to the transmission ratio at a region with a chromosome combination formed by one recombination from the SDR centromere combination, which could be present in FDR gametes (Figure 3, I and J). This result rejects the hypothesis that the SDR mechanism was involved in the production of 'Akiou'.

Since the SDR mechanism was rejected, the $2n$ gamete giving rise to 'Akiou' must have originated by FDR. In order to further verify this inference, the simplex PIA on the presumed

pericentromeric regions under the FDR scenario was selected for further analysis. The genomic regions (Red signs in Figure 7B) with a high level of the simplex inheritance of the simplex PIA ($\geq 95\%$) should be close to the centromere under the FDR scenario. It is worth noting that more than one presumable pericentromeric region was defined in many chromosomes according to this criterion in Figure 7B. Two mechanisms resulted from this phenomenon: (1) a portion of the regions is located between the centromeres and a recombination break, which is the closest to centromere; and (2) recombination and chromosome segregation during meiosis produce a parental chromosomal combination,

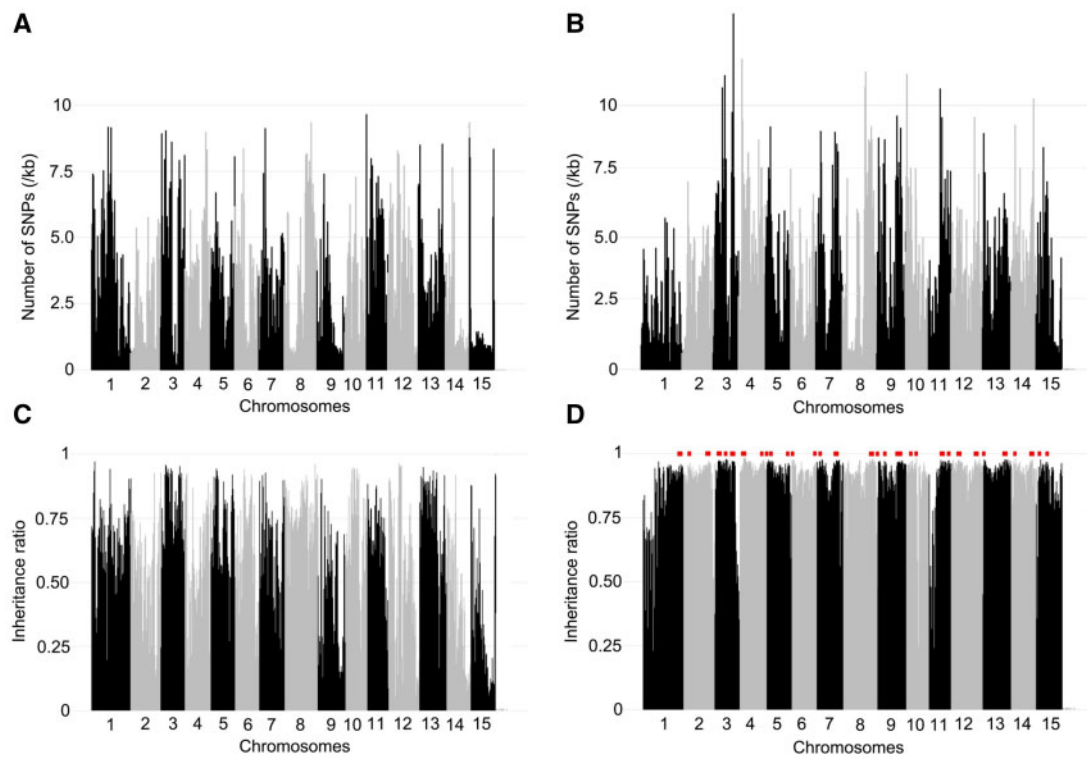


Figure 5 Genome-wide distribution of SNP density of MIA (A) and PIA (B) inherited to 'Akiou' per kb in every 1 Mb region along the genome; the proportions of MIA (C) and PIA (D) inherited to 'Akiou' in every 1 Mb region along the genome. Red lines in (D) indicate the presumable pericentromeric regions, with high inheritance proportions in the FDR scenario ($\geq 95\%$).

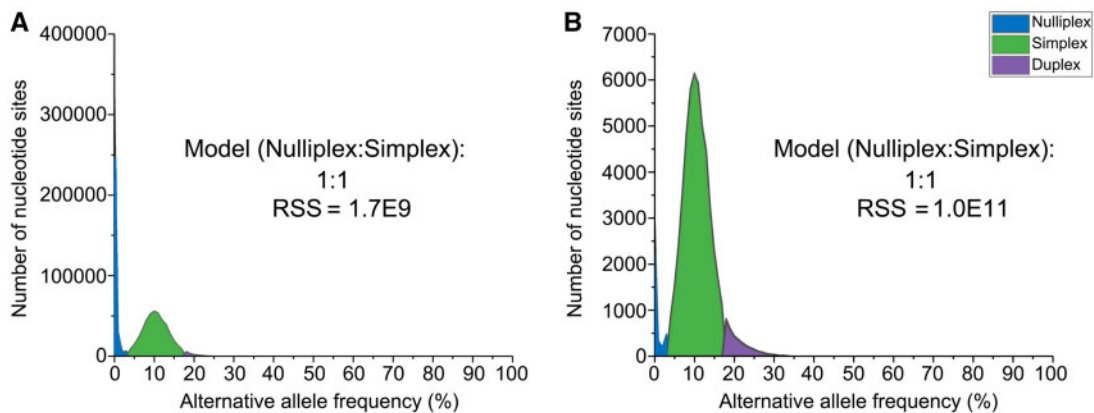


Figure 6 Inheritance of the simplex MIA and PIA to the progeny. (A) Alternative allele frequency distribution of the simplex MIA in 'Akiou'; (B) Alternative allele frequency distribution of the simplex PIA in 'Akiou'. The value in each figure represents RSS value of the goodness-of-fit of the nulliplex:simplex ratio against the 1:1 model.

which is same as that of centromere. According to Figure 7B, six presumed pericentromeric regions, including Chr 1: 58–64 Mb, Chr4: 5–18 Mb, Chr5: 34–37 Mb, Chr 8: 37–53 Mb, Chr11: 18–27 Mb, and Chr 15: 15–19 Mb, were selected to determine the inheritance of the simplex PIA in the parent to the progeny.

In the putative centromere regions based on the simplex inheritance pattern of FDR, PIA showed the FDR centromere pattern (nulliplex:simplex:duplex = 0:1:0) (Figure 9, A–F), rather than the one next to the FDR centromere (one recombination occurred between two homologous chromosomes here, and nulliplex:simplex:duplex = 1:4:1) (Figure 3I). This suggests that the regions are located between the centromere and a recombination break, which is the closest to centromere; whereas this pattern could

also be obtained with a combination of parental chromosomes mixed by recombination, as mentioned above. Collectively, regions with TR-FDRc were observed across the genome, but there were no regions with TR-SDRc in 'Akiou'. This result exclusively fits the broad sense FDR mechanism, as illustrated in cross-combination I (Figure 2).

Discussion

In this study, a new strategy for uncovering mechanism used to produce $2n$ gametes in polyploid plants was proposed. We showed that even without any information on the location of centromere, it is possible to discriminate between the two

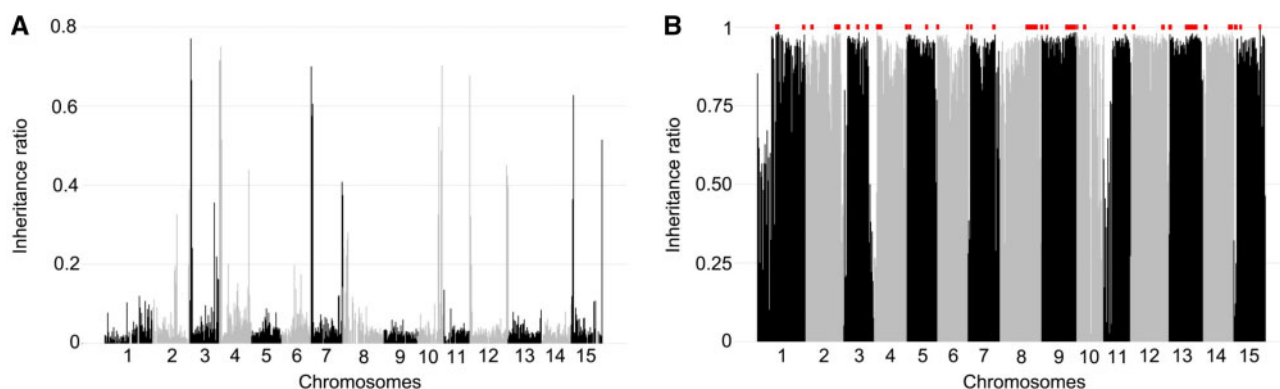


Figure 7 Genome-wide inheritance pattern of the simplex PIA. (A) Genome-wide distribution of duplex inheritance of the simplex PIA. (B) Genome-wide distribution of simplex inheritance of the simplex PIA. The y-axis represents the ratio of SNP density of duplex (A) and simplex (B) genotypes in the progeny, inherited from the simplex PIA. The ratio was calculated and plotted every 1 Mb, along the genome. Red lines in (B) indicate the presumable pericentromeric regions with a high inheritance proportion in the FDR scenario ($\geq 95\%$).

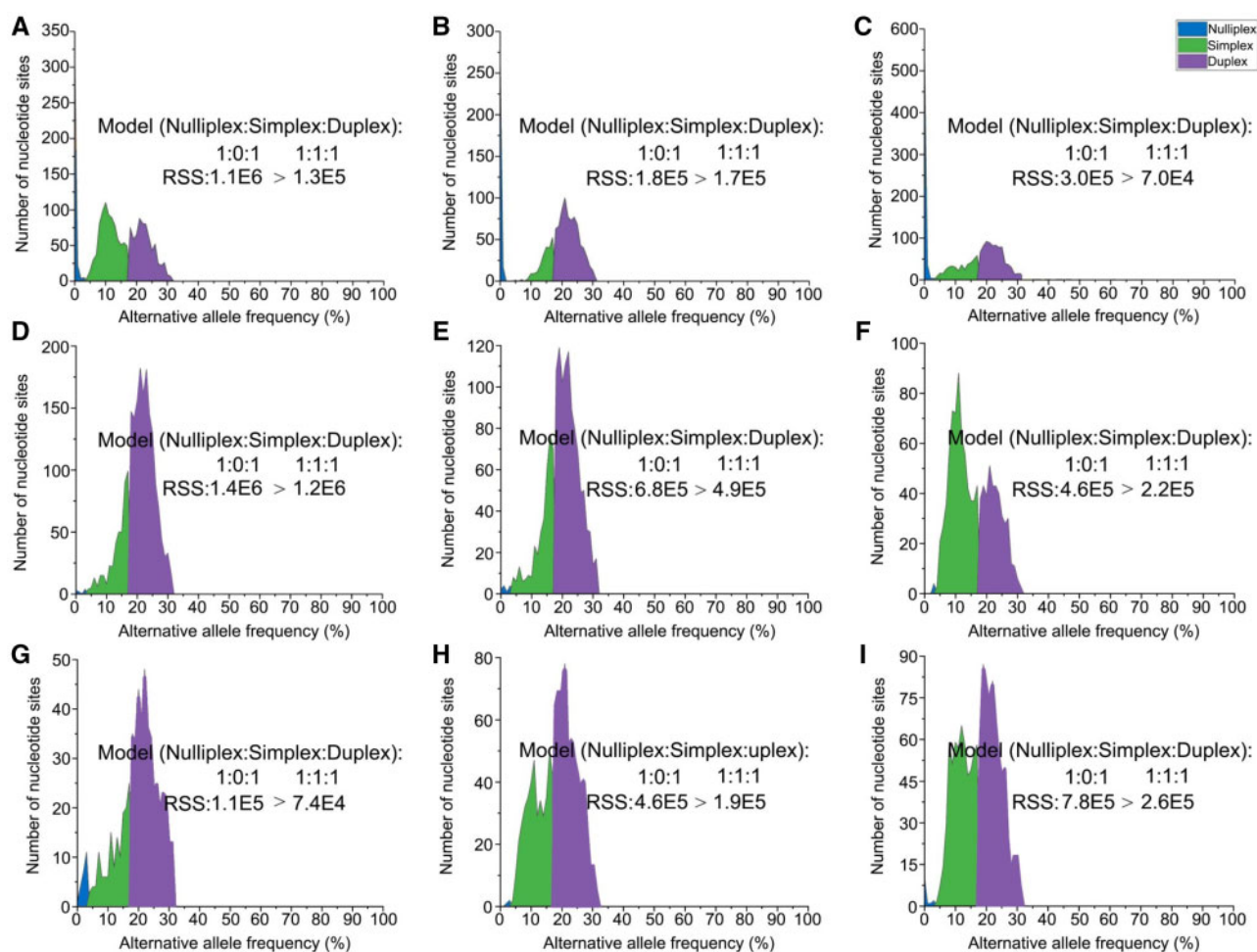


Figure 8 Inheritance of simplex PIA to progeny in the presumed pericentromeric regions (A) Chr2: 50.5–51.5 Mb, (B) Chr3: 3.3–4.1 Mb, (C) Chr3: 33.9–34.5 Mb, (D) Chr4: 0.1–2.9 Mb, (E) Chr7: 0–2 Mb, (F) Chr7: 41.9–42.6 Mb, (G) Chr10: 32.1–33.7 Mb, (H) Chr12: 0.5–1.3 Mb, and (I) Chr12: 50.7–52 Mb for the alternative SDR scenario. The former and latter values in each figure represent RSS values of the goodness-of-fit of the nullplex:simplex:duplex ratios to the 1:0:1 and 1:1:1 models, respectively.

possible mechanisms associated with the $2n$ gamete formation and the parental origin of the $2n$ gamete. Since the genotypes of simplex centromeric markers are not affected by ploidy or the occurrence of multivalents, the strategy presented in this study can be tested on the products of any hybridizations involving $2n$ gametes in polyploid species. The mechanisms resulting in

individual $2n$ gamete can be distinguished by analyzing the goodness-of-fit of the observed transmission to the expected transmission ratio at the centromere, assuming that any recombinations do not produce a chromosome combination expected at the centromere under the alternative hypothesis. This is particularly useful for identifying FDR-type gametes,

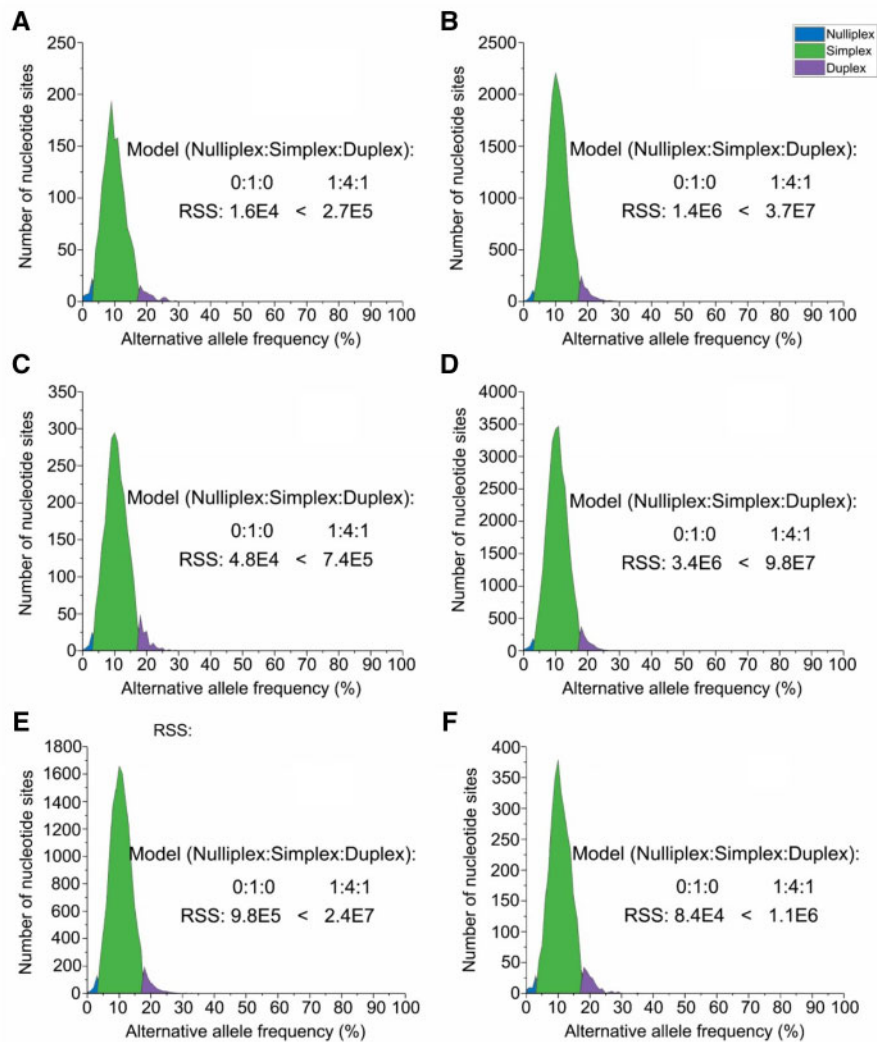


Figure 9 Inheritance of simplex PIA to the progeny in the presumed pericentromeric regions (A) Chr 1: 58–64 Mb, (B) Chr4: 5–18 Mb, (C) Chr5: 34–37 Mb, (D) Chr 8: 37–53 Mb, (E) Chr11: 18–27 Mb, and (F) Chr 15: 15–19 Mb for the broad sense FDR scenario. The former and latter values in each figure represent RSS values of the goodness-of-fit of the nulliplex:simplex:duplex ratio to the 0:1:0 and 1:4:1 models, respectively.

though there is also the potential to identify SDR-type gametes (Figure 3, G and H). This approach also allows to identify the centromeric region, given that a gamete has arisen by either the FDR or SDR scenario.

Quadrivalent formation in normal meiosis affects allele transmission pattern, whereas the effect in the transmission through $2n$ gametes has not been well characterized. Here, it was shown that increased quadrivalent formation increases duplex transmission of parental simplex alleles in $2n$ gametes (Supplementary Figure S3). In SDR gametes, the increased amount of duplex transmission in the quadrivalent is due to the situations where the chromosomes involved in crossing over go toward the same pole. We have no clear explanation for FDR gametes, but the increased duplex transmission in the FDR gametes could be related to the increased opportunities for recombination with quadrivalent formation (Sved 1964; Voorrips and Maliepaard 2012).

Because the duplex fraction in FDR gametes arose solely by recombination (Figures 3 and 4), the observed distribution of dosage doubling in ‘Akiou’ reflects the recombination landscape under the FDR scenario. Interestingly, allelic doubling was almost exclusively detected at the telomeres of the *D. oleifera* reference

genome (Figure 7A), suggesting macroscale collinearity between *D. kaki* and *D. oleifera*.

Despite strong inbreeding in the development of ‘Akiou’, as observed in its pedigree (Chijiwa et al. 2013) and genotype (Table 1), ‘Akiou’ has intermediate vigorousness between ‘Taishu’ (moderate) and ‘Fuyu’ (strong) (Chijiwa et al. 2013), and so does not suffer from the inbreeding depression common in modern PCNA breeding. This is probably a consequence of polyploid breeding. Progeny bred by the FDR-derived $2n$ gametes tends to be superior to that not derived from $2n$ gametes and that derived from SDR-type $2n$ gametes, due to the increased allelic diversity, resulting in heterosis (Yao et al. 2013), as FDR is generally more than twice as effective in transmitting heterozygosity as SDR (Peloquin et al. 2008). The assumed superiority of FDR-derived $2n$ gametes was confirmed with respect to yield in potato (Hutten et al. 1994).

The outstanding traits of ‘Akiou’, such as vigorousness, larger fruit size, and seedlessness, demonstrate that polyploid breeding is valuable in persimmon and likely to be advantageous in other breeding programs. The FDR gamete is likely to be a useful model to study the recombination landscape in polyploid species, as well as for breeding. Apart from some reports suggesting polysomic inheritance in the astringency-controlling locus based on genotype

segregation (Akagi et al. 2012; Nishiyama et al. 2018), no systematic characterization of chromosomal behavior in polyploid persimmon has been reported. Further analysis combining cytology, double reduction, and 2n gamete formation may be helpful for understanding the chromosomal evolution of *Diospyros*.

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P.S., S.N., H.A., J.F., and R.T. conceived the study. P.S. and S.N. designed the study and drafted the manuscript. H.A. and R.T. prepared the materials. P.S. and S.N. carried out the experiments and conducted the bioinformatics analysis. S.N. and R.E.V. developed and carried out the simulation. All authors interpreted the data and edited the manuscript.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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