Heterozygosity analysis of spontaneous 2n female gametes and centromere mapping of the diploid Hevea brasiliensis based on full-sib triploid populations

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Abstract

Key message

Unreduced megagametophytes via second division restitution was confirmed through heterozygosity analysis, and four candidate physical centromeres of rubber were located for the first time.

Abstract: 【Objective】The evaluation of maternal heterozygosity restitution (MHR) is vital in identifying the mechanism of 2n gametogenesis and assessing the utilization value of 2n gametes. 【Method】In this study, three different full-sib triploid populations were employed to evaluate the MHR of 2n female gametes of rubber clone GT1 and confirm their genetic derivation. 【Result】The results indicated that the 2n female gametes of GT1 are derived from second division restitution (SDR) and transmit more than half of the parental heterozygosity. In addition, low recombination frequency markers were developed, and four candidate physical centromeres of rubber were located for the first time. 【Conclusion】The confirmation of 2n female gametes of rubber clone GT1 are derived from SDR provides insights into the molecular mechanisms of 2n gametogenesis. Additionally, the identified centromere location will aid in the development of centromeric markers for the rapid identification of 2n gametogenesis mechanism.

Key words: 2n female gamete • SDR • centromere location • full-sib population • *Hevea brasiliensis*

Introduction

An unreduced gamete, also known as a 2n gamete, is a germ cell that possesses the same number of chromosomes as the somatic cell. Despite pre- and post-meiotic replication phenomena, 2n gametes are mainly formed through meiotic abnormalities. Meiotic irregularities can be classified into the first and second meiotic abnormalities, based on the occurrence stage. While based on genetic effects, 2n gametes can be categorized into first-division restitution (FDR) and second-division restitution (SDR). As named, homologous chromosomes of FDR-type gametes are restituted after normal first meiosis, equivalent to the loss of the first meiosis. Consequently, homologous chromosomes are not separated, but sister chromosomes are typically separated during the second meiosis (Fig. 1). For SDR-type gametes, homologous chromosomes are separated normally during the first meiosis, sister chromosomes are restituted after normal second meiosis or the entire second meiosis are absent (Fig. 1). Theoretically, in the absence of chromosomal crossover and recombination during meiosis, FDR- and SDR-type 2n gametes transmit heterozygous and homozygous genetic compositions, respectively. However, because of the existence of meiotic recombination, FDR-type 2n gametes usually transmit 70-80% of maternal heterozygosity

restitution (MHR) into offspring, while SDR-type 2n gametes only transmit 30-40% of maternal heterozygosity (Barone et al. 1995; Peloquin et al. 2008; Liesebach et al. 2015; Dong et al. 2015). The disparity in maternal heterozygosity transmission capacity between FDR and SDR emphasizes the importance of precise identification of 2n gamete sources for polyploid breeding.

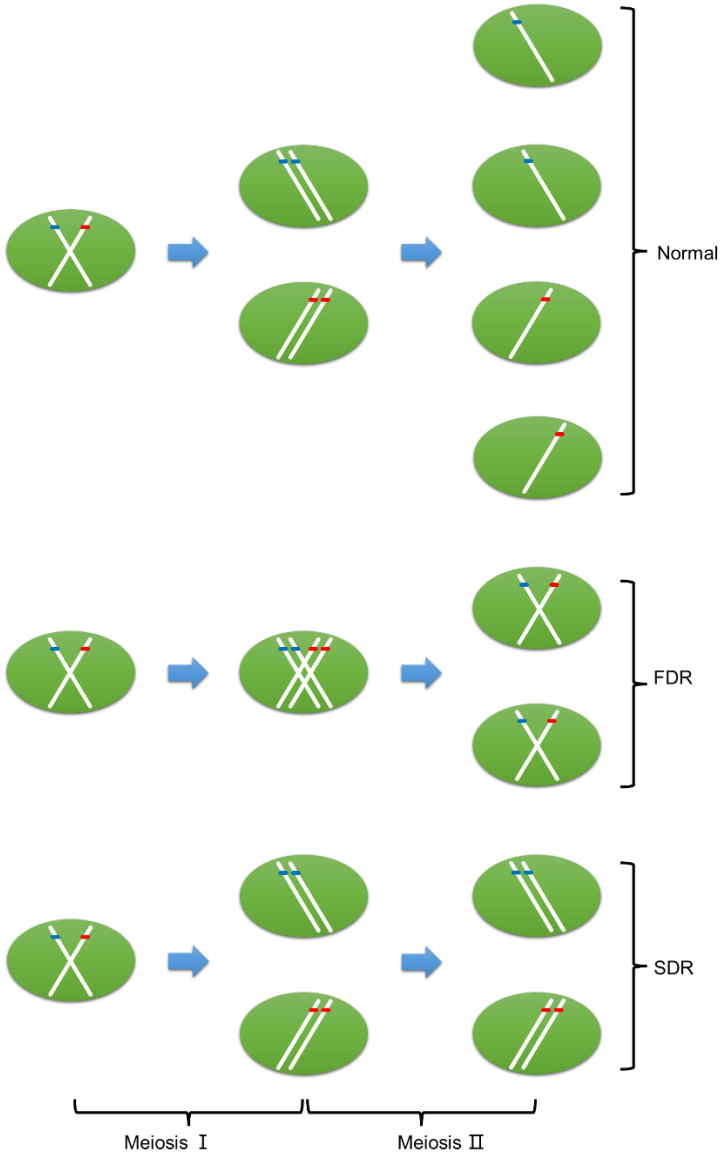

Fig. 1 Allele markers segregation during normal meiosis, FDR, and SDR

In flowering plants, 2n gametes usually combine with a reduced gamete to produce a triploid, and rarely with another 2n gamete to produce a tetraploid, because of the low probability for unreduced female and male gametes to meet. In *Hevea brasiliensis*, although natural triploids were discovered decades ago, the natural 2n gametes that led to their formation were not confirmed until recent years (Yao et al. 2016; Zhang et al. 2019). As far as we know, the first reported natural triploid of rubber tree was discovered about fifty years ago (Zheng et al.1981). Later, consciously select polyploids from offspring of different cross-mating conducted (Hu et al. 1982), and many triploids were separated from the progeny of maternal clone GT1 (which is a primary clone selected by Indonesia and widely planted in Southeast Asia). Thereafter, numerous triploids were identified using the high-throughput ploidy detection method called flow cytometry. Yao et al. (2016) first identified eight half-sib triploids offspring of

rubber clone GT1 came from 2n female gametes, and the rate of MHR varied between 27.78% and 75.00% with a mean of 51.69%. After that, a larger population (446 triploids) of half-sib triploid progeny of clone GT1 were screened, 76 triploids were determined derived from 2n female gametes, and 37 triploids of them maintain 14.29% to 66.67% of MHR with a mean of 41.38% (Zhang et al. 2019). Notably, triploid varieties "Yunyan77-2" and "Yunyan77-4" with cold-resistant and high-yield (Li et al. 2009) recently confirmed that come from 2n female gametes by molecular markers (Zhang et al. 2018) highlighted the potential of using 2n female gametes for polyploid breeding in rubber tree. Although both studies suggest that the 2n female gametes originated from SDR, the precision of MHR of 2n female gametes is low due to the small sample size and inadequate markers.

Centromeres are the genomic regions that organize and regulate chromosome behaviors during cell cycle, and their variations are associated with genome instability, karyotype evolution and speciation in eukaryotes (Zhou et al. 2022). Centromere malfunction frequently results in ploidy variation such as haploid and polyploid formation in plants (Keceli et al. 2020). Therefore, centromere-mediated ploidy changes have been implemented to expedite crop improvement through breeding (Ishii et al. 2016). Centromere mapping is important for distinguishing chromosome arms, identifying proximal and distal markers or genes (Bastiaanssen et al. 1996; Part et al. 2007), which is of great significance to improve existing genetic maps, locate quantitative trait loci (QTLs), and conduct molecular marker assisted breeding research (Ollitrault et al. 2020). In *Hevea brasiliensis*, multiple versions of genome have been published (Rahman et al. 2013; Tang et al. 2016; Lau et al. 2016; Pootakham et al. 2017; Liu et al. 2020; Cheng et al. 2023), nonetheless, the ignorance in locating the centromeres can be attributed to unconsciously neglecting or inadequacy of localization techniques.

In this study, three different full-sib triploid populations were used as materials to evaluate the MHR of 2n female gametes of GT1 and further confirm their genetic derivation. To identify the physical location of centromeres, SSR markers were formulated to probe regions with low recombination frequency. The impact of population size and the number of markers on the MHR analysis of 2n gametes are discussed.

Materials and methods

Plant materials

Full-sibling triploids of GT1×Reyan879 and GT1×IAN873 were obtained through artificial pollination. In brief, the conventional hand-pollination method was conducted in Mengla (Yunnan Province, China) in 2015 and in Ruili (Yunnan Province, China) in 2019, 2020, and 2021. In these years, capsules containing GT1 were harvested before falling, and the seeds were separated from the capsules. Subsequently, the seeds were sown in a nursery sand bed in Danzhou (Hainan Province, China), and the resulting seedlings were tested using flow cytometry to detect triploids. The triploid progeny population of GT1×PR228 was obtained via open pollination, and the parents were identified in previous study (Zhang et al. 2019). Leaves of the parents and triploids were collected and stored at −80 °C for DNA extraction.

Flow cytometry and chromosome numbers for ploidy determination

Flow cytometric analysis was performed using a CyFlow®Cube8 with an ultraviolet lamp to detect 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei (http://www.sysmex-partec.com). Fresh leaves of one-year-old

seedlings at the green stage were sampled, quickly sectioned in 500 μL of extraction buffer (LOT1709111, Sysmex), and nuclei were passed through a 30 μm filter. Next, the nuclei were mixed with 1.6 mL of DAPI staining buffer (LOT1709112, Sysmex) and incubated for 30 s at room temperature, after which the samples were analyzed by flow cytometry. The diploid parent GT1 was used as a control. ModFit LT 3.1 (Inc, Verity Software House) was used for visualizing the flow cytometry data.

Chromosome numbers of somatic cells in polyploids detected via flow cytometry were counted as described previously (Zhang 2013). Briefly, unexpanded bronze-colored leaves were sampled and soaked in paradichlorobenzene saturated solution for 4-6 hours. The leaves were then transferred to Carnot solution (V ethanol: V acetic acid = 3: 1) and fixed for 24 hours at 4 □. After three rinses with water, the fixed leaves were dissociated in the mixture of 38% HCI and Ethanol for 10-20 mins at room temperature. The dissociated leaves were then stained with Carbopol fuchsin solution and squashed on a microscope slide. Cells with clear chromosome images were observed under an Olympus BX53 microscope and photographed with a VP700c digital camera.

SSR mining

The reference genome sequence of rubber clone GT1 (Liu et al. 2020) is publicly available in the National Center for Biotechnology Information (NCBI). The reference genome sequence of rubber clone Reyan879 is unavailable in public databases. The MISA software tool (Thiel et al. 2003) for microsatellite identification was used to search for SSR loci from the two genome sequences. The SSR sequences of GT1 and Reyan879 were compared, and the sites with length differences in the same positions were found. Sites with base conversion and transversion in the flanking sequence between individuals and sites with single base repeat units and complex repeat units in the sequence were filtered out. Sites containing repeat units composed entirely of G/C bases were also removed. Primer3 (Untergasser et al. 2012) was used to design PCR primers, and primers with a length other than 20 bp and/or containing “AAAA,” “TTTT,” “CCCC,” “GGGG,” “AGAGAG,” “ACACAC,” “ATATAT,” “GCGCGC,” “GTGTGT,” “CACACA,” “CGCGCG,” “CTCTCT,” “TATATA,” “TGTGTG,” “TCTCTC” were removed. Finally, primers with the target product at 110-350 bp were selected for screening for polymorphism primers.

DNA extraction and SSR analysis

DNA was extracted from each stored leaf sample using the DNAsecure Plant Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer’s instructions. The fluorescently labeled TP-M13-SSR polymerase chain reaction (PCR) method (Schuelke 2000) was used with a forward primer labeled with a universal M13 primer tail (5-TGTAAAACGACGGCCAGT-3) at the 5′ end, and the universal M13 primer labeled with either 6-carboxyfluorescein (FAM, blue), hexachloride-6-carboxyfluorescein (HEX, yellow), 6-carboxy-x-rhodamine (ROX, green), or tetrachloro-6-carboxy rhodamine (TAMRA, red). The PCRs were performed using the following conditions: denaturation for 5 min at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at the optimal annealing temperature for each SSR marker, and 30 s at 72 °C; 8 cycles of 30 s at 94 °C, 30 s at 53 °C, and 30 s at 72 °C; and a final extension of 8 min at 72 °C. Capillary electrophoresis-based fluorescent SSR analysis was performed using the ABI3730xl DNA Analyzer (Genewiz Inc., Beijing, China). GeneMarker software V2.20 (SoftGenetics, LLC. College Station, PA, USA) was used to analyze the allelic configuration of each sample from the raw sequence data.

Genotype of 2n gametes and MHR analysis

The loci with "ab×cd" or "ab×cc" type genotype between the female parent and the male parent clone were selected for triploid genotyping and heterozygosity analysis. Theoretically, regardless of a null allele, allele insertion, or deletion, triploid hybrids have two alleles or double doses of one allele of the 2n gamete provider (Fig.2). Since the selected loci bear a completely different parental allelic configuration "ab×cd" or "ab×cc," the allelic configuration of triploid hybrids involves three different alleles as "abc" or two alleles as "aac," and the genotype of 2n gamete is consistent with that of the donor as "ab" or with two identical alleles from the donor as "aa". The "abc" type allelic configuration was read directly through GeneMarker software, and the "aac" type allelic configuration of triploids was assessed based on the measured allele dosage by the microsatellite DNA allele counting-peak ratio method (MAC-PR; Esselink et al. 2004).

The rate of maternal heterozygosity restitution (MHR) was calculated as follows:

$$\%\mathrm{HR} = n_{ab}/(n_{ab} + n_{aa} + n_{bb}) \times 100$$

where n is the number of 2n gametes, ab refers to heterozygous 2n gametes, and aa and bb refer to two types of homozygous 2n gametes (Xie et al. 2014).

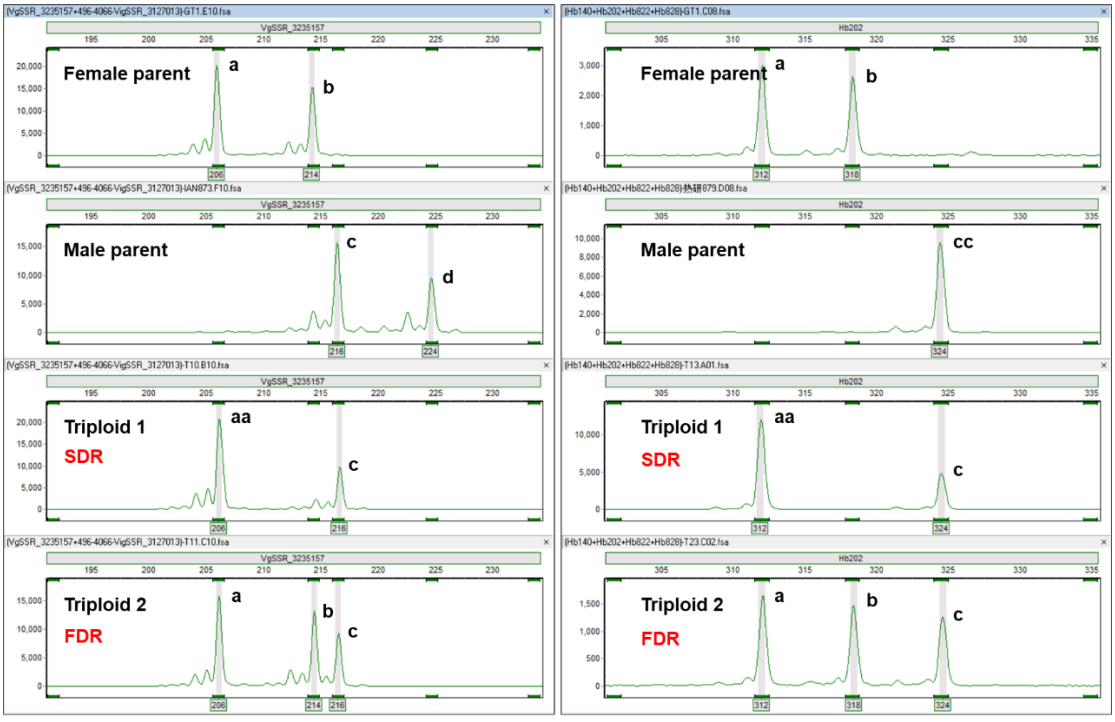

Fig. 2 Examples of the allele status of the female parent, male parent, and triploids. FDR-type and SDR-type loci. A. Parents with "ab×cd" loci, triploid 1 with SDR-type loci (aac), triploid 2 with FDR-type loci (abc); B. Parents with "ab×cc" loci, triploid 1 with SDR-type loci (aac), triploid 2 with FDR-type loci (abc).

Results

Triploid detection

A total of 36,787 female flowers were hand-pollinated to produce full-sib triploid progenies. 4,677 seeds were collected, resulting in 1,479 offspring, of which 75 were triploids detected through flow cytometric analysis and

chromosome counting (Table 1 and Fig. 3). Out of these triploids, only 58 from GT1×Reyan879 and 12 from GT1×IAN873 survived due to management issues and were consequently used for this study (Table 1). Moreover, 32 triploid progenies of GT1×PR228 that were identified in a previous study (Zhang et al. 2019) were utilized in the present study.

Table 1 Full-sib triploids obtained by hand-pollination

Hybrids	No. of hand-pollination	No. of seeds	No. of seedlings	No. of triploids	No. of triploids survived
GT1×Reyan879	29812	4325	1329	61	58
GT1×IAN873	6975	352	150	14	12
GT1×PR228					32

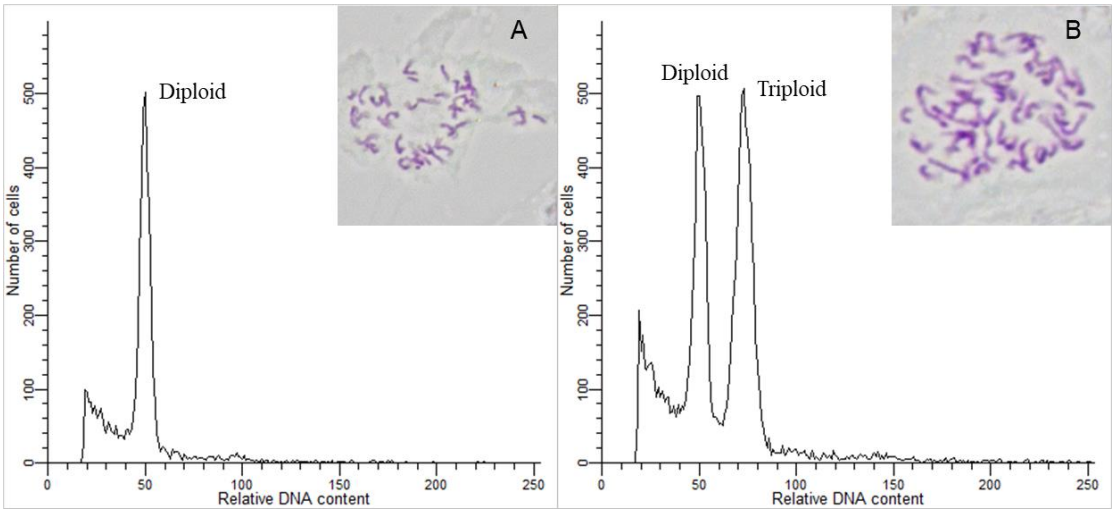

Fig. 3 Flow cytometric analysis and chromosome counting of diploid and triploid. A diploid, B mixture of diploid and triploid.

SSR marker screening

SSR marker screening involved selecting different numbers of primer pairs for polymorphism screening and genotyping in the full-sib triploid populations (Table 2). For the triploid progenies of GT1×Reyan879, a total of 2,224 pairs of primers were selected, including 1,211 that were mined specifically for this population using the SSR mining producer. In addition, 1,000 pairs of primer pairs were chosen randomly from SSRome (Mokhtar and Atia 2019), which included 750 genic and 250 non-genic SSRs. The remining thirteen pairs of primers were selected from published papers (An et al. 2009; Triwitayakorn et al. 2011; Li et al. 2012; Zhang et al. 2019; An et al. 2021; Wei et al. 2022). For the triploid progenies of GT1×IAN873, 1,020 pairs of primers were selected including 1,000 randomly chosen from SSRome, as well as 20 pairs selected from published papers, as mentioned above. Similarly, for the triploid progenies of GT1×PR228, 1,006 primer pairs were selected, including 1,000 randomly chosen from SSRome and six pairs selected from published papers.

Table 2 Number of pairs of primers selected and passed screening and used in this study

Hybrids	Sources	No. of pairs of primers selected for screening	No. of pairs of primers for genotyping	No. of pairs of primers for MHR analysis
GT1×Reyan879	Published papers	13	2	1
	SSRome	1000	20	11
	SSR mining	1211	54	43
GT1×IAN873	Published papers	20	2	1
	SSRome	1000	43	26
GT1×PR228	Published papers	6	2	0
	SSRome	1000	17	13

Genotype scanning was performed on the parents using selected primers, with 76, 45, and 19 pairs of primers displayed “ab×cd” or “ab×cc” type genotype in GT1×Reyan879, GT1×IAN873, and GT1×PR228 respectively, and then used for the MHR analysis of the three full-sib triploid populations. After triploids genotyping, any pairs of primers with an error rate greater than 10% in the populations, which caused by null allele, allele insertion, or allele deletion, were discarded. Finally, 55, 27, and 13 pairs of primers and their genotyping results were used for further analysis (Table S1). The physical position of the markers was obtained by BLAST against the Reyan879 genome using Tbtools (Chen et al. 2020), revealing that 55 markers for GT1×Reyan879 were distributed on 17 chromosomes, 27 markers for GT1×IAN873 were distributed on 14 chromosomes, while 13 markers for GT1×PR228 were distributed 10 chromosomes except two markers’ physical location unknown (Table S1).

Analysis of 2n gamete genotype and maternal heterozygosity

Triploid hybrids were genotyped through SSR markers and the genotype of 2n gametes were indirectly derived, results showed that the 2n gametes of all triploids are derived from the female parent GT1. However, there is one triploid in each of the GT1×Reyan879 and GT1×IAN873 populations which n gametes from unknown male parents other than the artificial pollinating male parent. These two triploids were excluded from subsequent analysis due to the inability to infer their 2n gamete genotypes. Finally, 57, 11, and 32 triploid progenies of GT1×Reyan879, GT1×IAN873, and GT1×PR228 were used for analysis of maternal heterozygosity.

According to the analysis of maternal heterozygosity, the triploid progenies exhibited different rate of MHR in the three populations, due to differences in the number of full-sib triploids. Specified, the maternal heterozygosity rate of triploid progenies from the GT1×Reyan879 ranged from 1.96% to 69.09%, with an average of 51.37%. At the 55 loci in the triploid progenies of the GT1×Reyan879, maternal heterozygosity rate ranged from 0.00 to 78.95%, with an average of 51.47%. At 16 of the 55 loci, maternal heterozygosity rate was less than 50%, and 6 markers had no maternal heterozygosity. The maternal heterozygosity rate of 11 triploids from the GT1×IAN873 ranged from 30.77% to 79.17%, with an average of 58.40%. At 27 loci in the triploid progenies of the GT1×IAN873, maternal heterozygosity rate ranged from 0% to 90.91%, with an average of 57.35%, and at 9 of these loci less than 50% with 1 marker having no maternal heterozygosity. The maternal heterozygosity rate of 32 triploids from the

GT1×PR228 ranged from 38.46% to 92.31%, with an average of 69.32%. At 13 loci in the triploid progenies of the GT1×PR228, maternal heterozygosity rate ranged from 48.39% to 87.50%, with an average of 69.18% (Table S2).

The unimodal distribution of heterozygosity rate in the 2n megaspores among the analyzed genotypes and loci suggests that the 2n gametes across different populations arise from a similar mechanism (Fig. 4). The presence of multiple loci with no maternal heterozygosity confirms that the 2n female gametes derived from the SDR mechanism, consistent with previous studies (Yao et al. 2016; Zhang et al. 2019). The locus VgSSR3126329 showed homozygosity in both the GT1×Reyan879 and GT1×IAN873 triploid populations supports the conclusion further.

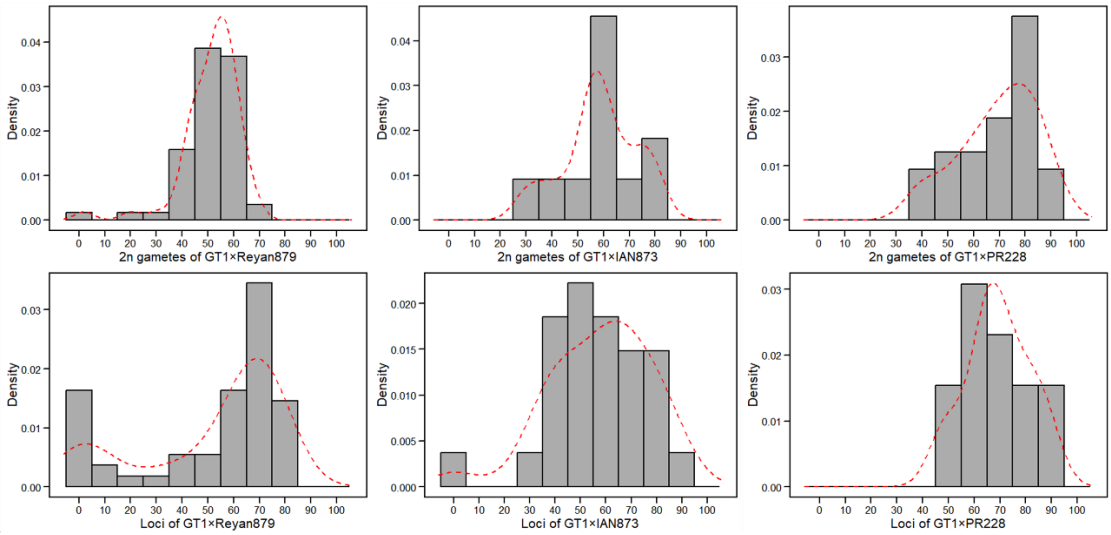

Fig. 4 Distribution and density trace of the maternal heterozygosity rate for 2n female gametes and loci in different populations

Low recombination frequency sites and physical position of centromeres

The low recombination frequency sites were identified based on the results of MHR analysis. This study recorded ten loci in five chromosomes with an MHR lower than 10%, which are regarded as low recombination frequency sites. Of these loci, Hb1068 in Chromosome2, VgSSR3126329 in Chromosome11, Hb182 in Chromosome12, Hb205, Hb697, and Hb207 in Chromosome13 had a maternal heterozygosity rate of 0%.

The theory of Half-tetrad analysis (HTA) which developed by Mendiburu and Peloquin (1979) and improved by Cuenca et al (2011, 2015) was used to localize the centromeres. In brief, when the 2n gametes produced by the mutant are completely from FDR or SDR, the heterozygosity of a specific marker in the 2n gamete population is related to the genetic distance between the marker and centromere. In particular, the completely homozygosity marker derived from SDR or completely heterozygosity marker derived from FDR is close to or at the centromere. Based on this theory, the locations of the six markers in this study are potential candidate centromere locations, as shown in Fig. 5.

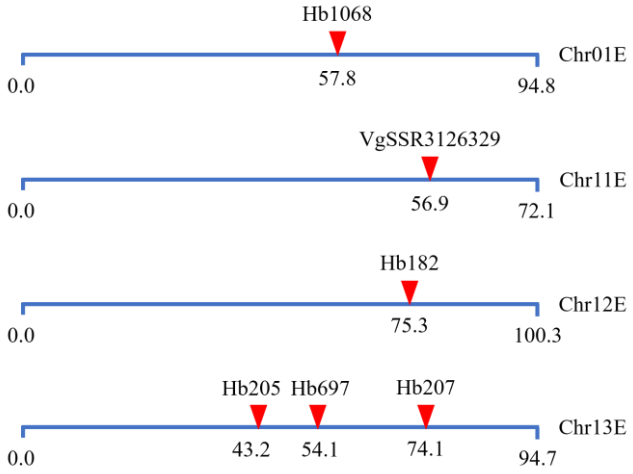

Fig. 5 Candidate centromere locations in the four chromosomes (size in Mb). Chr means Chromosome.

Discussion

The method of integrating HTA and MAC-PR analyses using SSR markers to conduct MHR analysis of triploid progenies is widely used in polyploid plant breeding, as it allows for the identification of the source and genetic pathway of 2n gametogenesis. For instance, using the HTA and MAC-PR analysis, Han et al (2018) identified that the origin of 21% of 87 triploids of *Populus tomentosa* originated from 2n female gametes, and the genetic composition analysis revealed that 2n gametes were mainly produced by SDR in female and FDR in males. In *Citrus*, using 16 Simple Sequence Repeat (SSR) and 18 Single Nucleotide Polymorphism (SNP) markers, Rouiss et al (2017) performed the maximum-likelihood method at the individual level via centromeric marker analysis, finding that 88% of the hybrids arose from SDR, 7% from FDR or pre-meiotic doubling (PRD), and 5% from post-meiotic genome doubling (PMD). In *Hevea brasiliensis*, using the same methods, Yao et al (2016) and Zhang et al (2019) both identified that open-pollinated triploid progeny of clone GT1 derived from SDR-type 2n female gametes. However, because of the small size of the population, insufficient of markers, and half-sib population, the MHR of 2n female gametes is not accurate which makes the conclusion not entirely reliable. In this study, large number of full-sib populations with a sufficient number of primers were used for the MHR analysis. The results showed that all the triploids originated from 2n female gametes, and the average of MHR ranged from 50% to 60% (the results of GT1×PR228 were not credible because of insufficient of markers). Especially, six markers performed totally homozygosity obtained for the first time, which strongly supported that 2n female gametes of GT1 produced by SDR. Consensually, FDR-type gametes transmit 70–80% of parental heterozygosity to progeny and SDR-type gametes transmit 30–40%. In this study, SDR-type gametes also transmit more than 50% of parental heterozygosity, which means they are equally valuable in polyploid breeding in *Hevea brasiliensis* (Zhang et al. 2019).

Although population size, number of markers, and recombination frequencies of markers are influencing the authenticity of genetic pathway identification of 2n gametogenesis, they have not been fully studied. While it is widely agreed that larger populations provide better results, the minimum number of genotypes required for accurate conclusions is still unclear. Cuenca et al (2011) pointed out that one and two genotypes identified by six markers are not sufficient to prove the conclusions that triploid lemon derived from FDR and triploid orange derived from

SDR. For markers, Dong et al (2014) indicated that to reduce the impact of recombination and determine the source of the chromosome sets in polyploid hybrids, a large number of random SSR markers or a small number of SSR markers with low recombination frequencies are required. Compared with random markers, low recombination frequency markers have the advantage of more accuracy, less expensive, and require less labour. After that, low recombination frequency markers are frequently used for MHR analysis and 2n gamete formation mechanism discrimination (Han et al. 2018; Geng et al. 2019). In this study, three populations with a different number of induvial and markers are employed to calculate the maternal heterozygosity rate of 2n female gametes derived from rubber clone GT1. For the population GT1×Reyan879, since it has the most individuals (57) and most markers (55, which is more than double the number of chromosomes of 18), we assume that it has the most accurate results of maternal heterozygosity rate of 2n female gametes and markers. Although the average maternal heterozygosity rate for 2n female gametes and markers in population GT1×IAN873, which had the least number of individuals (11) and markers (27, two-thirds of the double number of the chromosome), was not significantly different from that of GT1×Reyan879, their distribution intervals differed (1.96%-69.09% vs 30.77%-79.77% for 2n gametes, and 0-78.95% vs 0-90.91% for markers), suggesting that larger populations and more markers result in greater accuracy. In population GT1×PR228 which has fewer individuals (32) but the least markers (13), the average maternal heterozygosity rate of 2n female gametes and markers are significantly higher than population GT1×Reyan879. Based on the MHR analysis results of the above populations, we believe that the number of markers is more important than the number of individuals in MHR analysis, and two-thirds of the double number of chromosomes is sufficient.

Except for MHR analysis, the HTA analysis can also be used to map the centromere, because the rate of MHR of the 2n gametes is related to the genetic distance between the marker and centromere. Since Rhoades and Dempsey (1966) first attempted to map centromeres using HTA in maize, it has been used in centromere localization of many plants, such as potato (Mendiburu and Peloquin 1979) and *Brassica* (Mason et al. 2016). Based on the theory of HTA, ten centromere positions were identified by 100% heterozygosity transmitted from the 2n heterozygous gametes of the paternal parent into the tetraploid offspring in potato (Park et al. 2007). In *Citrus*, half-tetrad multilocus structure analysis was proposed to locate the centromere, nine genetic centromeres were mapped in the chromosomes and then physical locations of the citrus centromere are revealed by combining genetic and immunological assays (Cuenca et al. 2011; Aleza et al. 2015; Xia et al. 2020). In this study, four candidate physical locations of rubber centromeres were identified by six 0% heterozygosity loci directly for the first time, and more centromeric or near-centromeric markers are needed to map other centromeres. In particular, the chr13E has three putative centromere locations, and the three locations are far away from each other (Fig. 5). We speculate that there are three reasons for this result. The first is that ch13E is a polycentric chromosome. At present, there is no report on whether there is polycentric chromosome in *Hevea brasiliensis*. The second is that there are completely recombination free regions on this chromosome except for the centromere. If this is the case, these regions are of great value for developing low recombination frequency markers. The third reason is that these markers are in areas with extremely low recombination rates, and the population size in this study is not sufficient to identify them. These speculations require further research to confirm. The centromere or near centromere markers mined in this study

can also be low recombination frequency sites for further genetic pathway identification of 2n gametes in rubber trees.

In conclusion, we confirmed that 2n female gametes of rubber clone GT1 are derived from SDR, and that these gametes can transmit more than 50% of parental heterozygosity based on sufficient triploid progenies and markers. Further, we proved that the number of markers is more important than the number of individuals in MHR analysis based on three full-sib triploid populations. Additionally, we identified a set of low recombination frequency markers that could be valuable for further genetic pathway identification, and for the first time, we located four candidate physical centromeres of rubber.

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