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Male Parent Identification of Triploid Rubber Trees (*Hevea brasiliensis*) and the Mechanism of 2*n* Gametes Formation

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Abstract: Eight triploids were screened among offspring of the rubber tree clone GT1 × different clones by flow cytometry and chromosome counting. Twenty-five simple sequence repeat (SSR) markers were screened to identify the origin of 2*n* gametes, to determine the male parents of these triploids, and to evaluate the mechanism of 2*n* gamete formation using band configurations and microsatellite DNA allele counting peak ratios (MAC-PR). The results showed that 2*n* gametes originated from the maternal rubber tree clone GT1, contributing the extra genome copy present in the triploids. It was confirmed that GT1 is able to produce a 2*n* megagametophyte spontaneously. Many male parents were shown to provide pollen for formation of triploid rubber trees, including clones RRIC 103, Yunyan 277-5, and three other clones. The second division restitution (SDR) was likely the main mechanism involved in formation of megagametophytes in GT1, as the rate of maternal heterozygosity restitution (HR) of all eight triploids varied from 27.78% to 75.00%, with a mean of 51.46%, and all 25 markers varied from 0% to 100%, with a mean of 51.69%. Elucidation of the origin and formation of 2*n* gametes will help optimize further sexual hybridization of polyploid rubber trees.

Keywords: rubber trees; triploids; SSR; 2*n* gamete; male parents

1. Introduction

The rubber tree, *Hevea brasiliensis*, is the only cultivated species for latex [1,2], and has often been described as an out-breeding species that is pollinated by insects such as thrips and midges [3,4]. Natural rubber is one of the most important raw materials in industry, agriculture, defense, transportation, and daily life [5,6]. Demand for rubber is increasing with economic development; however, the regions where rubber trees are planted are limited due to the stringent environmental requirements for their growth [7]. In addition, alternatives to natural rubber are still limited because synthetic rubber produced from petroleum does not match its resilience, elasticity, and abrasion resistance [8,9].

Substantial efforts have been expended to solve the problem of the imbalance between rubber supply and demand. Efforts by breeders led to many rubber tree cultivar clones being selected and planted in non-traditional planting areas, such as Chinese rubber plantations. These were established in Hainan and Yunnan Provinces, in areas as far north as 22° N, while rubber plantations are typically located in latitudes that range from 10° N to 10° S [6,10]. Two new rubber tree cultivars, Yunyan 77-2 and Yunyan 77-4, were selected and confirmed as triploids which were largely planted in Yunnan,

China [11]. Some reports showed that these triploids had a relatively high rubber yield and good cold resistance [12,13]. These two triploid cultivars both were derived from GT1 × PR 107 [13,14]. In many studies, this parental cross was considered a special case regarding triploid formation, particularly because GT1 is male-sterile and PR 107 has very low fruit production, often considered to be female-sterile [11,13]. To date, no efforts have been made to explore these special rubber tree materials, and the mechanism of triploid formation in rubber trees has been forever obscure.

Generally, triploid plants can be created by crossing tetraploids with diploids or by combining unreduced ($2n$) gametes with reduced (n) gametes [15–18]. No rubber tree tetraploids have been reported in the wild; therefore, triploid rubber trees most likely arise from the combination of $2n$ gametes with reduced gametes. Unreduced gametes are crucial for triploid formation and can be formed via several mechanisms, namely, premeiotic doubling, first-division restitution (FDR), chromosome doubling during the meiotic interphase, postmeiotic restitution (PMR), and indeterminate meiotic restitution (IMR) [19–21]. Many studies have reported that FDR and SDR are the predominant mechanisms of $2n$ gamete formation based on their genetic consequences [22]. It has been theorized that $2n$ gametes from FDR transmit roughly 80% of parental heterozygosity to the progeny, while $2n$ gametes from SDR transmit about 40% [23–25].

In this study, the rubber tree clone GT1 was chosen as the mother tree. Its open pollinated half sib progeny were collected to screen the triploids. Then, the origin of $2n$ gametes and the male parents of these triploids were determined using SSR markers. The mechanism of $2n$ gamete formation was also studied. This study will be helpful for the study of rubber trees especially in polyploid breeding.

2. Materials and Methods

2.1. Plant Materials

The mother tree, GT1 (Original clone, $2n = 2x = 36$), was located in the flower park of the XiShuangBanNa, Yunnan Institute of Tropical Crops, China. Seventeen rubber trees were selected as candidate male parents which were nearby GT1 (Table 1). These materials derived from Wickham germplasm (PB 310, Yunyan 277-5, RRIC 103), and IRRDB' 81 (International Rubber Research and Development Board) germplasm (A_1-A_{12}). All rubber trees were identified by clonal inspectors.

Table 1. Plant materials used as candidate male parents of GT1's triploid offspring.

Rubber Trees (Number)	Pedigree/Source
PB 310 (1)	PB 5/51 × RRIM 600
Yunyan 277-5 (1)	PB 5/63 × Tjir 1
RRIC 103 (3)	RRIC 52 × PB 86
A_1-A_{12} (12)	Original clones

PB, Prang Besar Rubber Estate, Malaysia; RRIC, Rubber Research Institute of Ceylon.

2.2. Collection of Seeds and Sowing

The fruits of GT1 were bagged before seed dispersal from the fruit shell, and then seeds were collected and sown in sandy soil that had been disinfected using carbendazim.

2.3. Ploidy Analysis by Flow Cytometry

Flow cytometry was performed using an Accuri™ C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). About 20 mg of fresh young leaves were cut into pieces with a sharp blade in 0.5 mL of nuclear extraction solution (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 0.1% (*v*/*v*) Triton X-100, pH 7.0), and then filtered through 40-μm nylon mesh after being left to stand for 3 min. The suspension of released nuclei was stained with 50 μL of propidium iodide (PI) for 5 min. The leaf sample from a known diploid rubber tree was used as a control, which was

balanced by mixing with samples. A seedling was recorded as triploid when there were two peaks and the ratio of their peak values was 3:2. Samples were assessed independently three times for each putative triploid.

2.4. Chromosome Counting

The ploidy level of plantlets can be further confirmed by chromosome counting. Very young leaves which were reddish in color were collected from the seedlings and pretreated in a saturated solution of para-dichlorobenzene for at least 3 h, washed once using distilled water, and then fixed in Carnoy's fluid (ethanol/acetic acid, 3:1) for at least 24 h at 4 °C. Next, the materials were transferred to 1 mol/L HCl for 10–15 min at 60 °C, washed with water, and then immersed in distilled water for 10 min. These hydrolyzed materials were stained with carbolfuchsin solution. Chromosome counts of at least 10 cells with a well-spread metaphase per seedling were observed using a microscope (Olympus CX41; Olympus, Tokyo, Japan).

2.5. DNA Extraction and SSR Analysis

DNA was extracted from approximately 300 mg of each leaf sample using a DNeasy® Plant Mini Kit (Tiangen Biotech Co. Ltd., Beijing, China) following the manufacturer's instructions. The SSR primers used in this study were derived from many previous studies [26–28]. The fluorescently labeled TP-M13-SSR method [29] was adopted in this work. Three primers were included for the polymerase chain reaction (PCR), including a forward primer with the tail of a universal primer, M13 (5′-TGTAAAACGACGGCCAGT-3′), at the 5′ end, a reverse primer, and a universal primer, M13, fluorescently labeled with 6-carboxyfluorescein (FAM), 6-carboxy-x-rhodamine (ROX), hexachloro-6-carboxyfluorescein (HEX), or tetrachloro-6-carboxyrhodamine (TAMARA). PCR was carried out in a total volume of 20 μL containing 2 μL of DNA template, 10 μL of PCR Master Mixes (Bo Maide Biotech Company, Beijing, China), 0.08 μL of forward primer, 0.32 μL of reverse primer, and 0.4 μL of fluorescence primer. Amplification was performed using the following conditions: 94 °C for 5 min; followed by 11 cycles of 94 °C for 30 s, 30 s at the optimal annealing temperature for each SSR marker, and 72 °C for 30 s; 20 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 8 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 10 min. Finally, capillary electrophoresis fluorescence-based SSR analyses were conducted on an ABI 3730xl DNA Analyzer (Genewiz Inc., Beijing, China). Fragment sizes and peak areas were determined automatically using GeneMarker software v2.2 (Soft Genetics, LLC. College Station, PA, USA).

Selected SSR markers should be heterozygous for triploids and be polymorphic between maternal parent GT1 and candidate male parents. When one allele was shared between the two parents, the MAC-PR [30,31] was used to determine the allelic configuration. The origin of 2*n* gametes is determined by comparing SSR marker results among GT1, triploids, and candidate male parents. Theoretically, a triploid and its parent which provided 2*n* gamete should share two alleles for a locus when the null alleles and dropout alleles are neglected. So 2*n* gametes origin can be determined if one parent always can share possible two alleles with the triploid. Based on the knowledge of 2*n* gametes origin, the triploid's male parent is determined by comparing SSR marker results among GT1, triploids, and candidate male parents. A triploid and its male parent share at least one allele for a locus when the null alleles and dropout alleles are neglected. So the male parent can be determined only, if all other candidate males are excluded by mismatches.

Based on the results of 2*n* gametes origin and determined parents, the mechanism of 2*n* gamete formation was identified by comparing allelic configurations among the triploid, its female parent, GT1, and its male parent. The genotypes of 2*n* gametes were directly read when parental alleles displayed as completely different based on the allele number and type. The rate of maternal HR (HR%) was estimated as described by Xie et al. [32], calculated as:

$$HR\% = n_{ab}/(n_{ab} + n_{aa} + n_{bb}) \times 100 \quad (1)$$

where n_{ab} is the number of heterozygous 2*n* gametes, and n_{aa} and n_{bb} are two kinds of homozygous 2*n* gametes.

3. Results

3.1. Determination of Hybrid Ploidy Levels

A total of 969 seeds was obtained. After seed sowing and transfer of the young seedlings, 725 seedlings remained in the field. The ploidy levels of the hybrids were determined by the DNA content-associated peak of each triploid sample, which was shifted to a position indicating 1.5 times the DNA content of the diploid control (Figure 1a), and then the number of somatic chromosomes was further confirmed. The results showed that the triploid hybrids contained 54 chromosomes (Figure 1c), compared with 36 chromosomes in the diploids (Figure 1b). In these experiments, eight triploids were obtained, which were named Tri-1 to Tri-8.

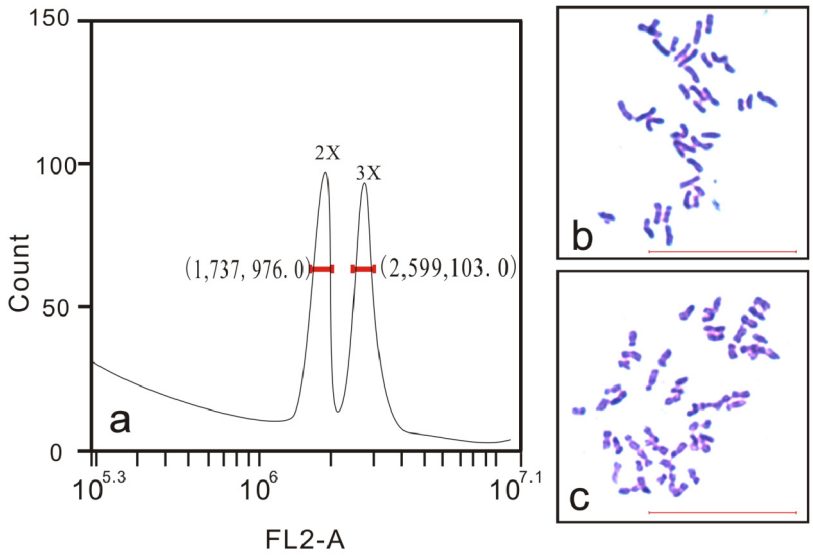

Figure 1. Ploidy level detection of offspring derived from GT1. (**a**) Flow cytometric analysis of diploid and triploid; (**b**) Chromosome number of diploid ($2n = 2x = 36$); (**c**) Chromosome number of triploid ($2n = 3x = 54$). Scale Bar = 50 μm.

3.2. Screen of SSR Markers

In total, 25 polymorphic SSR markers were screened in this study (Table 2). There were at least three alleles for a marker (RUB 16, 74, 138) and at most 10 alleles for a marker (RUB 112, 178) in all tested rubber tree materials. The markers location at the chromosomes was unavailable. Three identical clones RRIC 103 always had the same allelic configurations for all markers. A_2 and A_3 also had the same allelic configurations for all markers, suggesting that A_2 and A_3 were the same clones. So there were a total of 14 rubber tree clones as the candidate male parents in this study.

Table 2. Characteristics of screened 25 SSR markers in *Hevea brasiliensis*.

Marker	Sequence ID	Number of Alleles in This Study	SSR Motif	Reference
RUB 9	EC608804	4	$(AG)_n$	[26]
RUB 16	EC607870	3	$(ATC)_n$	[26]
RUB 19	EC606684	8	$(GAA)_n$	[26]
RUB 20	EC606350	4	$(GAT)_n$	[26]
RUB 28	EC606163	7	$(CT)_n$	[26]
RUB 33	EC605199	5	$(CT)_n$	[26]
RUB 65	EC605124	5	$(AAG)_n$	[26]
RUB 70	EC601354	6	$(GGA)_n$	[26]
RUB 74	EC606215	3	$(AGA)_n$	[26]
RUB 75	EC606169	4	$(GGA)_n$	[26]
RUB 90	CB376545	6	$(AGA)_n$	Unpublished
RUB 95	FJ919795	5	$(AT)_n(GT)_m$	[27]
RUB 98	EC609548	5	$(CG)_n$	[26]
RUB 102	EC606350	4	$(GAT)_n$	[26]
RUB 103	EC604892	5	$(CT)_n$	[26]
RUB 112	EC604918	10	$(ATT)_n$	[26]
RUB 138	EC606085	3	$(TTA)_n$	[26]
RUB 156	EC600469	6	$(AGA)_n$	[26]
RUB 161	FJ919800	4	$(CT)_n$	[27]
RUB 178	Pr012324089	10	$(GCTTCT)_n(CTT)_m$	[28]
RUB 179	Pr012324091	4	$(TCT)_n$	[28]
RUB 183	Pr012324095	7	$(CAA)_n$	[28]
RUB 184	Pr012324096	4	$(AGA)_n$	[28]
RUB 190	Pr012324102	8	$(TTA)_n$	[28]
RUB 199	Pr012324114	4	$(TCT)_n$	[28]

3.3. Origin of 2n Gametes

A hypothesis to be tested was that the 2*n* gamete was inherited from one of the 14 putative male parent clones. With Tri-1, for example, alleles 'abc' can be amplified using the marker RUB 183 (Figure 2a) and, therefore, one of the parents has to provide 2*n* gametes with the alleles 'ab', 'ac', or 'bc'. However, in possible parents, only one allele can be observed in PB 310 ('c') and A_2-A_5 ('d') and, therefore, these five samples can be ruled out as Tri-1's 2*n* gamete source. Marker RUB 184 can amplify alleles 'abc' in Tri-1; while only allele 'a' can be observed in A_1, A_6-A_8 and A_{12}; and only allele 'd' can be observed in A_2, A_3, and Yunyan 277-5 (Figure 2b); so candidate male parents A_1, A_2, A_3, A_6-A_8, A_{12}, and Yunyan 277-5 were excluded as Tri-1's 2*n* gamete source. According to this method, marker RUB 156 can exclude A_1 ('b') and Yunyan 277-5 ('a') (Figure 2c); and RUB 199 can exclude A_4, A_5 and A_9-A_{11} ('c'), A_2, A_3, A_{12} ('b') and RRIC 103 ('a') (Figure 2d). Thus, all 14 candidate male parent clones can be excluded as the origin of 2*n* gametes of Tri-1. Another alternative hypothesis was that 2*n* gametes came from the female parent GT1. For these markers female parent GT1 can always provide two possible alleles i.e., alleles 'ac' for markers RUB 183, RUB 156 and RUB 199 (Figure 2a, 2c, 2d respectively), allele 'bc' for marker RUB 184 (Figure 2b), suggesting that 2*n* gametes were inherited from the female parent GT1.

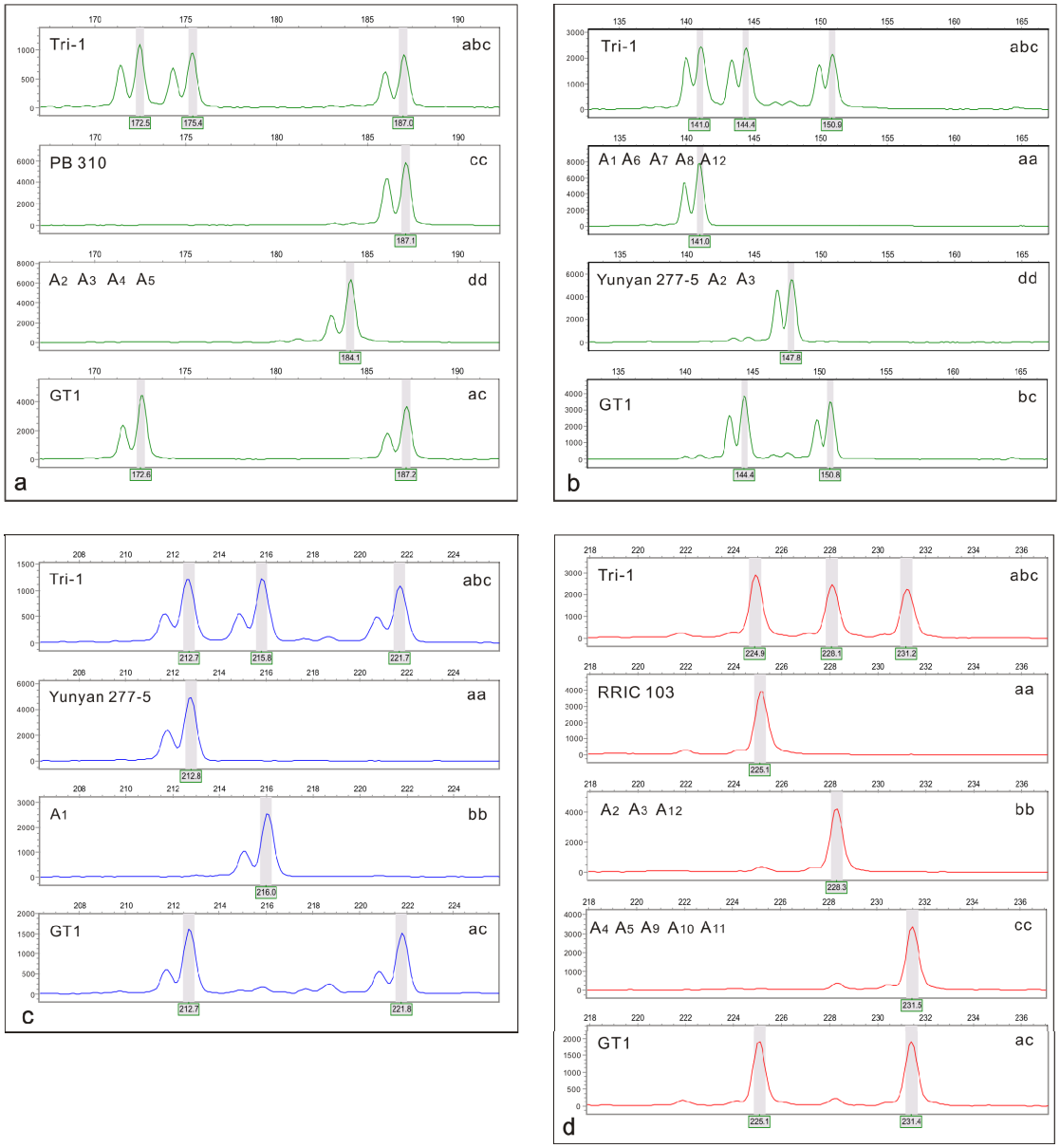

Figure 2. Alleles configurations of Tri-1, candidate male parents and GT1 for markers RUB 183, RUB 184, RUB 156 and RUB 199. (**a**) Marker RUB 183, alleles 'abc', 'cc', 'dd', 'ac' were amplified in Tri-1, PB 310, A_2-A_5 and GT1 respectively; (**b**) Maker RUB 184, alleles 'abc', 'aa', 'dd', 'bc' were amplified in Tri-1, (A_1, A_6-A_8, A_{12}), (A_2, A_3, Yunyan 277-5) and GT1 respectively; (**c**) Marker RUB 156, alleles 'abc', 'bb', 'aa', 'ac' were amplified in Tri-1, A_1, Yunyan 277-5 and GT1 respectively; (**d**) Marker RUB 199, alleles 'abc', 'cc', 'bb', 'aa', 'ac' were amplified in Tri-1, (A_4, A_5, A_9-A_{11}), (A_2, A_3, A_{12}), RRIC 103 and GT1 respectively.

Using the same approach to analyze all triploids, the results showed that all candidate male parents can be excluded as the 2*n* gametes origin (Table 3). For these markers female parent GT1 can always provide two possible alleles, suggesting that of these triploids' two alleles came from GT1, while one allele came from the pollen parent for a marker. The results proved that 2*n* gametes originated from female parent GT1 not the male parents.

Table 3. Allelic configuration comparing triploids, female parent GT1, and candidate male parents of rubber trees.

Markers	Triploids								FP			MP											
	Tri-1	Tri-2	Tri-3	Tri-4	Tri-5	Tri-6	Tri-7	Tri-8	GT1	PB310	Yunyan 277-5	RRIC 103	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
RUB9	bbc	bbc	bbc	bbc	abc	abc	bbc	bcc	bc	cc	cc	bb	aa	ab	ab	ab	ab	ad	ad	ad	aa	aa	aa	bb
RUB 16	abc	abc	abc	abc	aab	abc	bcc	abc	ac	cc	ab	bc	bb	bb	bb	bc	bc	bc	bc	bc	bb	bb	bb	ab
RUB 19	abd	bbc	bbf	bbf	aae	abf	bbf	abb	ab	ab	ab	bf	ef	ff	ff	ff	ff	fh	fh	fh	fg	fg	fg	cd
RUB 20	aac	bbc	bbc	bbc	aac	abd	abb	aaa	ab	ab	aa	ab	cd	cc	cc	cc	cc	cd	cd	cd	aa	aa	aa	cc
RUB 28	aab	aab	aab	bbe	abc	bbc	abb	abd	ab	ad	ad	aa	cc	ae	ae	af	af	bg	bg	bg	aa	aa	aa	aa
RUB 33	aab	aab	abb	abb	aaa	aaa	abb	aaa	ab	ac	aa	ac	aa	aa	aa	ad	ad	ae	ae	ae	ad	ad	ad	ae
RUB 65	abb	aab	aaa	aaa	abb	abb	aab	abb	ab	bc	ab	be	aa	aa	aa	aa	aa	aa	aa	aa	ad	ad	ad	aa
RUB 70	aab	aaa	abc	aaa	bbb	aab	aac	aab	ab	ad	ab	cf	bb	ac	ac	ab	ab	bb	bb	bb	be	ee	ee	ab
RUB 74	aab	aab	abc	aaa	abc	abc	aab	aab	ab	ab	bb	ab	cc	ac	ac	ac	ac	ac	ac	ac	aa	aa	aa	aa
RUB 75	aab	abd	abc	aac	aab	aaa	aaa	abb	ab	bb	bb	ab	ab	ac	ac	ab	ab	ac	ac	ac	aa	aa	aa	ad
RUB 90	aab	aaa	abc	aab	aab	aab	abf	aab	ab	ab	aa	bf	aa	ac	ac	ac	ac	bd	bd	bd	de	de	de	aa
RUB 95	abc	abc	aab	aab	aab	aab	abc	abb	ab	ac	ac	cd	ad	aa	aa	ac	ac	ac	ac	be	dd	dd	dd	ce
RUB 98	abc	aac	aab	abd	aab	aaa	aab	aab	ab	aa	ab	ab	aa	ad	ad	aa	aa	ae	ae	ae	aa	aa	aa	ac
RUB 102	aaa	bbc	bbc	bbc	aac	abd	abb	aaa	ab	ab	aa	ab	cd	cc	cc	cc	cc	cd	cd	cd	aa	aa	aa	ac
RUB 103	abd	abb	abd	bbd	aaa	abb	abc	aaa	ab	ac	ac	ac	aa	dd	dd	aa	aa	aa	aa	aa	ee	ee	ee	ad
RUB 112	aab	aab	abb	abe	aac	abc	aab	abd	ab	fg	fg	aj	bc	be	be	ah	ah	ag	ag	ag	fi	fi	fi	aa
RUB 138	bbc	bbb	abc	aac	bbc	aac	abb	aaa	ab	aa	aa	ab	ac	cc	cc	ac	ac	aa	aa	aa	ac	ac	ac	bc
RUB 156	abc	aab	acd	abc	abc	abc	aac	aac	ac	ac	aa	ac	bb	bd	bd	be	be	ce	ce	ce	ef	ef	ef	ab
RUB 161	aab	aaa	abc	abc	aac	abc	aaa	aab	ab	aa	aa	aa	cc	cc	cc	ac	ac	aa	aa	aa	cd	cd	cd	aa
RUB 178	aab	bcc	cce	ccf	aad	bcc	ccc	acc	ac	aa	ac	cj	bd	ef	ef	dg	dg	dh	dh	dh	ii	ii	ii	bd
RUB 179	aab	aab	bbc	abc	bbc	abc	abb	aab	ab	dd	aa	ad	cc	cc	cc	cc	cc	cc	cc	cc	cd	cd	cd	ac
RUB 183	abc	ccg	acd	acd	aaf	ace	acc	aaa	ac	cc	ac	af	ef	dd	dd	dd	dd	ad	ad	ad	af	af	af	bg
RUB 184	abc	abc	ccd	bcd	abc	abc	bcc	bcd	bc	cd	dd	bc	aa	dd	dd	ad	ad	aa	aa	aa	bd	bd	bd	aa
RUB 190	bbc	bbb	abc	abc	abc	bbc	aaa	aaa	ab	ad	ah	aa	cc	cc	cc	ef	ef	dd	dd	dd	gg	gg	gg	bc
RUB 199	abc	bcc	abc	abc	aab	acc	acc	aac	ac	ac	bc	aa	bc	bb	bb	cc	cc	bd	bd	bd	cc	cc	cc	bb

FP–female parent; MP–male parent.

3.4. Male Parent Identification of Triploids

When the origin of 2*n* gametes had been determined, the alleles were compared among the triploid, GT1, and candidate male parents. With Tri-1, for example (Table 3), when marker RUB 95 is used, Tri-1's configuration is 'abc', female parent GT1 provides 2*n* gametes in the form of alleles 'ab', so possible male parents have to provide the extra allele 'c'; this means that the possible male parents are PB 310, Yunyan 277-5, A_4-A_7, A_{12}, and RRIC 103. In contrast, for RUB 178, Tri-1's configuration is 'aab'; among all of the possible male parents, only A_1 and A_{12} can provide the extra allele 'b'. With the same approach, all 25 markers were used to determine the possible male parents. For every possible male parent, we recorded the number of markers (N) that shared at least one allele with Tri-1. This showed that A_{12} was the only male parent for Tri-1 due to the ratio (N/25) being 100% (Table 4), which meant that A_{12} could be determined to be Tri-1's male parent for all of these 25 SSR markers (Table 3).

Table 4. Number of SSR markers showing every possible male parent for Tri-1.

Rubber Trees	N	N/25 (%)
PB 310	11	44.00
Yunyan 277-5	14	56.00
RRIC 103	13	52.00
A_1	14	56.00
A_2	16	64.00
A_3	16	64.00
A_4	16	64.00
A_5	16	64.00
A_6	14	56.00
A_7	14	56.00
A_8	13	52.00
A_9	9	36.00
A_{10}	8	32.00
A_{11}	8	32.00
A_{12}	25	100.00

Male parents of all eight triploids determined in this way are shown in Table 5. Tri-1 and Tri-2 had the same male parent (A_{12}); Tri-3 and Tri-4 had the same male parent (A_2/A_3); Tri-5 and Tri-6 had the same male parent (A_1); Tri-7's male parent was RRIC 103; and Tri-8's male parent was Yunyan 277-5. For Tri-8, N/25 was 96.00%; the primer pairs RUB 112 had a special allele 'd' in Tri-8 (Table 3); however, it could not be found in Yunyan 277-5 and others candidate male parents, suggesting that 'd' may be allele dropout in Yunyan 277-5.

Table 5. Male parent's identification of all triploids.

Triploids	Male Parent	N_1/25 (%)
Tri-1	A_{12}	100.00
Tri-2	A_{12}	100.00
Tri-3	A_2/A_3	100.00
Tri-4	A_2/A_3	100.00
Tri-5	A_1	100.00
Tri-6	A_1	100.00
Tri-7	RRIC 103	100.00
Tri-8	Yunyan 277-5	96.00

3.5. Mechanism of 2n Gamete Formation

The rate of maternal HR of all eight triploids varied from 27.78% to 75.00% (Table 6), with a mean of 51.46%. Each marker's HR% was also calculated, showing that the rate of maternal HR of

all 25 markers varied from 0% (RUB 178) to 100% (RUB 9), with a mean of 51.69%. Among these 25 markers, 10 showed a heterozygosity rate of less than 50%. The result showed that SDR might be the mechanism of GT1's 2*n* megagametophyte formation.

Table 6. Genotypes of 2*n* megagametophytes and the rates of maternal HR for each 2*n* megagametophyte and each marker.

Markers	Triploid Samples								HR%
	Tri-1	Tri-2	Tri-3	Tri-4	Tri-5	Tri-6	Tri-7	Tri-8	
RUB 9	bc	bc	bc	bc	bc	bc	bc	bc	100.00
RUB 16	ac	ac	ac	ac	aa	ac	cc	ac	75.00
RUB 19	ab	bb	bb	bb	aa	ab	bb	-	28.57
RUB 20	aa	bb	bb	bb	aa	ab	-	aa	14.29
RUB 28	ab	ab	ab	bb	ab	bb	bb	ab	62.50
RUB 33	ab	ab	bb	bb	aa	aa	bb	aa	25.00
RUB 65	bb	ab	aa	aa	bb	bb	aa	-	14.29
RUB 70	-	aa	ab	aa	bb	aa	aa	-	16.67
RUB 74	ab	ab	ab	aa	ab	ab	-	aa	71.43
RUB 75	ab	ab	ab	aa	-	aa	aa	ab	57.14
RUB 90	ab	aa	ab	ab	ab	ab	ab	ab	87.50
RUB 95	ab	ab	ab	ab	ab	ab	ab	bb	87.50
RUB 98	ab	aa	ab	ab	ab	aa	-	-	66.67
RUB 102	aa	bb	bb	bb	aa	ab	-	aa	14.29
RUB 103	ab	bb	ab	bb	aa	bb	ab	aa	37.50
RUB 112	ab	ab	ab	ab	aa	ab	ab	-	85.71
RUB 138	bb	bb	ab	aa	bb	aa	-	aa	14.29
RUB 156	ac	aa	ac	ac	ac	ac	-	ac	85.71
RUB 161	ab	aa	ab	ab	aa	ab	aa	ab	62.50
RUB 178	aa	cc	cc	cc	aa	cc	cc	-	0.00
RUB179	ab	ab	bb	ab	bb	ab	bb	ab	62.50
RUB 183	ac	cc	ac	ac	aa	ac	cc	aa	50.00
RUB 184	bc	bc	cc	bc	bc	bc	-	bc	85.71
RUB 190	bb	bb	ab	ab	ab	bb	aa	aa	37.50
RUB 199	ac	cc	ac	ac	aa	ac	cc	aa	50.00
HR%	75.00	44.00	68.00	52.00	37.50	60.00	27.78	47.37	

HR, heterozygosity restitution.

4. Discussion

In this study using co-dominant SSR markers, the clone GT1 was unambiguously determined to be a 2*n* gamete donor for triploid rubber tree formation. We demonstrated the advantages of the selected SSR markers and the capabilities of determining the origin and ploidy levels of gametes. Ideal markers should be different and heterozygous in both parents with three different alleles in triploids, such as the RUB 16, RUB 19, RUB 95, RUB 98, RUB 103, RUB 156, RUB 183, RUB 184, and RUB 199 markers for Tri-1 used in this study. We can directly determine the allelic configuration via differences in alleles at a locus, and if the same alleles are present in both parents and offspring, MAC-PR should be used to determine the donor genome in triploids, such as demonstrated with RUB 178 for Tri-1. More crosses among *Hevea brasiliensis* clones are required to confirm whether other clones are related to 2*n* gamete formation. In other plants the 2*n* gametes probably originated from certain species or clones. Raboin et al. [33] reported that unreduced gametes were produced by partially sterile diploid cultivars and reduced gametes by fertile diploid cultivars in the banana. Chen et al. [34] reported that 2*n* eggs originated only from the maternal parent in *Citrus sinensis* × *Poncirus trifoliata*. The presence of 2*n* gametes in many other clones would have significant potential for rubber tree breeding to select desired triploids. If only 2*n* gametes are produced from the clone GT1, further efforts should focus

on male parent selection. Crosses between high-quality rubber tree cultivars to produce triploids can likely yield high-quality triploids.

SSR markers have been applied in many studies for parent identification. For example, this method was used to identify 220 open-pollination progeny of *Liriodendron* spp., of which 49 male parents were identified for 138 progeny [35]. SSR was also used to identify the male parent of 41 elite clones derived from sugarcane polycross families, showing the importance of using molecular marker technology in the identification and confirmation of male parents of high-performance clones in sugarcane breeding programs [36]. In our study, parents of all triploid clones were successfully identified. A large set of SSR markers should be adopted when using the exclusion method, as in this study. Additionally, if specific alleles can be amplified in one or two clones, such as the band 'b' was only amplified in A_1 and A_{12} in all candidate male parents by the marker RUB 178. These markers can improve the efficiency of male parent identification. The results highlight the usefulness of SSR markers in the identification of male parents of triploid rubber trees, and will provide guidance for parent identification and early selection of rubber trees, providing references for improving the efficiency of rubber tree breeding.

Without previous knowledge of the positions of markers relative to the centromeres, Park et al. [37] suggested that the rate of HR varies between 0% and 100% for SDR 2*n* gametes, and between 50% and 100% for FDR 2*n* gametes, under the hypothesis that only one crossover occurred between the locus and centromere. Xie et al. [32] reported that SDR was the mechanism of 2*n* megagametophyte formation in 'Nadorcott' tangor, because the rate of maternal HR varied from 0.00% to 87.80% for 22 SSR markers, with 13 exhibiting a heterozygosity rate <50%. In the present study, the rate of maternal HR of all 25 markers varied from 0% to 100%, with a heterozygosity rate <50% for 10 markers, suggesting that SDR may be the mechanism underlying 2*n* megagametophyte formation. Cuenca et al. [38] reported that maternal heterozygosity transmitted to each SDR 2*n* megagametophyte varied from 15.38% to 100%, with a mean value of 54.98%. In this study, based on all loci analyzed, the rate of maternal HR transmitted to each triploid hybrid ranged from 27.78% to 75.00%, with a mean of 51.46%. Therefore, compared with FDR, SDR is more likely the mechanism of 2*n* megagametophyte formation in the rubber tree clone GT1. Although one mechanism may be predominantly observed in certain clones, it was by no means ruled out that other mechanisms did not operate in these clones at the same time because substantial influences from the environment likely disturb the processes of meiosis and meiotic nuclear restitution. For example, high temperature can induce 2*n* female gametes in *Populus* [39,40]. However, gamete formation is controlled mainly by genes [32,41]. Some genes, such as *AtPS1* [42] and *JASON* [43], were reported to be involved in 2*n* gamete formation in *Arabidopsis*. However, the molecular mechanism in rubber trees remains unclear.

For practical application, the level of heterozygosity in the triploid progeny is of empirical importance regardless of the mechanism of gamete production. Therefore, use of SSR analysis is important as a determinant of maternal HR and as an indicator in early breeding projects involving rubber trees. In future studies, we will examine the selection of male parents based on allelic differentiation from GT1 to determine whether this will result in triploid progeny that are also highly heterozygous at loci controlling production traits.

5. Conclusions

This study proved that rubber tree clone GT1 can produce 2*n* megagametophyte spontaneously. This cultivar has a great value in rubber tree breeding especially in polyploidy breeding which should be used commensurately. Our study provided a good method to identify male parents in the rubber tree and it also can be used as a reference to other plant research. We speculated that the SDR is the mechanism underling the 2*n* megagametophyte formation in the rubber tree clone GT1.

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