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ORIGINAL ARTICLE

Analysis of genetic composition and transmitted parental heterozygosity of natural 2n gametes in *Populus tomentosa* based on SSR markers

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Abstract

Main conclusion **Natural 2n female gametes and transmission of parental heterozygosity by natural 2n gametes in *Populus tomentosa* are reported for the first time, which provides a new approach to polyploid breeding.**

Abstract Naturally occurring 2n pollen is widespread in *Populus tomentosa* and plays an important role in polyploid breeding. However, the competitiveness of 2n pollen is lower than that of haploid pollen during pollination and fertilization, so 2n pollen is less efficient at fertilizing haploid female gametes to produce polyploids. In theory, polyploids can also be obtained when 2n female gametes are fertilized by haploid pollen. Thus, the question becomes whether natural 2n female gametes exist in *P. tomentosa*, which can be answered by examining the genetic composition of natural 2n gametes. In this study, the origin of 87 triploids from the hybrid combination "X-2 × Z-5" was identified by SSR markers and 21% of natural 2n gametes were found to originate from female parents. Four SSR loci with low recombination rates were used to identify the genetic composition of natural 2n gametes. The results showed that the genetic composition of 2n female gametes was mainly characterized by SDR, while 2n male gametes were mainly produced by FDR. Moreover, the transmission of parental heterozygosity by natural 2n gametes, which is significantly different between female and male parents in FDR and SDR types, was analysed using 42 SSR primers. Here, we report naturally occurring 2n female gametes for the first time in *P. tomentosa* and reveal the genetic constitution and transmitted parental heterozygosity of these gametes. Our results provide a foundation for theoretical research into 2n gametes and their application in new polyploid breeding strategies.

Keywords *Populus tomentosa* · Natural 2n gametes · First division restitution · Second division restitution · Transmission of heterozygosity

Abbreviations

SDR	Second division restitution
FDR	First division restitution
hDNA	Heteroduplex DNA
K–S	Kolmogorov–Smirnov
HR	Homologous recombination
CO	Crossing-over

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Introduction

The spontaneous formation of polyploids is the main force driving species formation and diversification in the process of plant evolution (De and Geelen 2013). Polyploids are widespread in nature, and approximately 70% of angiosperms have undergone polyploidization at least once during evolution (Masterson 1994). The main manner in which polyploids occur in nature is polyploidization by fertilization of 2n gametes (Harlan and Dewet 1975; Veilleux 1985). In plants, the previous studies have shown that natural 2n

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gametes are mainly formed through FDR and SDR (Chen et al. 2008; Ferrante et al. 2009; Honsho et al. 2016). Because the heterozygosities of the natural 2n gametes produced by FDR and SDR are different, FDR- and SDR-derived 2n gametes transmit different parental heterozygosities (70–80 and 30–40%, respectively) (Barone et al. 1995; Mendiburu and Peloquin 1977).

In *Populus*, Nilsson-Ehle observed natural triploid European aspen in Sweden in 1935 (Nilsson-Ehle 1936); thereafter, natural triploids of *P. balsamifera*, *P. tremuloides*, and *P. alba* were discovered in the former Soviet Union, Finland, Canada, and the United States (Vanbuijtenen et al. 1958; Every and Wiens 1971; Tamm and Iarvekiul'G. 1975). Because larger pollen grains were observed when Müntzing (1936) studied the origin of natural triploid *P. tremula*, Müntzing believed that triploid aspen was derived from a zygote produced by a normal egg cell and an unreduced male gamete. However, when Zhang et al. (2009) and Liesebach et al. (2014) researched the origins of natural 2n gametes in *Populus* using SSR markers, they found spontaneous triploid poplars originating from 2n female and male gametes. In addition, in the *P. tomentosa* group, Kang (1996) also discovered natural triploids and found a certain proportion of larger pollen grains, which were unreduced 2n pollen from diploid *P. tomentosa* (Kang et al. 1997), thus suggesting that natural triploids may derive from these 2n pollen grains. Similarly, Zhang et al. (2007) observed the size of the pollen of *P. tomentosa* by microscopy and found large pollen grains, which were 2n and occurred at frequencies of 0.6–21.9%. In the previous studies, natural triploids have been proven to be widespread in poplar, especially in *Populus* groups from sect. Leuce Duby (Zhu et al. 1998). However, the possibility that these natural triploids were produced through the fertilization of 2n female gametes by normal haploid pollen cannot be excluded, and natural triploids are more likely to originate from 2n female gametes (Zhu et al. 1998). Nevertheless, there is no report about the existence of natural 2n female gametes in *P. tomentosa* for now.

If all triploids are produced by natural 2n pollen in *P. tomentosa* without induction, then the genetic constitution of these natural 2n gametes and their transmission of parental heterozygosity in *P. tomentosa* remain of interest. The objective of this study was to investigate these issues. We used three female plants with high fertility and general combining ability (GCA) and six male plants with high pollen production as materials (Bai 2015) to design hybrid experiments. The correlation between the occurrence frequency of natural 2n pollen and the ratio of triploid offspring was evaluated and used to explore the possibility of the occurrence of natural 2n female gametes. We used SSR markers to identify the origins and the genetic constitution of natural 2n gametes, and analysed the transmission of heterozygosity by 2n gametes. This study provides theoretical and technical evidence revealing the characteristics of genetic variation in poplar and specifically in triploid hybrid poplar; the evidence provided here can be used to further formulate breeding strategies for triploid poplar and similar triploid trees.

Materials and methods

Plant materials

In this study, we collected the strong flowering shoots from the female plants 'X-1', 'X-2', and 'X-3' with higher fertility and GCA (Bai 2015) and male plants 'Z-1', 'Z-2', 'Z-3', 'Z-4', 'Z-5', and 'Z-6' in the National Gene Bank of *P.* × *tomentosa* Carriere in early February 2016, and these parent plants are all *P. tomentosa* clones collected throughout the species' entire natural distribution range. For this experiment, the shoots were subjected to hydroponics in batches under the same environmental conditions. We collected pollen after its release by male flowers and performed controlled pollination. As a result, we obtained 3984 full-sib hybrid seedlings (Table 1).

Observation of 2n pollen

The size of artificially induced 2n pollen grains is far greater than that of normal pollen grains, approximately 37 μm in *P. tomentosa* (Zhang and Li 1992). The natural 2n pollen of *P. tomentosa* is also much larger than normal pollen (Kang 2002a). Identification of 2n pollen is easy due to this obvious difference in size. The collected pollen from each clone was examined in three temporary smears, and the proportion of 2n pollen was estimated by observing the size of pollen grains. Five views were randomly selected to calculate the percentage of 2n pollen.

Ploidy levels of progeny plants

Ploidy level was determined by flow cytometry in the full-sib progeny, as described previously (Galbraith et al. 1983; Loureiro et al. 2007). Approximately 0.5 g of young leaf was chopped using a sharp razor blade in a 55 mm plastic Petri dish containing 1 mL of modified Galbraith's buffer (45 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 30 mmol L^{-1} sodium citrate, 20 mmol L^{-1} 3-[*N*-morpholino]-propanesulfonic acid, 0.5% Triton X-100, and 1% polyvinylpyrrolidone 10, pH 7.0). The separation buffer containing the nuclei was filtered into a 1.5 mL centrifuge tube using a 40 μm nylon filter. Propidium iodide (PI; 100 μL, 50 μg/mL) staining solution was added, and the tube was shaken to mix the solution evenly and then incubated in an ice bath for 30 min (Galbraith et al. 1983). A CyFlow Ploidy Analyzer (Partec Company Ltd., Germany)

Table 1 Characteristics of *P. tomentosa* hybrids and 2n pollen from male plants

Female	Male	Seed number	Sprouting amount	Sprouting proportion (%)	Survival seedling			Counted pollens	
					Number of survival	Seedling survival rate (%)	Number of triploid progeny	Ratio of triploid progeny (%)[a]	Total sample size of counted pollens	Occurrence frequency of 2n pollen (%)[a]
X-1	Z-1	4998	1424	28.49	995	69.87	9	0.90	5200	2.42
	Z-2	1906	192	10.07	137	71.35	4	2.92	3880	3.10
	Z-3	3418	342	10.00	247	72.22	3	1.22	4853	3.70
X-2	Z-4	2800	170	6.07	137	80.58	2	1.46	5647	2.80
	Z-6	3654	1106	31.03	803	72.60	1	0.12	3986	0.00
	Z-5	5310	1584	29.83	1210	76.38	87	7.19	3160	4.30
X-3	Z-6	6810	252	3.70	205	81.34	0	0.00	4100	0.00
	Z-5	5124	345	6.73	250	72.46	0	0.00	4520	4.30
Total		34,020	5415	15.92	3984	73.57	106			

[a]The data obeyed a normal distribution characteristic through the K–S test ($p > 0.1$)

was used to analyse the DNA content. Somatic cell chromosome counting was further validated using carbol fuchsin staining (Kang 2002b).

DNA extraction and SSR analysis

DNA was extracted from each leaf sample using the DNeasy Plant Mini Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. A forward primer with a universal M13 primer tail (5-TGTAAA ACGACGGCCAGT-3) at the 5′ end and a universal M13 primer fluorescently labelled with 6-carboxy-*x*-rhodamine, 6-carboxy-fluorescein, tetramethyl-6-carboxyrhodamine, or 5-hexachlorofluorescein were used for the previously described fluorescently labelled TP-M13-SSR polymerase chain reaction (PCR) method (Schuelke 2000). The PCR conditions were as follows: 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. The final products were used for SSR analysis based on capillary electrophoresis fluorescence on the ABI 3730XL DNA Analyzer, and the results were analysed by the GeneMarker 1.75 software (Hulce et al. 2011).

Screening of polymorphism primers

We randomly selected 1365 pairs of SSR primers for screening from the SSR database (http://web.ornl.gov/sci/ipgc/ssr_resources.htm) released by the International *Populus* Genome Consortium (IPGC). M13 fluorescent dye-labelled primer was added to the 5′ end of all forward primers. All primers were synthesized by Beijing Datsing Biotechnology Co., Ltd. (Beijing, China). These primers were used in TP-M13-SSR PCR to screen for polymorphic SSR markers applied to identify the origins of the 2n gametes, at which the allelic configurations of the parents (hybrid combination 'X-2 × Z-5') were heterozygous and contained no alleles in common. The origins of the female or male 2n gametes were determined by the codominance and dosage effect characteristics of the SSR markers (Liesebach et al. 2014). SSR primers with a stable dosage effect, at which the allelic configuration of the female parent 'X-2' was heterozygous and different from that of the male parent 'Z-5', were used to detect the genetic constitution of the 2n female gametes. SSR primers with a stable dosage effect, at which the allelic configuration of the male parent 'Z-5' was heterozygous and was different from that of the female parent 'X-2', were used to detect the genetic constitution of the 2n male gametes. To avoid the influence of homologous recombination (HR) when distinguishing the genetic constitution of the 2n gametes, SSR primers with low HR rates were selected for this

study (Dong et al. 2014a). SSR loci with low HR rates can be obtained by screening SSR markers near centromeres, because the area near each chromosome centromere tends to have a low HR rate (Dong et al. 2014b). The SSR loci with the lowest persistent hDNA frequency for each chromosome were localized near the putative centromeric regions identified on the basis of the distribution of cytosine methylation in a previous study (Vining et al. 2012). The ratio of the distance from the location of each SSR locus to the centromere and the chromosome arm length is calculated; smaller ratios indicate that the locus is closer to the centromere, and thus, the HR rate of the locus is lower.

Statistical analysis

The normal distribution K–S test and Pearson correlation analysis between the ratio of triploid offspring and the frequency of 2n pollen were performed using SPSS (version 19.0) and OriginPro software (version 8.5), respectively. Comparisons of heterozygosity transmission between the source of gender of the 2n gametes in both F-type and S-type were evaluated based on Duncan's multiple range test at the 5% level to determine whether there were any significant differences.

Results

The relationship between triploid progeny and natural 2n pollen

We obtained 3984 full-sib progeny plants after sowing and thinning (Table 1). The results indicated that the relative DNA content in triploid plants was 1.5-fold higher than that in diploid plants (Fig. 1a, b). The nuclei were further analysed by chromosome counting, and this technique

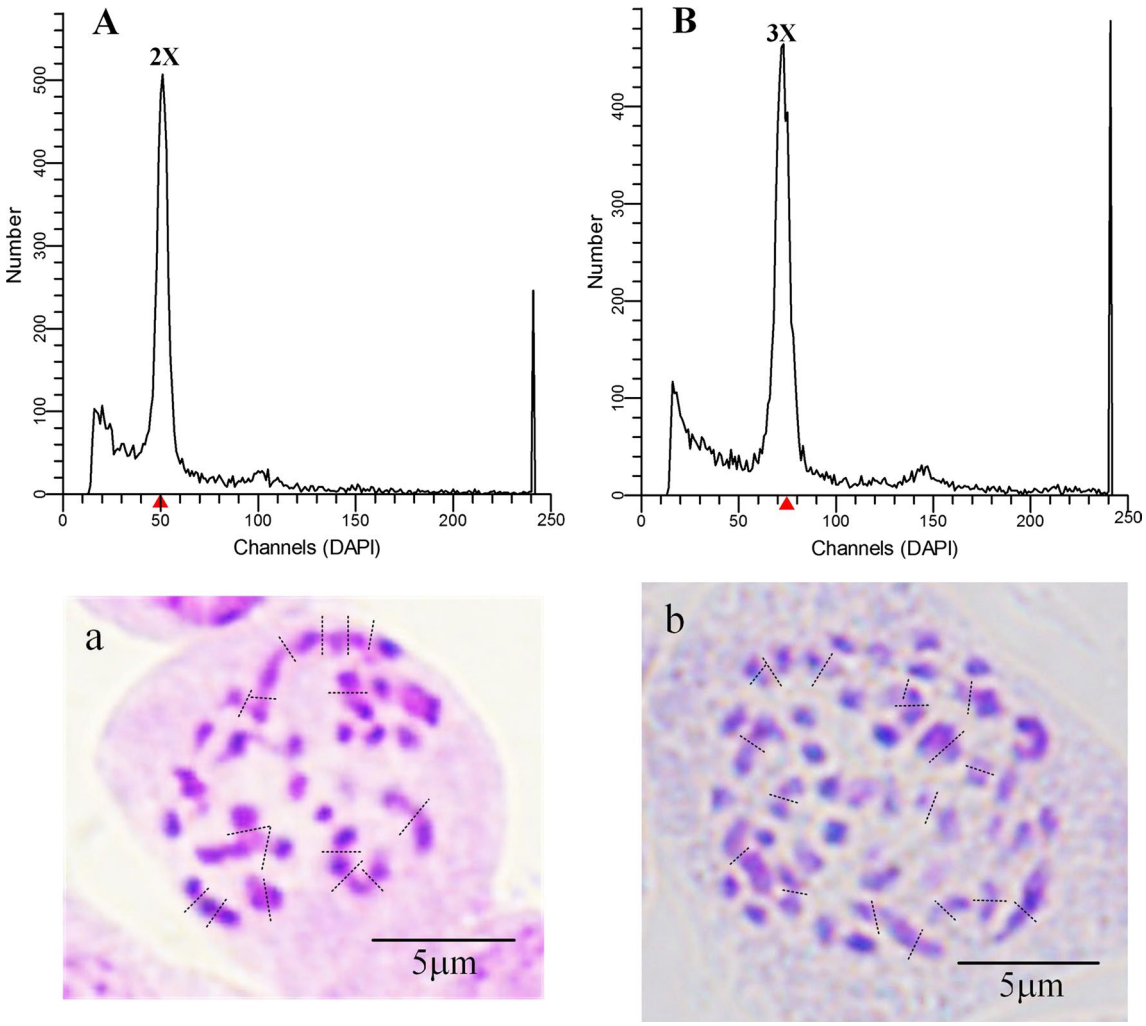

Fig. 1 Histograms presenting the flow cytometric analysis results. **a** Diploid and **b** triploid plants. Chromosome counting. **c** Chromosomes of a diploid plant with 2n = 2x = 38. **d** Chromosomes of a triploid plant with 2n = 3x = 57. Scale bar 5 μm

indicated that the diploid plants contained 38 chromosomes (2n = 2x = 38) (Fig. 1a) and the triploid plants contained 57 chromosomes (2n = 3x = 57) (Fig. 1b).

The overall triploid rate was 2.66%. The triploid rate of hybrid combination with the male parent 'Z-6' was lower than those of crosses with other males, reaching 0.12 and 0% in 'X-2 × Z-6' and 'X-3 × Z-6', respectively. A total of 87 triploids (7.19%), which is greater than the proportion of triploids in the other crosses, were detected among the full-sib progeny of hybrid combination 'X-2 × Z-5'. However, we did not find triploid in hybrid combination 'X-3 × Z-5'.

Large pollen grains were observed in the natural pollen of *P. tomentosa* (Fig. 2a), and the occurrence frequency of natural 2n pollen in the male parent 'Z-5' was 4.30%, which is higher than those in other male parent. However, the triploid rate in the hybrid combination 'X-2 × Z-5' was higher than that in 'X-3 × Z-5' (Table 1). A Pearson correlation analysis between the triploid rate and the frequency of 2n pollen in each male parent was performed for these normally distributed data ($p > 0.1$) according to the K–S test (Table 1). The results showed no significant correlation between these factors ($r = 0.1869$, $p = 0.5197$) (Fig. 2b).

Identification of the origins of *P. tomentosa* natural hybrid triploids

To identify the origins of natural 2n gametes from crossing combination 'X-2 × Z-5', we found that five pairs of SSR primers (GCPM_2012-1, GCPM_1575-2, GCPM_1297-1, GCPM_1072-2, and GCPM_1692-1) were heterozygous in their allelic configurations of female 'X-2' and male 'Z-5' and did not have common alleles (Table 2). To avoid the effects of HR, the GCPM_2012-1 locus, which is closest to the centromeres at 36.24% of the length of the chromosome arm, was used to identify the origin of the natural hybrid triploids.

Either two or three different alleles from the parents were found in the allelic configurations of the triploid offspring (Fig. 3), i.e., the alleles of the heterozygous triploid progeny were present in either a bi-allelic or tri-allelic pattern. The origin of the two different alleles or the double dosage alleles indicates the origin of the 2n gametes. If the alleles in question were inherited from the female parent, then the triploid offspring derived from a 2n female gamete; otherwise, the triploid derived from a 2n male gamete. In Fig. 3, parents 'X-2' and 'Z-5' were heterozygous with 'ab' and 'cd' genotypes, and each had two distinct alleles (size in bp) at the GCPM_2012-1 locus. The allelic configurations found in the triploid hybrids were 'abc', 'abd', 'aad', and 'bbd' ('aac' and 'bbc' were not detected), which must have originated from 'ab', 'aa', and 'bb' 2n female gametes, and 'acd', 'bcd', 'bcc', 'add', 'bdd', and 'acc' genotypes, which originated from 'cd', 'cc', and 'dd' 2n male gametes. Based on the 87 triploid plants assayed from the 'X-2 × Z-5' cross, we found that 18 triploid plants were derived from 2n female gametes, accounting for 21% of all the triploids found in 'X-2 × Z-5'. Sixty-nine triploids from 2n male gametes were found in 87 triploid plants (Table 3). According to variance

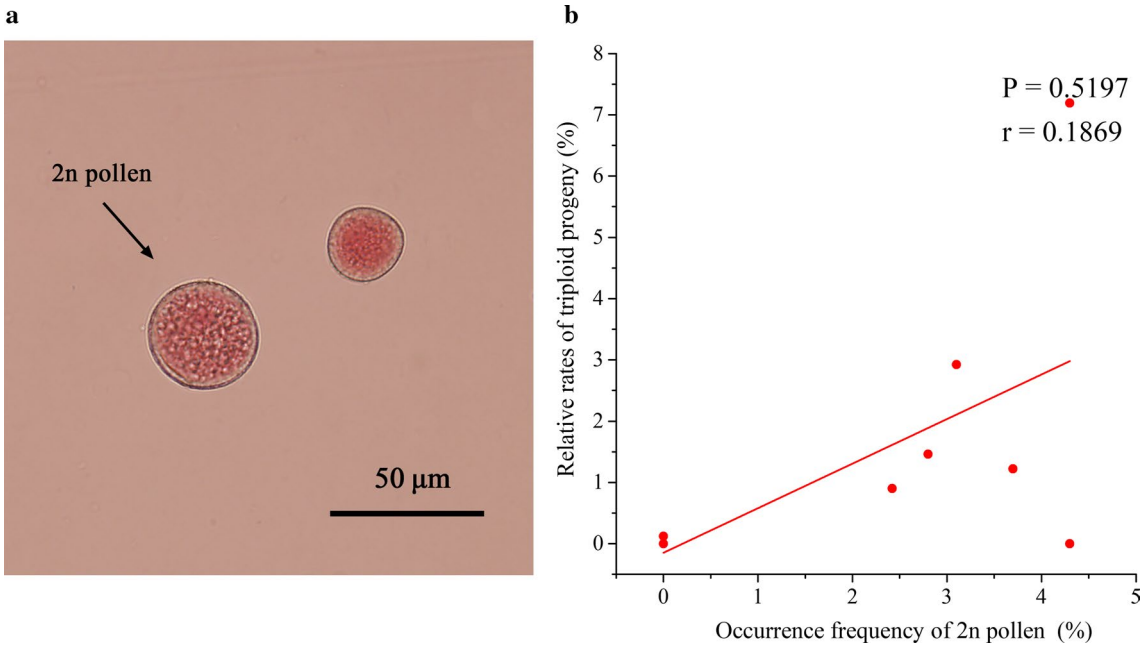

Fig. 2 **a** Occurrence of natural 2n pollen in *P. tomentosa*, 2n pollen (arrow). **b** Pearson's correlation analysis of the occurrence frequency of natural 2n pollen and the ratio of triploid offspring

Table 2 Details of the SSR loci at which the alleles detected were different between the female and male parents

Markers	Chr[a]	Location (Mb)	Primer sequences (5′→3′)	Detected alleles		Ratio[b] (%)
				Female parent (bp)	Male parent (bp)	
PMGC_2606	Chr05	18.44	Forward: AATTTACATTTCTTTATCATC ACC	182	164/182	31.56
			Reverse: GCTGTCTAACATGCCATTGC			
GCPM_2687-2	Chr03	9.35	Forward: CAGCAAAATCATCACAAATC	226	222/226	28.03
			Reverse: AGGGTTTGGTAGAGAAGACC			
GCPM_1445-1	Chr09	9.98	Forward: TGATGTTGTGTGAGAGGAAA	174	174/183	75.97
			Reverse: AGCCCAGTAAACAAACTGAA			
GCPM_1522	Chr18	16.63	Forward: CTACCATCTTGGAGCTATCG	184	181/184	95.89
			Reverse: CTTGAACCAGCTCAAGAAAC			
GCPM_1343-1	Chr10	12.27	Forward: AATGGGTTTTTGTTTGTGTC	171	157/171	53.45
			Reverse: AGGCTTTTAAGATTCCCCTA			
GCPM_161-1	Chr19	11.04	Forward: AAGATATTGATCGTGGATGC	196	186/196	29.57
			Reverse: TACTTCAAGCTCAAGGGGTA			
GCPM_1604-1	Chr09	12.62	Forward: AGAATCATGTGCAAATTA ACAA	188	190/204	97.70
			Reverse: CACAAGGAATTTATGTGT CTCA			
GCPM_1920-1	Chr18	16.04	Forward: AGTTTGAATCATGCTGGTCT	146	146/154	89.33
			Reverse: TTTACACTCATTGAACCGAA			
GCPM_1157-1	Chr14	17.49	Forward: CCTCCTACCACATATTCCAA	170	173/175	64.75
			Reverse: AGTGGTTAAAATGCGAGTGT			
GCPM_1720-1	Chr05	21.81	Forward: ATAGGCCAATGTAGACCTGA	177	177/190	62.48
			Reverse: TGTCTTTCTTTGCCATTTCT			
GCPM_1571-1	Chr13	16.09	Forward: AAGAAGGGTGGTAAGCTTTT	172	181/186	96.87
			Reverse: TCCGTAGATCTCTCTCCAAA			
GCPM_461-1	Chr12	0.52	Forward: TGGGTTCATAAGAAAATTCC	174	174/178	91.68
			Reverse: TTAATGGTGAGGATTTGAGTG			
LG_XVI-7	Chr16	7.49	Forward: ACAAATCAAAGTCACAGCCT	362/366	350/362	6.38
			Reverse: ATAGTGTTCAATCGGACCTG			
GCPM_1416	Chr07	5.93	Forward: TCTTGAGAGGGCACTAGAAG	235/237	235	25.88
			Reverse: TGACAATTAGAATGGAACCC			
ORPM_329	Chr08	7.38	Forward: CATGGCTG GGCTTAACTTGT	221/224	224	56.59
			Reverse: CCGGGGTTCTTAACACTCAA			
GCPM_1072-2	Chr06	4.20	Forward: AGGAAAACAAAGGAGAGGAG	152/157	141/146	72
			Reverse: ATGCTTAAAAGGGGATCTCT			
GCPM_2849-1	Unknown	–	Forward: ATGTCACAACCAAAAAGAGG	106/116	116	–
			Reverse: AATACCTTTACCCGTGGATT			
GCPM_1263-1	Chr01	41.93	Forward: TGCATTAAGACATCACTTGC	235/244	235/247	74.03
			Reverse: TTCGCTTCTGTATTTCCTGT			
LG_XIX-23	Chr19	2.18	Forward: CAAGATCGGTGAGGATTTAC	351/360	360	75.78
			Reverse: AAAGAGCAAATCCACTGGTA			
GCPM_868-1	Chr09	11.78	Forward: CTTCATCACTGGAATTGGAT	200/212	212	93.63
			Reverse: CAATCAAATGTTGCTCACAG			
LG_XI-2	Unknown	–	Forward: GGAAGGAGAGGAGAGGATAA	231/243	231	–
			Reverse: ACCTTCCCTTACTGGATAGC			
GCPM_2088-2	Chr18	16.88	Forward: TGGTGGAGGCTAGGATAGTA	231/252	219	98.67
			Reverse: GCCCAAACTCTTATTTGATG			
GCPM_561-2	Chr17	11.20	Forward: AATCTGGGTTGTGATGAGAG	232/236	236	31.94
			Reverse: GAGGGCTCATGTACAGAAAG			

Table 2 (continued)

Markers	Chr[a]	Location (Mb)	Primer sequences (5′→3′)		Detected alleles		Ratio[b] (%)
					Female parent (bp)	Male parent (bp)	
GCPM_3588-1	Chr02	2.44	Forward:	TCGACCAACTATGTTTGACA	187/190	190	86.06
			Reverse:	AGTTGTAACTTTGGCCTGAA			
GCPM_1260	Chr02	3.44	Forward:	CACAGGAACCTGGTTATCAT	141/156	141	80.34
			Reverse:	CTGGCATTCCTTCTAAGCTA			
GCPM_2012-1	Chr 14	9.5	Forward:	GGTGATGAAGATCTGGGATA	123/132	126/135	36.24
			Reverse:	ACCCAAATTACAGAACAACG			
GCPM_1676-1	Chr 17	13.54	Forward:	CAACAAAATCAATCGCTCTC	149/157	149/161	64.44
			Reverse:	ACCCTAGCAAAATCAACAAA			
GCPM_2321-2	Chr 19	15.36	Forward:	TTCCTTTGCATGTCCTTTAT	169/178	169/180	92.17
			Reverse:	TGTAACACACGGTTCTACCA			
GCPM_1575-2	Chr 04	4.76	Forward:	GGGATGAATCAGGAGATGTA	122/127	116/120	67.17
			Reverse:	GTGATGCTCTGATACCACCT			
GCPM_1297-1	Chr 04	4.76	Forward:	GGGATGAATCAGGAGATGTA	136/142	128/132	67.17
			Reverse:	GAAGAAACCTGTGGGTGATA			
GCPM_1224-1	Chr 09	11.04	Forward:	AAAATGAATTGGCAGAAAGA	196/216	196/205	84.69
			Reverse:	GCTTCTTAGCTCAACCAGAA			
GCPM_1900-1	Chr 16	9.73	Forward:	CATCTGCAGAAATCATCT CTAA	156/162	156/166	26.62
			Reverse:	TAAAGGCCAATAGAAAATCG			
GCPM_734	Chr05	24.35	Forward:	GGCAATTTAGGTACAACAGC	180/184	180/190	85.78
			Reverse:	ACAAGCGAATGCTAATTGAT			
PMGC_2571	Chr10	13.01	Forward:	TCTCGCAGATTCATGTAACCC	104/120	104/116	56.78
			Reverse:	GACTGTATGTTGACCATGCCC			
GCPM_1153-1	Chr 11	11.14	Forward:	TTCCTTTCACACAATGACAA	177/180	180/183	10.79
			Reverse:	TTTAAAAACTGGGTCCGTAA			
LG_XIX-11	Chr19	8.77	Forward:	CAACATGAAATGAGCTGCTA	396/403	403	2.5
			Reverse:	TCCACATGATGTCTGATTTG			
LG_XVI-6	Chr 16	7.30	Forward:	ATAGCGATCATCAAAGGAAA	130/133	115/130	8.75
			Reverse:	AAATATTCATGTGGAGGCAC			
LG_XVI-9	Chr16	7.60	Forward:	CTCGCAGCTCTTCTCATAGT	234	218/225	5
			Reverse:	CCTACCCATTTATGACCAAA			
GCPM_1524-1	Chr 04	13.39	Forward:	TTCAATGGAAAGGGATAATG	170/175	170/173	7.66
			Reverse:	TCATTTGTAAAACATCACGC			
GCPM_67	Chr16	6.97	Forward:	TGAAGCCCTCACTACTCATT	207/209	209/215	12.88
			Reverse:	CCCCAATCTTTTGTTTATTG			
GCPM_3474-1	Chr 08	16.81	Forward:	GATCCGAAAACAACAACAAT	121/133	121	1.12
			Reverse:	ACCCCTTTCTCTTCTCAATC			
PMGC_223	Chr02	16.23	Forward:	CGATGAGGTTGAAGAAGTCG	189	186/189	7.26
			Reverse:	ATATATGTACCGGCACGCCAC			

All SSR primer sequences were from the International *Populus* Genome Consortium (IPGC) and were BLAST searched against the publicly available genome sequence of *Populus trichocarpa* v.3.0 (DOEJGI, http://www.phytozome.net/poplar) to determine the chromosome numbers and locations of these SSR loci

[a]Chromosome number for the SSR locus

[b]The ratio of the locus–centromere distance and the physical length of the arm; absence of this value (–) indicates that the details of a single sequence from the SSR primer could not be confirmed when blasted against the publicly available genome sequence of *Populus trichocarpa* v.3.0

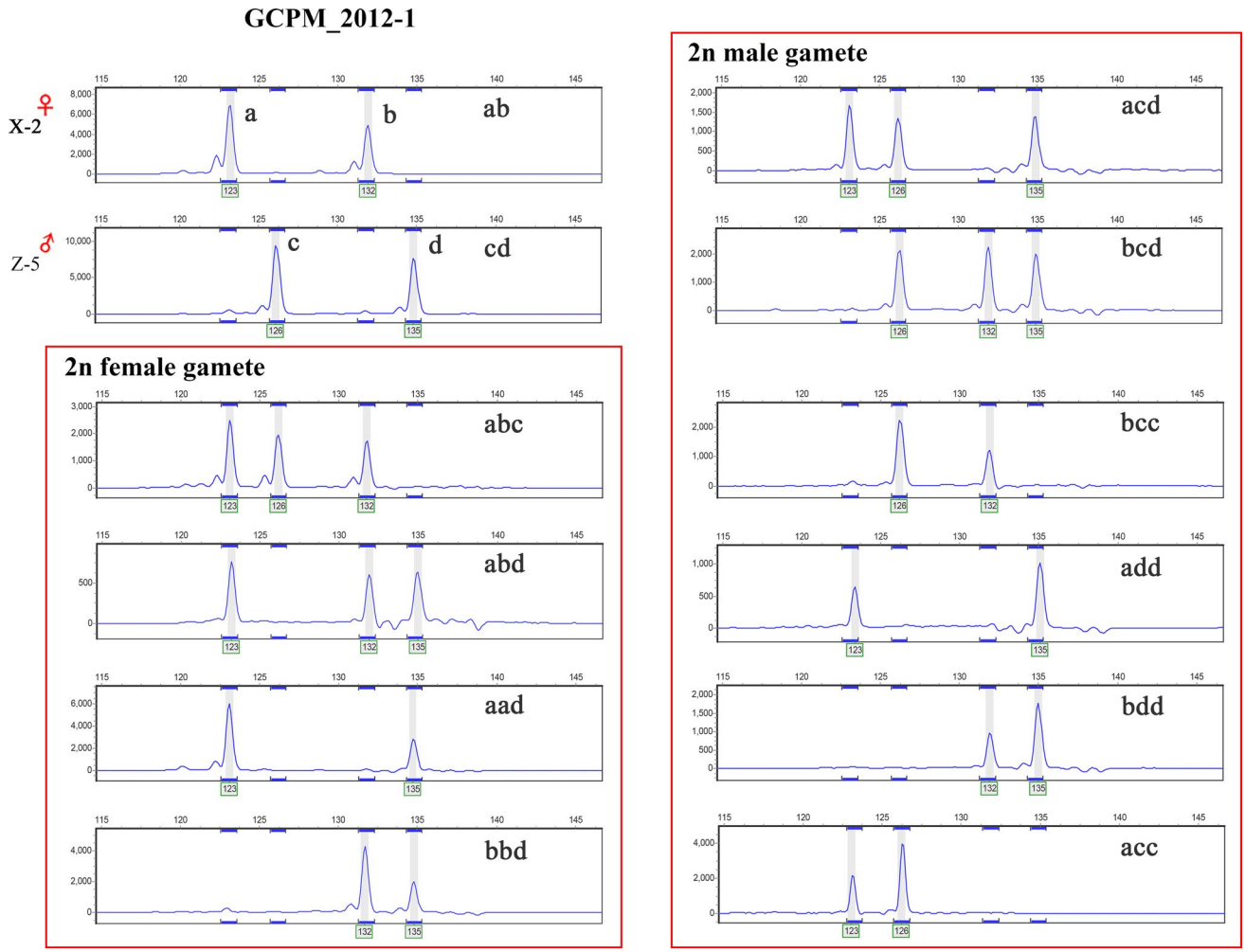

Fig. 3 Allelic configurations detected at the GCPM_2012-1 locus for the hybrid parents and triploid progeny in hybrid combination 'X-2×Z-5'.♀ represents female parent, and ♂ represents male parent

analysis, this ratio (21%) is significantly lower than that of triploids derived from 2n male gametes at the $p < 0.05$ level.

Detection of genetic constitution of natural 2n gametes

Screening of SSR suitable primers

We obtained 149 pairs of SSR primers at which the allelic configurations were different between the parents 'X-2' and 'Z-5'. Furthermore, we screened these 149 pairs for suitable loci at which the allelic configuration was heterozygous in the female or male parent and the alleles in the two parents differed. As a result, 42 pairs of SSR primers that met the above standards were selected (Table 2). On this basis, we screened for SSR markers with low HR rates to identify the genetic constitution of the 2n gametes. All 42 selected SSR markers were BLAST searched against the publicly available genome sequence of *Populus trichocarpa* v3.0 (Phytozome v8.0; http://www.phytozome.net) to determine their physical positions (Table 2). Two pairs of primers (LG_XI-2 and GCPM_2849-1) could not be located in the chromosome, but all other primers were found and their positions with respect to the centromere were calculated. Therefore, we obtained four pairs of primers for which the ratio of the locus–centromere distance to the chromosome arm length was between 1.12 and 10.79% (Table 2). These four pairs of SSR markers were located on chromosomes 4, 8, 11, and 16 (Fig. 4). The primers GCPM_1153-1, GCPM_1524-1, and LG_XVI-9 were used to identify the genetic constitution of the 2n male gametes, and the genetic constitution of the 2n female gametes was identified using the primers GCPM_1153-1, GCPM_1524-1, and GCPM_3474-1. The absolute distances between SSR loci (GCPM_1153-1, GCPM_1524-1,

Table 3 Results of microsatellite genotyping of 87 triploid offspring in cross combination 'X-2×Z-5'

Offspring ID	Diploid gamete	Genotype at centromere-associated markers			FDR/SDR	Number of marker genotypes of diploid gametes		Percentage of transmitted heterozygosity (%)
		Marker 1	Marker 2	Marker 3		Heterozygous	Homozygous	
87-1	♂	He	He	He	FDR	19	11	63.3
87-2	♀	He	Ho	He	FDR	18	10	64.3
87-3	♂	Ho	He	He	FDR	20	10	66.7
87-4	♂	Ho	Ho	Ho	SDR	9	21	30.0
87-5	♂	He	He	He	FDR	21	9	70.0
87-6	♀	Ho	Ho	Ho	SDR	8	20	28.6
87-7	♂	Ho	Ho	Ho	SDR	10	20	33.3
87-8	♀	Ho	He	He	FDR	19	9	67.9
87-9	♂	He	Ho	He	FDR	22	8	73.3
87-10	♂	He	He	He	FDR	24	6	80.0
87-11	♂	He	He	He	FDR	19	11	63.3
87-12	♂	Ho	Ho	Ho	SDR	11	19	36.7
87-13	♂	He	Ho	He	FDR	12	18	40.0
87-14	♂	He	He	He	FDR	25	5	83.3
87-15	♂	He	He	He	FDR	20	10	66.7
87-16	♂	He	Ho	He	FDR	22	8	73.3
87-17	♂	He	He	He	FDR	19	11	63.3
87-18	♀	Ho	Ho	Ho	SDR	9	19	32.1
87-19	♂	He	Ho	He	FDR	20	10	66.7
87-20	♂	He	He	He	FDR	21	9	70.0
87-21	♂	Ho	Ho	Ho	SDR	13	17	43.3
87-22	♂	He	He	He	FDR	23	7	76.7
87-23	♂	He	Ho	He	FDR	18	12	60.0
87-24	♂	Ho	Ho	Ho	SDR	12	18	40.0
87-25	♂	He	He	He	FDR	19	11	63.3
87-26	♂	He	Ho	He	FDR	22	8	73.3
87-27	♂	He	He	He	FDR	19	11	63.3
87-28	♂	Ho	He	He	FDR	21	9	70.0
87-29	♂	He	He	He	FDR	24	6	80.0
87-30	♀	Ho	Ho	Ho	SDR	10	18	35.7
87-31	♂	He	Ho	He	FDR	20	10	66.7
87-32	♂	He	He	He	FDR	19	11	63.3
87-33	♂	He	Ho	He	FDR	23	7	76.7
87-34	♂	He	He	He	FDR	19	11	63.3
87-35	♂	He	Ho	He	FDR	21	9	70.0
87-36	♂	Ho	He	He	FDR	22	8	73.3
87-37	♂	He	He	He	FDR	18	12	60.0
87-38	♂	He	Ho	Ho	SDR	12	18	40.0
87-39	♂	He	He	He	FDR	23	7	76.7
87-40	♀	Ho	Ho	Ho	SDR	8	20	28.6
87-41	♂	Ho	He	He	FDR	19	11	63.3
87-42	♀	Ho	Ho	Ho	SDR	9	19	32.1
87-43	♂	He	Ho	He	FDR	21	9	70.0
87-44	♂	He	He	He	FDR	20	10	66.7
87-45	♀	He	He	He	FDR	20	8	71.4
87-46	♀	He	Ho	He	FDR	22	6	78.6
87-47	♂	He	Ho	Ho	SDR	9	21	30.0

Table 3 (continued)

Offspring ID	Diploid gamete	Genotype at centromere-associated markers			FDR/SDR	Number of marker genotypes of diploid gametes		Percentage of transmitted heterozygosity (%)
		Marker 1	Marker 2	Marker 3		Heterozygous	Homozygous	
87-48	♂	He	He	He	FDR	22	8	73.3
87-49	♂	Ho	Ho	Ho	SDR	10	20	33.3
87-50	♂	He	He	He	FDR	21	9	70.0
87-51	♂	He	Ho	He	FDR	23	7	76.7
87-52	♀	Ho	Ho	Ho	SDR	10	18	35.7
87-53	♂	He	Ho	He	FDR	20	10	66.7
87-54	♂	Ho	Ho	Ho	SDR	11	19	36.7
87-55	♂	He	He	He	FDR	20	10	66.7
87-56	♂	He	He	He	FDR	19	11	63.3
87-57	♂	He	Ho	He	FDR	21	9	70.0
87-58	♂	He	Ho	Ho	SDR	12	18	40.0
87-59	♂	He	Ho	He	FDR	20	10	66.7
87-60	♀	He	Ho	Ho	SDR	11	17	39.3
87-61	♂	He	He	He	FDR	20	10	66.7
87-62	♂	He	He	He	FDR	21	9	70.0
87-63	♂	He	Ho	He	FDR	20	10	66.7
87-64	♂	He	He	He	FDR	22	8	73.3
87-65	♂	He	Ho	He	FDR	24	6	80.0
87-66	♂	He	He	He	FDR	23	7	76.7
87-67	♂	He	Ho	He	FDR	21	9	70.0
87-68	♀	Ho	Ho	Ho	SDR	7	21	25.0
87-69	♂	He	Ho	He	FDR	22	8	73.3
87-70	♂	He	He	He	FDR	19	11	63.3
87-71	♂	He	Ho	He	FDR	22	8	73.3
87-72	♂	Ho	Ho	He	SDR	13	17	43.3
87-73	♂	Ho	Ho	Ho	SDR	9	21	30.0
87-74	♀	He	Ho	He	FDR	21	7	75.0
87-75	♂	Ho	He	He	FDR	19	11	63.3
87-76	♀	Ho	He	Ho	SDR	9	19	32.1
87-77	♂	He	Ho	He	FDR	20	10	66.7
87-78	♂	He	He	He	FDR	21	9	70.0
87-79	♂	He	He	He	FDR	20	10	66.7
87-80	♂	He	Ho	He	FDR	19	11	63.3
87-81	♀	Ho	Ho	Ho	SDR	10	18	35.7

LG_XVI-9, and GCPM_3474-1) and the centromere was 0.89, 1.11, 0.4, and 0.19 Mb, respectively (Fig. 4).

Detection of the genetic constitution of 2n gametes

A total of 87 natural triploid plants derived from 2n gametes in the hybrid combination 'X-2 × Z-5' were used as the materials, and we found that triploid plants with two kinds of allelic configurations, FDR-derived (F-type) and SDR-derived (S-type), were detected by each pair of SSR primers. In the F-type allelic configuration, both heterozygous parental alleles were carried by the 2n gametes and were thus maintained in the these loci; in the S-type, the allelic configuration of the 2n gametes was homozygous in these loci (Fig. 5). However, through further research, we found that the allelic configurations were different in these SSR loci used for detecting the genetic constitution of the 2n gametes. Based on the number of F- and S-type results at the SSR loci, all triploid plants were divided into four categories (3F0S, 2F1S, 1F2S, and 0F3S, respectively). Considering the 3F0S triploids as an example, the results in triploid plants showed 3 F-type allelic configurations and 0 S-type allelic configurations at the three SSR loci. Because the allelic configurations detected at the three

Table 3 (continued)

Offspring ID	Diploid gamete	Genotype at centromere-associated markers			FDR/SDR	Number of marker genotypes of diploid gametes		Percentage of transmitted heterozygosity (%)
		Marker 1	Marker 2	Marker 3		Heterozygous	Homozygous	
87-82	♀	He	Ho	He	FDR	23	5	82.1
87-83	♀	Ho	Ho	Ho	SDR	11	17	39.3
87-84	♀	Ho	Ho	Ho	SDR	8	20	28.6
87-85	♂	He	He	He	FDR	22	8	73.3
87-86	♂	He	Ho	Ho	SDR	13	17	43.3
87-87	♂	He	He	He	FDR	21	9	70.0
		Ratio of the number of triploids						
$N=18$	♀	21%[b]			FDR ($N=6$)	123	45	73.2[a]
					SDR ($N=12$)	110	226	32.7[d]
$N=69$	♂	79%[a]			FDR ($N=56$)	1157	523	68.9[b]
					SDR ($N=13$)	144	246	36.9[c]

Male or female diploid gamete contribution, transmission of homozygous or heterozygous genotypes at centromere-associated marker loci, genetic constitution of 2n gametes, the number of homozygous or heterozygous genotypes, and percentage of transmitted heterozygosity via diploid gametes derived from available SSR markers are shown

♀ represents female, and ♂ represents male. Marker 1, Marker 2, and Marker 3 represent GCPM_1153-1, GCPM_1524-1, and LG_XVI-9, respectively, when the 2n gametes were generated by the male parent, and GCPM_1153-1, GCPM_1524-1, and GCPM_3474-1, respectively, when the 2n gametes were generated by the female parent. *Ho* homozygous, *He* heterozygous, *N* represents the number of triploid progeny plants; Various lowercase letters indicate significant differences based on Duncan's multiple range test at the 0.05 probability level

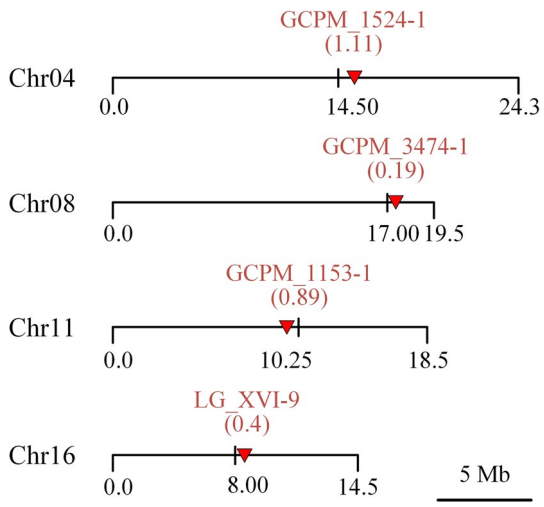

Fig. 4 Chromosomes (size in Mb) of *Populus* and details of the four pairs of SSR loci localized near the putative centromeric regions represent a region of the putative centromere; the vertical bars represent the locations of SSR loci. The figures in brackets represent the locus–centromere distance (Mb). Scale bar 5 Mb

SSR loci were consistent in 3F0S triploid plants, determining that 3F0S triploids were derived from F-type 2n gametes was easy and accurate. Similarly, 0F3S triploids were derived from S-type 2n gametes. However, the low HR rates at the three SSR loci create a very low probability that HR events, which could cause F- and S-type loci to coincide, would occur at the same time. Therefore, the allelic configurations 2F1S and 1F2S at the tested SSR loci were not consistent with our expectations. We tentatively assigned the triploids with 2F1S to the same genetic constitution as those with 3F0S, which were derived from F-type 2n gametes. In the same way, the triploids with 1F2S were considered the same as those with 0F3S, which were derived from S-type 2n gametes. Capillary electrophoresis for the SSR markers was used to infer the genetic constitution of the 2n gametes (Fig. 5). The number of triploid plants derived from 2n female gametes with SDR was significantly higher than that with FDR (12 vs 6). Conversely, the number of triploid plants derived from 2n male gametes with FDR was significantly higher than that with SDR (56 vs 13) (Table 3).

Transmission of heterozygosity by natural 2n gametes

In this study, 42 pairs of SSR polymorphic primers were used to analyse the transmission of heterozygosity by 2n gametes. A total of 28 pairs of primers, at which the allelic configuration in the female parent 'X-2' was heterozygous and different from that of the male parent 'Z-5' (Table 3), were used to analyse the transmission of heterozygosity by natural 2n female gametes. Thirty pairs of primers, at which

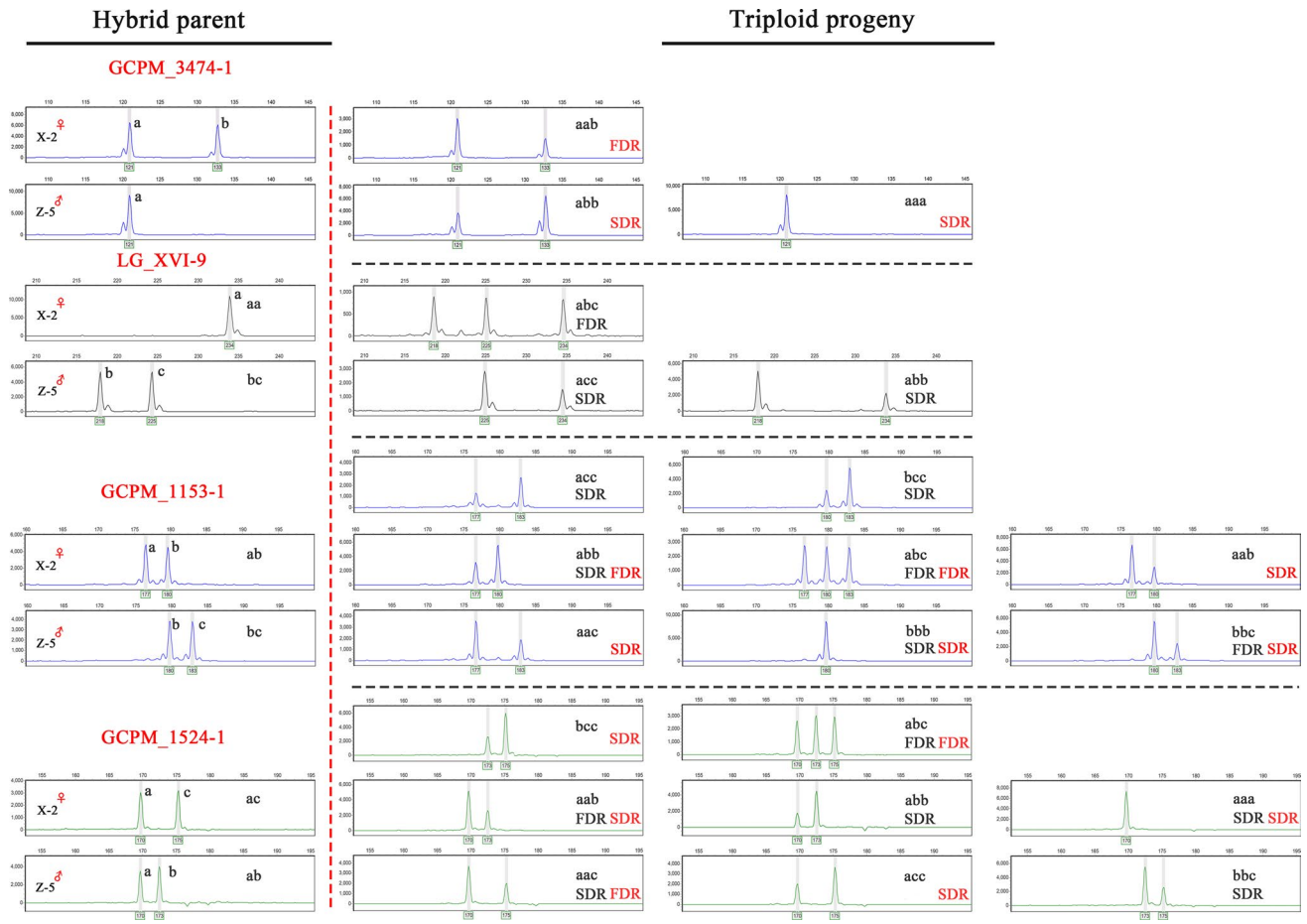

Fig. 5 Capillary electrophoresis for the SSR markers was used to infer the genetic constitution of the 2n gametes. The allelic configurations of the triploid hybrids were divided into two types, which were designated F-type and S-type (black fonts represents the genetic constitution of the 2n male gametes, and red font represents the genetic constitution of the 2n female gametes); ♀ represents female, and ♂ represents male

the allelic configuration in the male parent 'Z-5' was heterozygous and different from that of the female parent 'X-2', were used to analyse the transmission of heterozygosity by natural 2n male gametes.

The numbers of heterozygous and homozygous marker genotypes of 2n gametes, the percentages of transmitted heterozygosity, and single individual amount are presented in Table 3. Based on Duncan's multiple range test ($p < 0.05$), we found significant difference in heterozygosity transmission between the source of genders of the 2n gametes in both the F-type and the S-type. The average percentage of transmitted heterozygosity from female parental heterozygous marker loci by F-type 2n gametes was 73.2%, which was significantly higher than that of the male parent (68.9%). The average percentage of transmitted heterozygosity from female parental heterozygous marker loci by S-type 2n gametes was 32.7%, which was significantly lower than that of the male parent (36.9%).

Discussion

Detection of natural 2n female gametes in *P. tomentosa*

Zhang et al. (2009) analysed the origin of a natural tetraploid poplar by SSR markers and found natural 2n female gametes in *P.* × *euramericana* (Dode) Guinier for the first time. Moreover, Liesebach et al. (2014) identified the origins of 34 triploid progeny plants, whose female parents were *P. deltoides*, *P. tremula* × *P. tremuloides* or *P. tremula*, and *P.* × *canescens* using SSR marker analysis, and the results showed that seven natural triploid progeny had derived from 2n female gametes. In this study, we found that the triploid rates in different hybrid combinations ('X-2 × Z-5' and 'X-3 × Z-5'), in which female parents differed significantly (7.19 vs 0%) and male parents were the same, were significantly different. In addition, we found no significant correlation between the triploid rate and the frequency of 2n

pollen grains by correlation analysis, implying that triploids may not arise entirely from 2n pollen. In general, the existence of 2n female gametes could not be ruled out. A total of 87 natural triploid progeny were detected in the hybrid combination 'X-2 × Z-5'. By analysing allelic configuration at the GCPM_2012-1 locus, we found that 18 triploids were derived from 2n female gametes, accounting for 21% of the 87 triploids, which is consistent with the results of Zhang et al. (2009) and Liesebach et al. (2014), who found natural 2n female gametes in other poplar species. Natural 2n female gametes in *P. tomentosa* are reported for the first time in this study, providing a new approach to studying the origins of natural 2n gametes and polyploid breeding.

Genetic constitution of natural 2n gametes in *P. tomentosa*

The genetic constitution of individual loci can be effectively analysed by SSR markers, but based on our knowledge of HR, we know that HR events exchange genetic material among homologous chromosomes and lead to misjudgement of the origins of 2n gametes (Dong et al. 2014a, b). Therefore, we selected four SSR markers with low HR rates to identify the genetic constitution of 2n gametes. To avoid linkage disequilibrium, four pairs of SSR markers distributed across four different chromosomes were used to distinguish between FDR and SDR in this study. Therefore, an influence of tight linkage does not exist, and the information regarding the four markers is truly independent. We found that both parents produced both F and S-type 2n gametes. These results indicated that chromosome separation in the process of sporogenesis was disrupted in the first and second stages of meiosis, producing natural 2n female and male gametes in *P. tomentosa*.

The origins of six triploids were FDR-derived female gametes, while most of the 18 triploid plants derived from 2n female gametes that had undergone SDR. Liesebach et al. (2014) found seven triploids derived from natural 2n female gametes, and the genetic constitution of six of them was SDR. Further investigation is warranted to determine why the genetic constitution of natural 2n female gametes indicates a predominant influence of SDR.

Analysis of transmission of parental heterozygosity by natural 2n gametes in *P. tomentosa*

Because 2n gametes derive from different genetic constitutions, the transmission of parental heterozygosity is expected to differ depending on the 2n gametes involved (Vorsa and Rowland 1997). In the previous studies of the transmission of parental heterozygosity by 2n gametes, Hermsen (1984) found that FDR-derived gametes transmitted 80.2% of parental heterozygosity and SDR-derived gametes transmitted 39.6%, assuming one crossover per chromosome. Based on an analysis of the chromosomal pachytene stage, FDR (82.7%) was more than twice as effective as SDR (34.7%) in the transmission of heterozygosity under six different cytogenetic assumptions (Peloquin et al. 2008). In addition, molecular marker technology provides a convenient and accurate technical means for the transmission of parental heterozygosity by 2n gametes. When RFLP and RAPD markers were used to estimate parental heterozygosity transmission by natural 2n gametes, the results were consistent with those of a previous study, which had reported that FDR was 1.50–2.25 times as effective as SDR in the transmission of heterozygosity (Barone et al. 1995; Vorsa and Rowland 1997). Based on an analysis of parental heterozygosity transmission by 2n gametes in *Populus*, 2n gametes with different genetic constitutions transmitted parental heterozygosity at different levels (Liesebach et al. 2014; Dong et al. 2015). In this study, the transmission of parental heterozygosity by natural 2n gametes in *P. tomentosa* was analysed for the first time using 42 pairs of SSR primers, and obvious differences were present between F- and S-type 2n gametes. In addition, FDR and SDR transmitted 73.2 and 32.7% of the parental heterozygosity in natural 2n female gametes, respectively. However, 68.9 and 36.9% of the parental heterozygosity in natural 2n male gametes were transmitted by FDR and SDR, respectively, and these results were consistent with those of the previous studies. In addition, we found significant differences in heterozygosity transmission between the source of gender of the 2n gametes in both the F-type and the S-type, which could depend on the individual fluctuations independently from a certain sex in the chromosome behavior during the meiotic processes. Furthermore, the differences of heterozygosity transmission between FDR and SDR are assigned to the difference of HR between homologous chromosomes of different individuals (Dong et al. 2015).

Application of natural 2n gametes to *P. tomentosa* breeding

These 2n gametes play an important role in the *P. tomentosa* breeding process (Bingham 1980). To date, 2n pollen has been found in *Populus canescens* Moench, *P. nigra* L., *P. tomentosa* Carr., *P. tremula* L., and others in the genus *Populus* (Müntzing 1936; Seitz 1954; Bradshaw and Stettler 1993; Kang 1996; Zhu et al. 1998). The allotriploid *P. tomentosa* clone B301 is a new variety that was created by crossbreeding with natural 2n pollen (Zhu et al. 1995). Notably, 2n pollen grains have poor competitiveness due to their slow germination, causing difficulty in their effective application to breeding. However, because no competition exists, 2n female gametes have a higher application value (Zhang and Kang 2006). If female plants that produce natural 2n female gametes were used in crossbreeding, the

efficiency of breeding for polyploidy would be improved. This research suggests the application of *P. tomentosa* plants producing natural 2n female gametes to control pollination with male parents for the breeding of triploid hybrids. Thus, the discovery of natural 2n female gametes in *P. tomentosa* will promote the application of 2n female gametes to the genetic improvement of *P. tomentosa* and promote the rapid development of poplar polyploid breeding.

Author contribution statement ZQH and XYK conceived the study; ZQH, XNG, and KD conducted the experiments; FYB provided materials; ZQH and CPX analysed the data; XYK and PQY supervised the research; ZQH and XYK wrote the manuscript; and all authors read, edited, and approved the manuscript.

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