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Original article

Using SSR markers to study the mechanism of 2n pollen formation in *Populus* × *euramericana* (Dode) Guinier and *P.* × *popularis*

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Keywords:
tree breeding / flowering / genetics / silviculture / biotechnology / reproduction

Abstract

• Using cytological methods and SSR DNA marker analysis, this study revealed the formation mechanisms and the genetic constitutions of the 2n pollen in *Populus* × *euramericana* (Dode) Guinier and *P.* × *popularis*.

• In *P.* × *euramericana* (Dode) Guinier, four abnormalities in microsporogenesis were observed: parallel spindle, fused spindle, tripolar spindle and premature cytokinesis. The first three can lead to first-division restitution (FDR) 2n pollen formation and the last one can form second-division restitution (SDR) 2n pollen. The SSR marker analysis results of parents and their tetraploidy filial generation confirmed that the genetic constitution of 2n pollen produced by *P.* × *euramericana* (Dode) Guinier was FDR.

• In *P.* × *popularis*, three of these abnormalities were observed: parallel spindle, fused spindle and premature cytokinesis. The SSR marker analysis results showed the genetic constitution of 2n pollen produced by *P.* × *popularis* was SDR. Natural 2n female gametes in *P.* × *euramericana* (Dode) Guinier are reported for the first time. SSR analysis indicated that natural 2n female gametes of *P.* × *euramericana* (Dode) Guinier did exist and were fertile, which could be FDR genetic constitution.

• The results from this study showed a great potential for using 2n gametes to produce polyploid poplar clones, which can be used effectively for polyploid breeding for poplar species in the section *Aigeiros*.

Mots-clés :
amélioration génétique / floraison / génétique / sylviculture / biotechnologie / reproduction

Résumé – Utilisation de marqueurs SSR pour étudier le mécanisme de formation du pollen 2n chez *Populus* × *euramericana* (Dode) Guinier et *P.* × *popularis*.

• En utilisant des méthodes cytologiques et des analyses avec des marqueurs SSR, cette étude a révélé les mécanismes de formation et la constitution génétique du pollen 2n chez *Populus* × *euramericana* (Dode) Guinier et *P.* × *popularis*.

• Chez *P.* × *euramericana* (Dode) Guinier, quatre anomalies dans la microsporogénèse ont été observées : en parallèle broche, broche fusion,tripolaire broche et une cytokinèse prématurée. Les trois premiers peuvent entraîner une première division restitution (FDR) de formation du pollen 2n et le dernier peut former une deuxième division de restitution (SDR) du pollen 2n. Les résultats de l'analyse des marqueurs SSR des parents et de leur descendanta tetraploides ont confirmé que la constitution génétique du pollen 2n produit par *P.* × *euramericana* (Dode) Guinier a été FDR.

• Dans *P.* × *popularis*, trois de ces anomalies ont été observées : en parallèle broche, broche fusion et cytokinèse prématurée. Les résultats de l'analyse de marqueurs SSR ont montré que la constitution génétique du pollen 2n produit par *P.* × *popularis* a été SDR. Les gamètes naturelles femelles 2n chez *P.* × *euramericana* (Dode) Guinier ont été signalés pour la première fois. Les analyses SSR ont montré que les gamètes naturels femelles 2n de *P.* × *euramericana* (Dode) Guinier existaient et étaient fertiles, cela pourrait être la constitution génétique FDR.

• Les résultats de cette étude ont montré le grand potentiel de l'utilisation des gamètes 2n pour produire des clones de peuplier polyploïdes, cela peut être utilisé efficacement pour la sélection de polyploïdes pour des espèces de peuplier de la section *Aigeiros*.

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1. INTRODUCTION

Polyploid breeding is important in poplar breeding because polyploid trees generally display characteristics of heterosis over normal diploids. For example, triploid white poplar trees not only grow fast but also have low lignin and high cellulose contents (Kang, 2006; Zhu, 1995). Thus, polyploid poplar varieties are beneficial for pulp and paper production and valuable for general environment protection. Triploid and aneuploid hybrids were found in the F_1 hybrid offspring of *Populus trichocarpa* × *P. deltoides* and the authors deduced they were derived from partial or complete nondisjunction during female gametogenesis (Bradshaw, 1993). A few triploids were found in the poplar cultivar clones in the section *Aigeiros* and can also be inferred from unreduced gametes (Zhang, 2004). So, utilization of 2n gametes, if possible, may be an effective way to obtain poplar triploids in a breeding program.

Many abnormal meiotic processes can lead to 2n gamete formation in plants, such as premeiotic doubling, omission of the first or second meiotic division, abnormal spindle, and abnormal cytokinesis (reviewed by Bretagnolle, 1995). The genetic constitution of 2n gametes generally divides into two categories, first-division restitution (FDR) and second-division restitution (SDR). A FDR 2n gamete contains non-sister chromatids, while a SDR 2n gamete contains two sister chromatids (Tang, 2002).

Poplar can produce 2n gametes naturally or by artificial induction (Huang, 2002; Johnsson, 1940; Mashkina, 1989). Elucidation of the cytological mechanisms of 2n gamete formation has seldom been carried out in poplar. Understanding of the cytological mechanism of 2n gamete formation is vital in their utilization in breeding because genetic consequences are different in terms of the transmission of parental heterozygosity and epistasis retained in the two types of 2n gametes. It has been calculated that a 2n gamete derived from FDR with crossing over will transmit roughly 80% of parental heterozygosity to its progeny while a SDR 2n gamete will transmit about 40% in potato (Hermsen 1984; Peloquin 1982).

The objective of this study was to detect and elucidate the mechanisms of 2n pollen formation in diploid poplar *Populus* × *euramericana* (Dode) Guinier and *P.* × *popularis* by cytological observation and using SSR DNA molecular markers. The *P.* × *popularis* were the hybrid offspring of *P. simonii* × (*P. pyramidalis* + *Salix matsudana* mixed pollen) and cultivated by the Chinese Academy of Forestry. The results from this research may offer a more effective method for polyploid breeding in poplar in the section *Aigeiros*.

2. MATERIALS AND METHODS

2.1. Plant materials

Four male (EA1, EA2, EA3 and EA4) trees and one female (A) tree of *Populus* × *euramericana* (Dode) Guinier and one male (P) tree of *P.* × *popularis* were used in this study. They were all confirmed as diploids. Controlled pollination between EA1 to EA 4 and A, respectively, and between P and A were performed in a greenhouse by using a typical "twig and pot water culture" (Jafari Mofidabadi, 1998) during the spring of 2004. Hybrids were produced using pollen radiation (2 100 Rad, 339.7 Rad min^{-1}) (Kang, 2000) and multiple pollinations. Young embryos of the hybrids were excised 25 to 35 d after the first pollination and cultured on ½ MS medium (half strength of MS macronutrients; other ingredients were not changed) with 30 gL^{-1} sucrose and 7 gL^{-1} agar. Two months later, hybrid plants were transplanted into the greenhouse and three weeks later they were transplanted into the field.

2.2. Microsporogenesis observation

Sixty anthers at different stages of development were randomly collected from twigs of each male tree in the greenhouse, and fixed in 3:1 alcohol: glacial acetic acid for 24 h. These anthers were examined for microsporogenesis using the standard acetocarmine squash method. The frequency of abnormal meiosis was calculated from more than 7 500 microsporocytes among 30 anthers, replicated 5 times, about 1 500 microsporocytes for each time. The expected 2n pollen rate was calculated as: (2×dyads +1×triads)/(2 × dyads + 3 × triads + 4 × tetrads) and the observed 2n pollen rate was the number of large pollen grains (d > 40 µm)/ 400 observed pollen grains under the microscope, replicated 5 times. A total of about 2 000 pollen grains was used for the calculation. To assess the divergence between expected and observed 2n pollen frequencies, a Chi-square (χ^2) test was performed.

2.3. Flow cytometry analyses

A polyploid scan was carried out for all 97 hybrid offspring derived from the five crosses (34 from A × EA1, 18 from A × EA2, 25 from A × EA3, 4 from A × EA4, 16 from A × P) using flow cytometric analysis. Crude nuclei were prepared from about 500 mg of fully expanded field-grown leaves by chopping each sample for 30 s with a sharp razor blade in 1 mL extraction buffer (Dolezel, 1989). A 1.0-mL extraction buffer was added and gently blended for about 2 min. The nuclei suspension was then filtered in a 50-µm nylon filter. The crude nuclei were sedimented by a 5-min centrifugation of the filtrate at 800 rpm. The pellet was resuspended in 1 mL of 50 µg mL^{-1} propidium iodide (PI)/Triton X-100 staining solution with RNaseA (Robinson, 2006) for at least 30 min in the dark. The samples were then analyzed on a FACSCalibur Flow Cytometer (BD Biosciences, USA) with an argon laser emitting at 488 nm for excitation of propidium iodide. The mean nuclear DNA content of each plant sample was based on 10 000 scanned nuclei. The chromosome number and 2C DNA content of the known diploid poplar tree 61# (2× = 38 and 2C DNA = 1.01 pg) were used as internal criteria. For each putative polyploid tree, the sample was independently characterized three times. Only measurements with coefficients of variation smaller than 8% were accepted. The ploidy levels of the hybrid offspring were inferred by comparing the DNA content of the sample with the internal diploid.

2.4. Chromosome counting

The fresh root apical meristem tissues from water-cultured cuttings for each of the 3 putative polyploidy trees were collected for

Table I. Expected and observed rates of 2n pollen grains.

Code of poplar	Sporads				Expected rate of 2n pollen %	Observed rate of 2n pollen %	χ^2
	Dyad	Triad	Tetrad	Total			
EA1	539	341	6 629	7 509	4.96	0.03	
EA2	163	1818	5 528	7 509	7.69	0.09	
EA3	689	682	6 354	7 725	7.14	10.08	60.128**
EA4	2189	4093	1 691	7 973	36.17	29.41	
P	308	484	7 308	8 100	3.15	2.35	

** Indicated significant difference between expected frequency of 2n pollen and the observed frequency of 2n pollen from pollen sample at $P < 0.01$.

somatic chromosome counts. Conventional squashes of root tip somatic cells were used under a light microscope equipped with a digital camera system. The somatic cell squashes were prepared from root apical meristem tissues as described by Li (1996) with minor modifications. Briefly, actively growing root tips were cut and placed into a saturated solution of 1,4-dichlorobenzene for 3 h at room temperature to shorten the chromosomes. Then the pretreated root tips were fixed in 3:1 ethanol-acetic acid solution for 24 h at room temperature. The fixed root tips were washed in flowing deionized water for 10 min, then digested in cytolase (95% ethanol: hydrochloric acid = 1:1) solution at 30 °C for 5 to 10 min. Chromosomes were stained with Carbol fuchsin. Thirty cell nuclei per individual were analyzed for chromosome counting.

2.5. SSR analysis

Leaf samples were collected from individual plants, placed in liquid nitrogen, freeze-dried and ground before storing in a freezer at −70 °C. DNA from 300-mg stored leaf samples of each of the parental trees and one 4× and its three 2× siblings (as controls) of the A × EA4 cross and two 3× and their three 2× siblings of the A × P cross was extracted using a DNeasy Plant Mini Kit (Tiangen Biotech (Beijing, China) Co.,Ltd.), following the manufacturer's protocol.

Gene Amp PCR reagent kits (Tiangen Biotech (Beijing, China) Co.,Ltd.) were used to perform a polymerase chain reaction (PCR). The genomic DNA sample was amplified using SSR primers in 25-μL reactions containing 2.5 μL of 1× GeneAmp PCR Buffer (10 mM Tris-Hcl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2), 1 μL of 10 mM SSR primers, respectively, 1 unit of Taq DNA polymerase, 2.0 μL of 25 mM dNTPs, and 30 ng of the template DNA sample. The PCR was conducted according to the method of Huang (2004).

The A with EA4 and A with P as two pairs of cross parents were detected with 120 SSR primer pairs from the International Populus Genome Consortium (IPGC, http://www.ornl.gov/sci/ipgc/ssr_resource.htm) to identify polymorphic loci, respectively. Then the identified polymorphic loci were used to detect the inheritance pattern of SSR loci among the one 4× hybrid and its three 2× siblings(as controls) of the A × EA cross and two 3× hybrids and their three 2× siblings of the A × P cross.

3. RESULTS

3.1. Cytological determination of 2n pollen formation

Normal microsporogenesis in *P.* × *euramericana* (Dode) Guinier and in *P.* × *popularis* is simultaneous cytokinesis, and formed a tetrad in a tetrahedral arrangement (Figs. 1a, 1b). However, parallel spindles, tripolar and fused spindles, and premature cytokinesis II (Figs. 1c–1f) were observed during the meiotic division. Tripolar and fused spindles were the variations of parallel spindles and could result in dyads and triads. Premature cytokinesis II could result in dyads.

The abnormal rates of meiosis in *Populus* × *euramericana* (Dode) Guinier and in *P.* × *popularis* were examined among more than 7 500 meiotic cells. The expected 2n pollen frequencies were not accordant with the observed 2n pollen frequencies by χ^2 test (Tab. I).

3.2. Detection of polyploid offspring of 2n pollen

The flow cytometry analyses of hybrid offspring did not find polyploid individuals among hybrids from the crosses A × EA1, A × EA2 and A × EA3. One tetraploid (4×, 75#) hybrid from the A × EA4 cross and two triploid(3×, 65# and 73#) hybrids from the A × P cross were detected. The DNA content value of 75# was the 2C value = 1.85 ± 0.13 pg (the second value is the standard deviation), 73# 2C value = 1.59±0.19 pg, 65# 2C value = 1.37 ± 0.02 pg and the internal criterion 61# 2C value = 1.01 pg.

It is not always possible to obtain an unambiguous picture of all the chromosomes of a poplar. The chromosome counting results of average chromosome number from 30 nuclei per individual was: for 75#, 2n = 68; for 73#, 2n = 56; for 65#, 2n = 55, and for the diploid control 61#, 2n = 36. The maximal chromosome number for those individuals was: for 75#, 2n = 76; for 73#, 2n = 57; for 65#, 2n = 57, and for the diploid control 61#, 2n = 38.

As shown in Figure 2a, 75# (4×) has about double the DNA content of the internal diploid control 61#. The chromosomes of 75# (Fig. 3a) were also about double those of the 61# ones (Fig. 3b). 65#(3×) and 73#(3×) had about 1.5 times more DNA content compared with 61#(2×) (Fig. 2b, 2c), respectively. They also had about 1.5 times more chromosomes than 61#(2×) (Figs. 3c, 3d).

3.3. Determination of mechanisms of 2n pollen formation by SSR markers

Five pairs of SSR primers with polymorphic loci (Tab. II) out of 120 primers were detected, and each locus had more

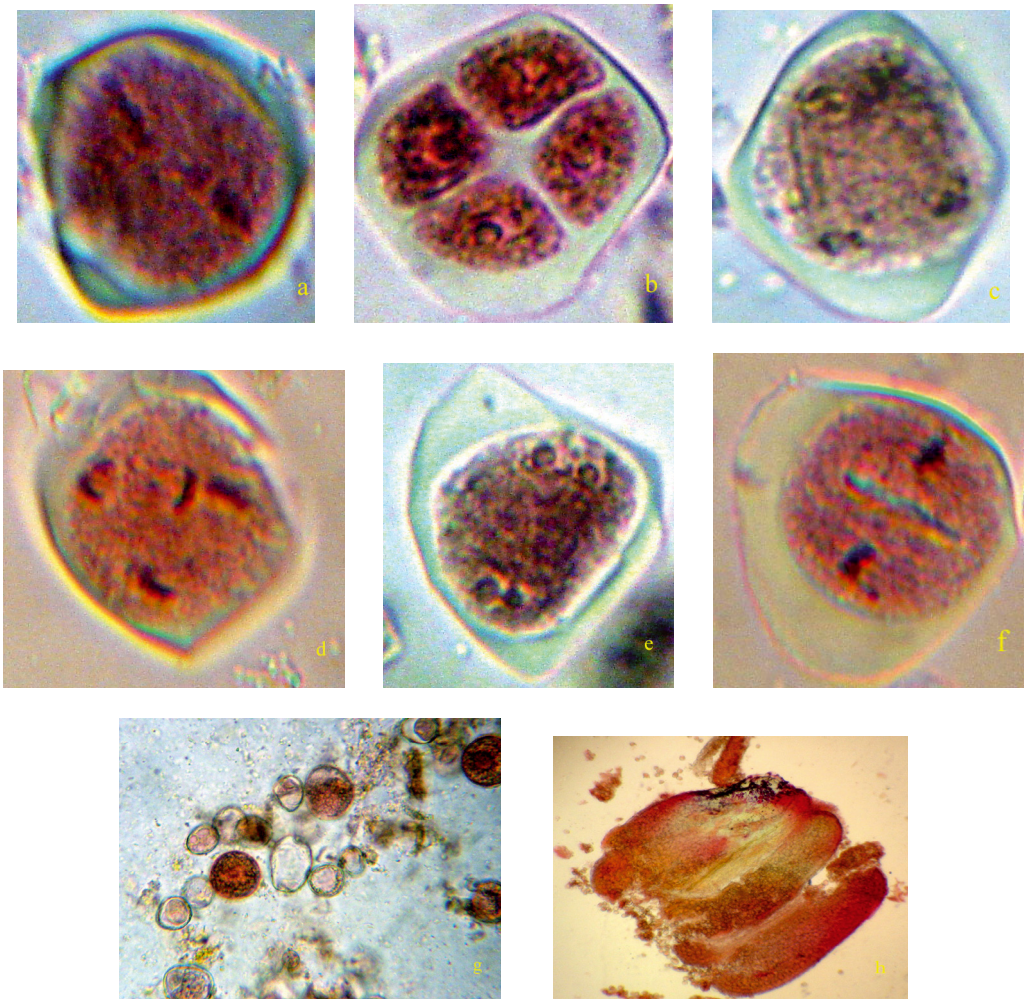

Figure 1. Cytological mechanisms of 2n pollen formation in *Populus* × *euramericana* (Dode) Guinier (2n = 2× = 38) and in *P.* × *popularis* (2n = 2× = 38). (a) Normal anaphase II, simultaneous cytokinesis; (b) normal tetrads; (c) anaphase II, parallel spindle; (d) anaphase II, tripolar spindle; (e) telophase II, fused spindle resulted in fusion of adjacent polar nuclei; (f) metaphase II, prematural cytokinesis; (g) aborted pollen before mature; (h) withered anther wall before matural opening.

Table II. SSR primers selected for cross A × EA4.

Code	Name	Left primer	Right primer	Length (bp)	Motif
14	GCPM_2453-1	ACACCAAGAGCTGTAGCATT	ACAACATggcctAACTCATC	200	ggt
41	GCPM_3345-1	AACTCTCagaAAAGGGTGGT	tacaaGgtagCCtggacATC	216	tta
47	GCPM_3559-1	CAGCGACCTAACAATTAACC	AGAGATAGGTggAGAAATTGAC	229	ag
68	GCPM_432-1	GGAGCACTTCATCCATAGTC	TGTGGACCTTAAACCACTTC	221	taa
105	ORPM_29	TGGTGATCCAGTTTTGGTGA	GTCCTTGCAAGCCATGAA	245	ac

than one different alleles (bands) between A and EA4. Another 3 pairs of SSR primers with polymorphic loci (Tab. III) out of the same 120 primers were also detected and each locus had more than one different alleles between A and P. They were used to analyze the hereditary pattern of allelic loci between the parent generation and the filial generation in the two crosses.

For the pollen parent *P.* × *euramericana* (Dode) Guinier EA4, both alleles of each of 6 polymorphic loci transmitted to its tetraploid offspring 75# while only a single one of those alleles transmitted to its diploid offspring 421#, 422# and 423# (Figs. 4a–4e and Tab. IV). This result was convincingly demonstrated on the SSR primer GCPM_2453-1 locus 1 (Fig. 4a and Tab. IV).

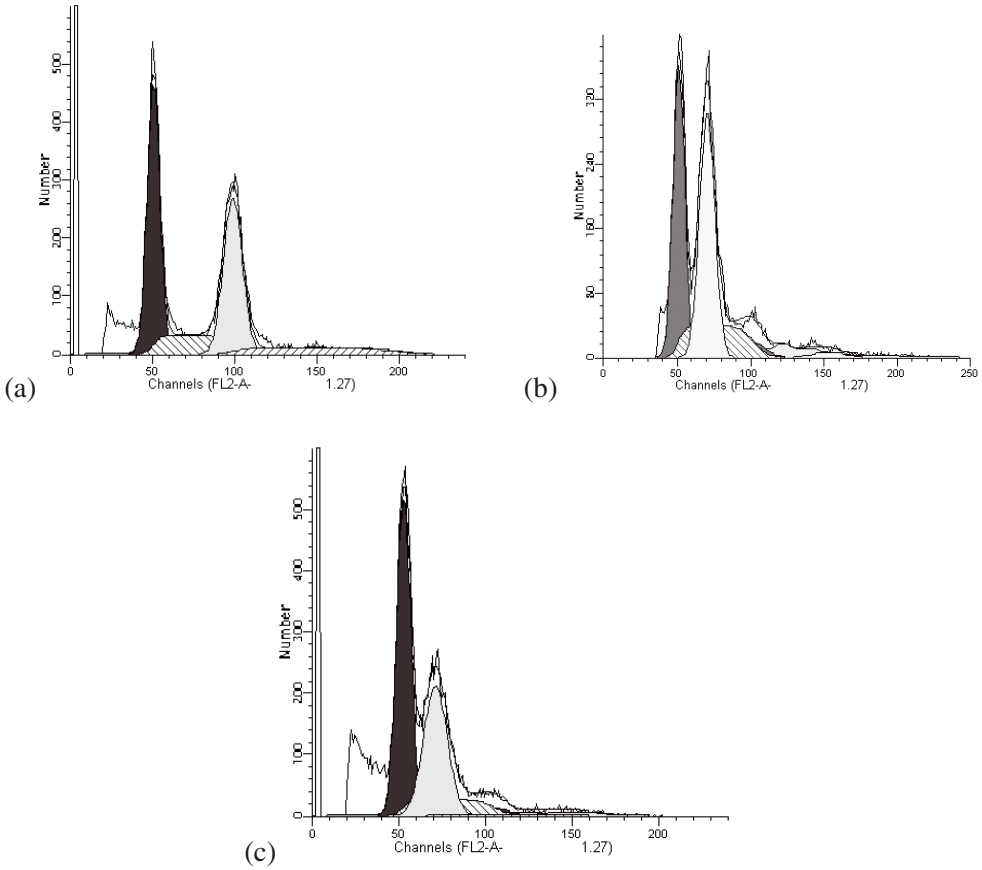

Figure 2. The DNA content of polyploid filiar generation of 2n pollen. (a) Flow cytometry of nuclear DNA content of tetraploid hybrid 75# and diploid control (left black peak for control (2×), right gray peak for 75# (4×)). (b) Flow cytometry of nuclear DNA content of triploid hybrid 65# and diploid control (left gray peak for control (2×), right white peak for 65# (3×)). (c) Flow cytometry of nuclear DNA content of triploid hybrid 73# and diploid control (left black peak for control (2×), right gray peak for 73# (3×)).

Table III. SSR primers selected for cross A × P.

Code	Name	Left primer	Right primer	Length (bp)	Motif
13	GCPM_2434-2	AGAGAGAGAggTATGAGGGC	gTTCggtaaagGtgatgga	203	tct
68	GCPM_432-1	GGAGCACTTCATCCATAGTC	TGTGGACCTTAAACCACTTC	221	taa
105	ORPM_29	TGGTGATCCAGTTTTGGTGA	GTCCTTGCAAGCCATGAA	245	ac

Table IV. Segregation of alleles at loci where the male. *Populus* × *euramericana* (Dode) Guinier parent EA4 is heterozygous.

Code	SSR primer	locus	EA4 (♂)	A (♀)	75# (4×)	421 (2×)	422 (2×)	423 (2×)
14	GCPM_2453-1	1	AB	CDE	ABCDE	ADE	ADE	ADE
41	GCPM_3345-1	2	AB	AC	ABC	AC	AC	AC
47	GCPM_3559-1	3	AB	B	AB	B	AB	AB
68	GCPM_432-1	4	AB	A	AB	A	A	A
105	ORPM_29	5	A0	C0	A0C0	C0	C0	C0
		6	B0	DE	B0DE	BE	BE	BE

These letters do not necessarily correspond to discrete alleles (e.g. the "E" band for GCPM_2453-1 may be the non-specific amplification) and ORPM_29 primer detects two loci, 0 means a null allele.

Table V. Segregation of alleles at loci where the male *P.* × *popularis* parent P is heterozygous.

Code	SSR primer	locus	P (♂)	A (♀)	65# (3×)	73# (3×)	321 (2×)	322 (2×)	323 (2×)
14	GCPM_2453-1	1	AB	CAD	CAB	CAB	AD	AD	AD
68	GCPM_432-1	2	AB	C	AC	BC	AC	AC	AC
105	ORPM_29	3	00	C0	C0	C0	00	C0	00
		4	AB	BD	AD	AD	BB	AB	AB

These letters do not necessarily correspond to discrete alleles(e.g. the "C" band for GCPM_2453-1 may be the non-specific amplification) and the ORPM_29 primer detects two loci, 0 means a null allele.

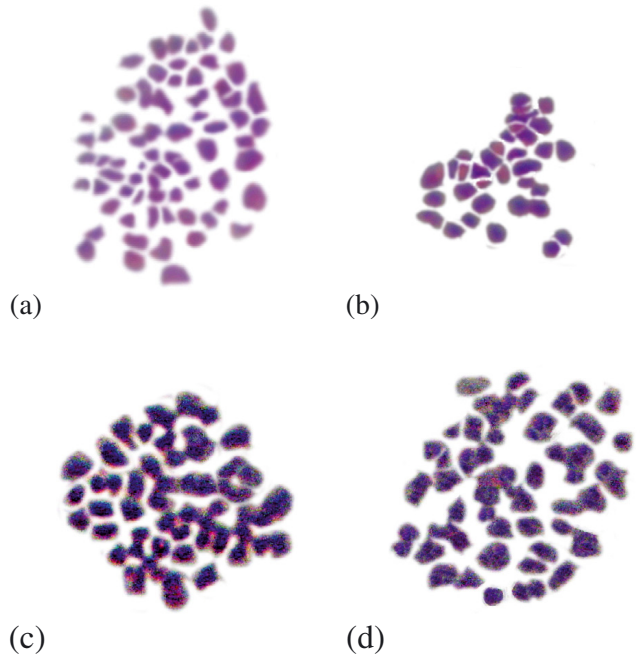

Figure 3. The chromosomes of polyploid poplar hybrids and their diploid control. (a) Tetraploid 75# (4×); (b) Diploid 61# (2×); (c) Triploid 65# (3×); (d) Tri-polyploid 73# (3×).

For the seed parent *P.* × *euramericana* (Dode) Guinier A in cross A × EA4, nearly the same hereditary situation was displayed. Both alleles of each of the 3 polymorphic loci (Tab. IV, locus 1, 2, 6) of *P.* × *euramericana* (Dode) Guinier A transmitted to its tetraploid offspring 75#, while only a single one of those alleles transmitted to its diploid offspring421#, 422# and 423#(Figs. 4a, 4b, 4e; Tab. IV).

For the pollen parent *P.* × *popularis* P in cross A × P, only one of the two alleles on each of the 4 polymorphic loci were transmitted to its triploidy offspring 65# and 73# as well as its diploidy offspring(Figs. 5a–5c and Tab. V). Triploid 65#, for example, inherited a single allele from each parent, exactly the same as its diploid siblings, although this allele differed from the one that was inherited by its triploid sibling 73# on primer GCPM_432-1 locus 2.

4. DISCUSSION

4.1. Mechanisms of 2n pollen formation

In this research, we studied the cytological mechanisms of 2n pollen formation of poplar, and also the genetic constitution of the 2n pollen, FDR versus SDR or both. Previously, studies of 2n pollen of poplar mainly focused on the discoveries (search for polyploidy), induction and utilization (Kang, 2006; Mashkina, 1989; Zhu, 1995). Bradshaw (1993) and Zhang (2004) inferred the triploid poplar derived from 2n gametes. Few authors have studied the mechanisms or the genetic constitution of 2n pollen of poplar.

Abnormal meiotic division led to 2n pollen formation. These abnormal meiotic phenomena were mainly due to the following: (1) Synaptic mutant: in potato, a synaptic mutant resulted in the homologous chromosomes failing to pair and cross-over rate reduction (Douches, 1988). This mechanism can form FDR 2n gametes finally. (2) Parallel spindle: in potato, spindles did not appear at the normal 60° angle, but paralleled the long axis of the mother cell and pulled the two groups of chromosomes to move to the same pole of the cell, and gathered them in one daughter cell at anaphase II. A dyad was formed and developed into two FDR 2n pollens (Conicella, 1991; Mok, 1975). (3) Fused spindle: at metaphase II, spindles gathered together to form a fused spindle. This mechanism led a mother cell to form a dyad and then two FDR 2n pollen were produced (Ramanna, 1979; Veilleux, 1982). (4) Tripolar spindle: at meiosis II, two spindles fused at one pole of the mother cell and another two spindles underwent normal division at the opposite pole. This formed one FDR 2n pollen and two 1n pollen consequently (Conicella, 1996). (5) Nuclear fusion: at anaphase II and telophase II, two adjacent daughter nuclei fused together to form two FDR 2n pollen (Chen, 1997). (6) Premature cytokinesis: cytoplasm divided early after meiosis I; however, sister chromatids divided normally. The result of this mechanism is the formation of two SDR 2n pollens (Mok, 1975). Many plants have more than one of the above mechanisms to form 2n pollen. Potato has at least four: parallel spindle, fused spindle, premature cytokinesis-1 and premature cytokinesis-2 (Oliveira, 1995). Persimmon (*Diospyros kaki* L.) has three: parallel spindle, fused spindle and tripolar spindle (Tang, 2002). The parallel spindle phenomenon was observed and postulated to form 2n pollen in Chinese white poplar (*Populus tomentosa* Carr.) (Kang, 2002).

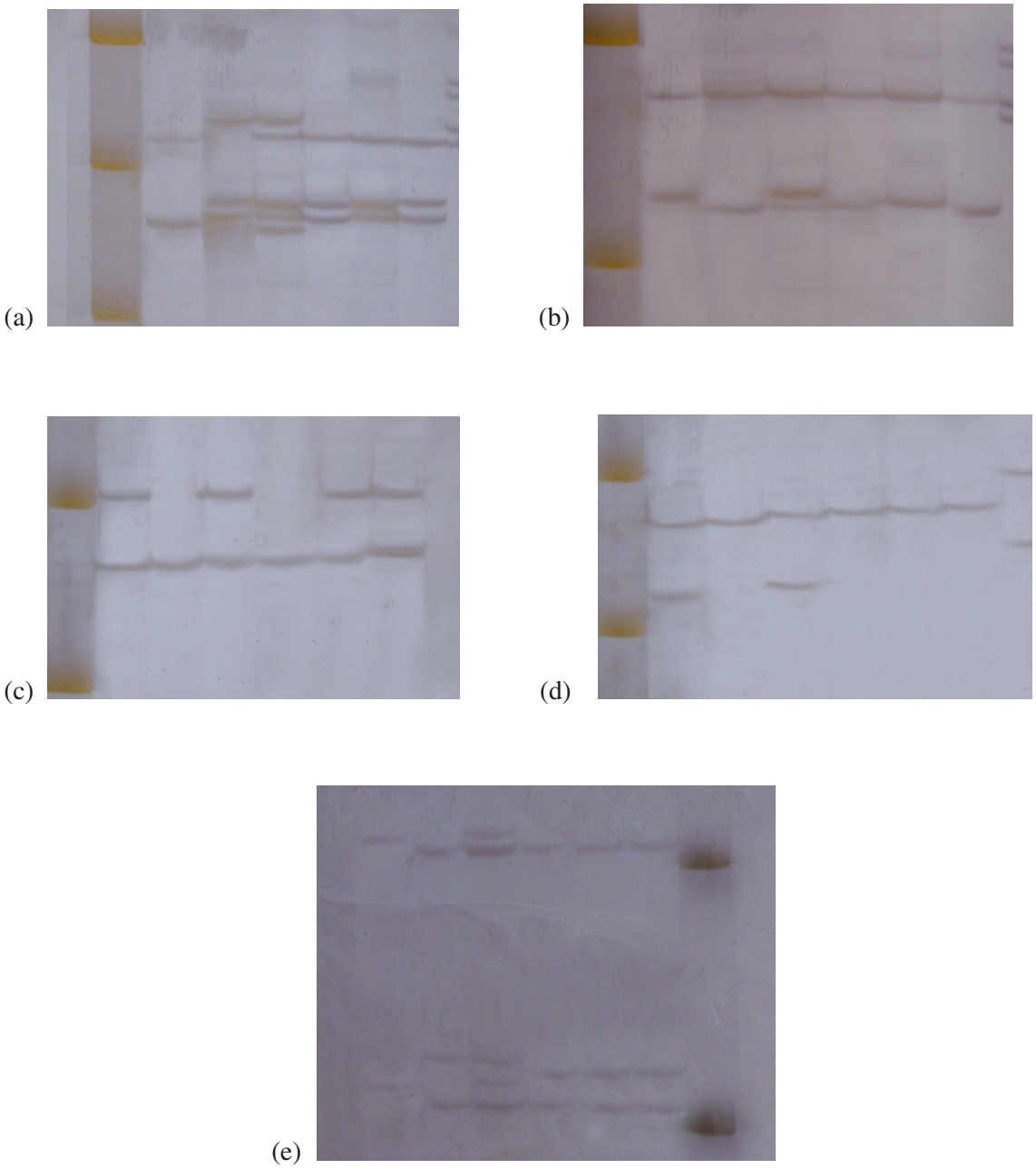

Figure 4. Autoradiogram of SSR bands from A (*Populus* × *euramericana* (Dode) Guinier), EA4 (*Populus* × *euramericana* (Dode) Guinier), and their tetraploid, diploid hybrids, showing that 2n pollen from EA4 was FDR genetic constitution. (a) Primer: 14: GCPM_2453-1, line order from left to right: Marker (from up to down: 400 bp, 300 bp, 200 bp), EA4 (from up to down: A band, B band), A (from up to down: C band, D band, E band), 75# (4×), 421 (2×), 422 (2×), 423 (2×). (b) Primer: 41: GCPM_3345-1, line order from left to right: Marker (from up to down: 300bp, 200bp), EA4 (from up to down: A band, B band), A (from up to down: A band, C band), 75# (4×), 421 (2×), 422 (2×), 423 (2×); (c) Primer: 47: GCPM_3559-1, line order from left to right: Marker (from up to down: 300 bp, 200 bp), EA4 (from up to down: A band, B band), A (from up to down: B band), 75# (4×), 421 (2×), 422 (2×), 423 (2×). (d) Primer: 68: GCPM_432-1, line order from left to right: Marker (from up to down: 300 bp, 200 bp), EA4 (from up to down: A band, B band), A (from up to down: A band), 75# (4×), 421 (2×), 422(2×), 423(2×). (e) Primer: 105: ORPM_29, line order from left to right: EA4 (from up to down: A band, B band), A (from up to down: C band, D band, E band), 75# (4×), 421 (2×), 422 (2×), 423 (2×), Marker (from up to down: 300 bp, 200 bp).

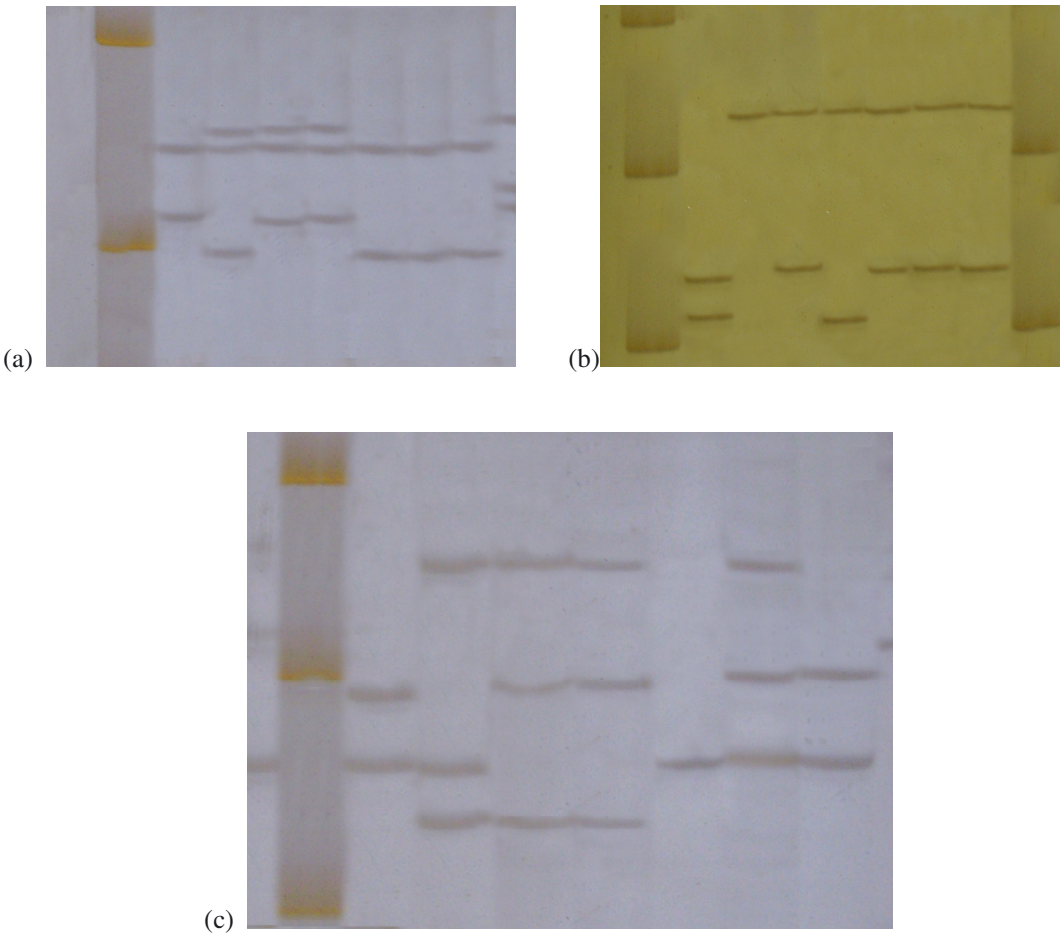

Figure 5. Autoradiogram of SSR bands from A (*Populus* × *euramericana* (Dode) Guinier), P (*P.* × *popularis*), and their triploid/diploid hybrids, showing that 2n pollen from P was SDR genetic constitution. (a) Primer: 13: GCPM_2434-2, line order from left to right: Marker (from up to down: 300 bp, 200 bp), P (from up to down: A band, B band), A (from up to down: C band, A band, D band), 65# (3×), 73# (3×), 321 (2×), 322 (2×), 323 (2×). (b) Primer: 68: GCPM_432-1, line order from left to right: Marker (from up to down: 300 bp, 200 bp, 100 bp), P (from up to down: A band, B band), A (from up to down: C band), 65# (3×), 73# (3×), 321 (2×), 322 (2×), 323 (2×). (c) Primer: 105: ORPM_29, line order from left to right: Marker (from up to down: 400bp, 300 bp, 200 bp), P (from up to down: A band, B band), A (from up to down: C band, B band, D band), 65# (3×), 73# (3×), 321 (2×), 322 (2×), 323 (2×).

In this study, we observed four of these abnormalities: parallel spindle, fused spindle, tripolar spindle and premature cytokinesis in *P.* × *euramericana* (Dode) Guinier, as well as three of these abnormalities: parallel spindle, fused spindle and prematural cytokinesis in *P.* × *popularis*. These abnormalities can be classified as having abnormal spindle fiber orientation during meiosis II and early division of cytoplasm after meiosis I. Abnormal spindle fiber orientation including tripolar spindles, fused spindles and parallel spindles could result in dyads and triads. The dyad developed into two 2n pollen and the triad grew into one 2n pollen and two 1n pollen. The 2n pollen formed in these ways were genetically equivalent to FDR. Premature cytokinesis II could result in dyads. The 2n pollen grains formed via this abnormal approach were genetically equivalent to SDR.

The tetraploid and triploid offspring were detected by flow cytometry, and chromosome counting from the hybrids of two controlled crosses confirmed that the 2n pollen from *P.* × *euramericana* (Dode) Guinier and *P.* × *popularis* do exist and can be fertilized to form polyploid hybrids.

The locus of the ORPM_29 primer has already been mapped on the *Populus* framework map (Tuskan et al., 2004) and the authors found that some SSR primer pairs produced multiple independent loci. In our research, the ORPM_29 primer pair detects two loci. It is possible that a primer pair detects multuple SSR loci since poplar is considered as an ancient polyploid and large duplications are found in the poplar genome(Sterck, 2005).

Further comparisons of SSR polymorphic loci between parents and their polyploid and diploid offspring have elucidated that *P.* × *euramericana* (Dode) Guinier could produce FDR 2n pollen and *P.* × *popularis* could produce SDR 2n pollen. So, the 2n pollens of poplar had FDR and/or SDR genetic constitution.

4.2. Biological reasons for high percentage of 2n pollen

In a previous study on persimmon, the expected 2n pollen percentage accorded with the observed 2n pollen percentage (Tang, 2002). However, the expected 2n pollen percentage did not accord with the observed 2n pollen percentage in this research (Tab. I). This finding indicated that the process from meiotic division to pollen release was not a random course. It involved the genotype of each tree and its development environment besides statistical error.

Generally, the abnormal pollen rate was under 10%, such as EA1,EA2,EA3 and P. However, EA4 had a high percentage of 2n pollen (Tab. I). Further observation in EA4 found that some microspores aborted (could not be stained) before maturity and some of the mature anther walls withered instead of opening (Figs. 1g, 1h). The aborted pollen grains were most of the 1n pollen (small pollen) and a few of the 2n pollen (big pollen). Most 2n pollen grains were successfully released. This release resulted in a high percentage of 2n pollen in the collected pollen (observed pollen). The explanation for this finding could be the imbalance of genetic material in the aborted pollen because of the unique genotype of hybrid origin of *P.* × *euramericana* (Dode) Guinier and the abnormal environmental factors during its meiosis process, such as high temperature.

4.3. Role of radiation during the fertilization of 2n pollen

Mixed pollen had to be used for pollination because the static electric adsorption among pollens made it very hard to separate 2n pollen from 1n pollen. The 1n pollen germinated earlier and grew faster than 2n pollen on the poplar stigma. Thus, it fertilized the female gamete while the 2n pollen had no such opportunity. This result may well explain why in the normal control cross only the triploids inherited both maternal alleles along with a single paternal allele (Bradshaw, 1993). The proper dose of radiation can restrain the 1n pollen growth while the 2n pollen can grow normally. Kang (2000) found the proper dose of radiation for white poplar was 1 470 to 1 680 rad and got some triploid white poplars. In this research, we radiated the mixed pollen with a series of doses (1 200, 1 500, 1 800, 2 100, 2 400, 2 700 and 3 000 rad) and investigated the germination of 1n pollen and 2n pollen(data not shown) and the seed germination (data not shown). We found the proper radiation dose for poplar in the section *Aigeiros* was 1 800 to 2 400 rad. The three polyploid trees were the offspring of pollen with 2 100 rad of radiation. SSR loci analysis showed the tetraploidy 75# was derived from the paternal 2n gamete (Fig. 4 and Tab. IV). So, the proper dose of radiation can assist 2n pollen instead of 1n pollen to fertilize the female gamete successfully.

4.4. Formation of 2n female gametes in poplars of the section *Aigeiros*

The tetraploid 75# had all the paternal and maternal parent allele bands and was totally different from its diploid siblings (Figs. 4a– 4e and Tab. IV). These results suggested that the 2n pollen from the EA4 paternal parent was transmitted via the FDR formation mechanism during hybridization with the A maternal parent. These results also demonstrated that maternal parent A could generate 2n ovum naturally during hybridization with paternal parent EA4. Natural 2n female gametes of poplar in the section *Aigeiros* have not been reported before. Our research indicated that natural 2n female gametes did exist and were fertile and could be FDR genetic constitution. Further investigations are required to determine their cytological mechanisms and utilization. The possible approaches might be investigation of the female gametogenesis process but this would be more difficult than for male gametogenesis.

4.5. Polyploidy identification using molecular markers

For polyploidy identification, the most directly experimental evidence is based on the chromosome number. However, the chromosome counting method is very laborious and time-consuming. It is very difficult to get a clear and not overlapped chromosome picture in poplar because of its small chromosomes and their high number. Recently, some codominant molecular markers were used to identify polyploidy. Bradshaw (1993) used RFLPs to detect triploidy in poplar. Besnard (2008) applied SSRs to analyze the Olive complex as an alternative approach to chromosome counting to determine polyploidy level of trees. However, SSRs or RFLPs cannot detect polyploidy via SDR 2n gametes according to our research (Fig. 5 and Tab. V). They can identify polyploidy via FDR 2n gametes (Fig. 4 and Tab IV). So, SSRs or RFLPs can only distinguish part of the polyploids.

4.6. Implications of polyploidy for genetic research and tree breeding

The formation of 2n gametes has played an important role during the evolution of plant sexual polyploidization (Thompson, 1992). The EST data suggested that poplar is an ancient polyploidy (Sterck, 2005). This research proved that poplar 2n gametes can be fertilized to form new polyploids, which is valuable information for our understanding of the evolution history of poplar species. The approach used in this study can be used to further study the origin of poplar and other plant species.

P. × *euramericana* (Dode) Guinier is an interspecific hybrid in the section *Aigeiros* and *P.* × *popularis* is an intersectional hybrid between the section *Aigeiros* and the section *Tacamahaca*. The result that 2n pollen can produce polyploids under controlled pollination in these two hybrids will open a new approach for polyploid breeding in those two sections.

The discovery of natural 2n female gametes will have great breeding value for poplar in the section *Aigeiros*. The 2n female gamete has natural superiority over the 2n male gamete because it does not need to compete with other 1n female gametes during fertilization. This discovery offers the possibility

of poplar polyploid breeding in the section *Aigeiros* via 2n female gametes.

The identification of the genetic constitution of 2n gametes in this paper is a good starting point for future research on the utilization of 2n gametes. The FDR 2n gamete has different genetic effects from the SRD 2n gamete; thus, SSR detection of FDR or SDR 2n gametes provides a new method of early selection in poplar improvement programs.

Triploid poplar clones in the section *Aigeiros* have already played an important role in poplar plantations, especially in China (Zhang, 2004). Thus, utilization of 2n gametes to produce polyploid poplar clones in the section *Aigeiros* has great potential.

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