Abnormal meiosis in an intersectional allotriploid of *Populus* L. and segregation of ploidy levels in 2x × 3x progeny

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Abstract

Triploid plants are usually highly aborted owing to unbalanced meiotic chromosome segregation, but limited viable gametes can participate in the transition to different ploidy levels. In this study, numerous meiotic abnormalities were found with high frequency in an intersectional allotriploid poplar (*Populus alba × P. berolinensis* ‘Yinzhong’), including univalents, precocious chromosome migration, lagging chromosomes, chromosome bridges, micronuclei, and precocious cytokinesis, indicating high genetic imbalance in this allotriploid. Some micronuclei trigger mini-spindle formation in metaphase II and participate in cytokinesis to form polyads with microcytes. Unbalanced chromosome segregation and chromosome elimination resulted in the formation of microspores with aneuploid chromosome sets. Fusion of sister nuclei occurs in microsporocytes with precocious cytokinesis, which could form second meiotic division restitution (SDR)-type gametes. However, SDR-type gametes likely contain incomplete chromosome sets due to unbalanced segregation of homologous chromosomes during the first meiotic division in triploids. Misorientation of spindles during the second meiotic division, such as fused and tripolar spindles with low frequency, could result in the formation of first meiotic division restitution (FDR)-type unreduced gametes, which most likely contain three complete chromosome sets. Although ‘Yinzhong’ yields 88.7% stainable pollen grains with wide diameter variation from 23.9 to 61.3 μm, the pollen viability is poor (2.78% ± 0.38). A cross of ‘Yinzhong’ pollen with a diploid female clone produced progeny with extensive segregation of ploidy levels, including 29 diploids, 18 triploids, 4 tetraploids, and 48 aneuploids, suggesting the formation of viable aneuploidy and unreduced pollen in ‘Yinzhong’. Individuals with different chromosome compositions are potential to analyze chromosomal function and to integrate the chromosomal dosage variation into breeding programs of *Populus*.
Introduction

Polyploidization is an important driving force in plant speciation and evolution [1,2]. During the evolution process of plants, crosses between different ploidy levels contribute to chromosomal introgression and genetic diversity [3–5]. Interploidy hybridization is also a useful pathway for the creation of new germplasms and crops varieties [6,7]. However, polyploids with odd numbers of chromosome sets, especially triploids, are usually unstable, which can either be sterile to be aborted in the evolution process due to their reproductive barrier, or contribute to production of polyploid gametes, depending on the species [8–10].

Polyploids with odd ploidy levels have difficulty carrying out regular meiosis owing to their genetic imbalance. Irregular chromosome pairing and unequal meiotic division commonly occur, resulting in unbalanced chromosome segregation and chromosome elimination [11]. Therefore, each spore after asymmetrical cytokinesis contains incomplete chromosome sets, i.e., aneuploidy, and the aneuploid unbalanced chromosomal composition causes high levels of sterility in gametes. Nevertheless, some aneuploid gametes can take part in fertilization to produce aneuploid progeny [12–16]. Additionally, meiotic nuclear division restitution in odd polyploids can also produce unreduced viable gametes, although the production of unreduced gametes is low frequency [17]. The production of viable aneuploidy and unreduced gametes suggests that polyploids with odd ploidy levels also have value in acting as a bridge for the induction of chromosomal number variation [17–19].

Aneuploids are particularly valuable for chromosome engineering breeding and cytogenetic research. Monosomic, nullisomic, trisomic and tetrasomic wheat lines have all been established to analyze chromosomal function and evolutionary relationships and to locate particular genes [20]. In *Arabidopsis thaliana* (L.) heynh., triploids have produced a group of aneuploids by self-pollination [21], and phenotypic variation is strongly associated with chromosome composition and dosage variation in *A. thaliana* aneuploids [22]. Chromosome manipulation based on aneuploids has attracted increasing attention for improving efficiency in plant breeding.

Poplars are dioecious, species widely cultivated across the Northern Hemisphere as an important source of fiber, biomass and lumber [23]. They also have been considered a model tree for molecular biological and genetic research in woody plants, owing to the relatively small genome size and short reproductive cycle [24]. Utilization of chromosome number variation is an important approach in *Populus* breeding programs, and many allotriploid poplar cultivars, including triploid *P. tomentosa* Carr., *P. alba* L. × *P. berolinensis* Dippel, ‘Yinzhong’, *P. tremula* L. × *P. tremuloides* Michx. ‘Astria’, *P. × euramericana* (Dode) Guiner CL. ‘Zhong-\(\text{-}46\)’, *P. × canadensis* Moench cv. ‘Sacrau 79’, and *P. × euramericana* (Dode) Guiner cv. ‘Wuhei-1’ [25–28], have been widely used in plantations owing to their favorable growth performance and pulpwood characteristics [29,30]. A series of methods for polyploid induction have been developed to increase the induction efficiency and heterozygosity of polyploid progeny [31–37]. However, chromosomal function and the relationship between chromosome composition and commercial phenotypic variation are poorly characterized in *Populus* owing to the lack of aneuploid germplasms.

*P. alba* × *P. berolinensis* ‘Yinzhong’ is an artificially synthesized male allotriploid hybrid. The parents *P. alba* and *P. berolinensis* are both diploid, belonging to two different sections (sect. *Populus* and sect. *Aigeiros*, respectively) within the genus *Populus* [38]. It is speculated that ‘Yinzhong’ is derived from the union between a normal gamete and a spontaneous unreduced gamete from one of the parents [26]. Generally, the sect. *Populus* is reproductively isolated from other sections [39]. Internal transcribed spacer (ITS) sequence analysis showed that sect. *Aigeiros* and sect. *Populus* had different evolution ratios [40], suggesting relatively large
genetic divergence between the two sections. In this study, we analyzed meiotic abnormalities and pollination variation of the intersectional allotriploid 'Yinzhong'. A cross between the triploid and a diploid female clone was conducted to produce progeny with ploidy level segregation, aiming to further analyze chromosomal function in *Populus* and integrate the potential for chromosomal dosage variation into *Populus* breeding programs.

**Materials and methods**

**Plant materials**

Floral branches of allotriploid 'Yinzhong' were collected from a plantation in Tongliao City (Inner Mongolia Autonomous Region, People’s Republic of China). The plantation was established by the Forestry Research Institute of Tongliao City, the Inner Mongolia Autonomous Region, People’s Republic of China. We got the permission from the institute to collect the branches for research. For sexual hybridization, a diploid female clone of *P. tomentosa* Carr. × *P. bolleana* Lauche, TB03 (2n = 2x = 38), collected from the campus of China Agricultural University, was used as the female parent. TB03 has usually been used as the female parent in test crosses of section Populus, because it has good fertility and no spontaneous unreduced egg production was found [41,42]. Floral branches of 'Yinzhong' and TB03 were cultured in water in a greenhouse (10–20˚C) to force floral development.

**Cytological observations of microsporogenesis**

Floral buds of 'Yinzhong' undergoing meiotic development were collected and immediately fixed in an ethanol-acetic acid fixative solution (3:1 v/v) at 4˚C for 24 h, and then they were stored in 70% ethanol until analysis.

Microsporocytes were pressed out of anthers and stained in 1% aceto-carmine on a microscope slide. To increase chromosome stainability, preparations were heated slightly over a flame before observation. All preparations were observed using a microscope (BX51: Olympus), and photos were taken with an attached video camera (DP70: Olympus). Meiotic pairing configurations were recorded based on 21 early metaphase I cells. The frequency of precocious chromosome migration in metaphase I, laggard chromosomes or bridge formation in anaphase I, the presence of micronuclei in telophase I and II, and the number of parallel, fused and tripolar spindles in anaphase II were analyzed together with the variation of sporad types. The meiotic index was calculated as the ratio of the number of normal sporads to the total number of sporads analyzed per hybrid multiplied by 100 [43].

**Indirect immunofluorescence of microtubules**

Indirect immunofluorescence testing was used to analyze the change in the microtubule cytoskeleton according to Wang et al. [44,45]. Anthers were fixed in 4% paraformaldehyde in PEM buffer (50 mM Pipes, 5 mM EGTA, 2 mM MgSO₄, pH 6.9) for 45 min. After three 5-min rinses in PEM buffer, the anthers were treated with 10% dimethylsulfoxide (DMSO) and 1% Triton X-100 for 15 min each. The anthers were then rinsed in PEM buffer, followed by three 5-min rinses in PBS buffer (137 mM NaCl, 2.7 mM KCl, 7 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3). Subsequently, microsporocytes were squeezed out from the anthers and transferred to a slide coated with 0.1% poly-L-lysine (Sigma-P1274: Sigma-Aldrich, St Louis, MO, USA). The cells were incubated in a monoclonal anti-α-tubulin antibody (Sigma-T9026: Sigma-Aldrich) diluted 1:100 with the PBS buffer for 2 h at 37˚C in a moist chamber. Following further washing with the PBS buffer, the microsporocytes were incubated in FITC-conjugated antimouse IgG (Sigma-F0257: Sigma-Aldrich) diluted 1:100 with the PBS buffer for 2 h at 37˚C in a dark
chamber. After a final wash in the PBS buffer, the microsporocytes were mounted using mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vectashield® H-1200; Vector Laboratories, Inc., Burlingame, CA, USA). The preparations were observed and photographed with a confocal laser scanning microscope (TCS-SP5: Leica, Solms, Germany).

**Analysis of pollen size and viability**

A pollen sample was collected from freshly dehiscent anthers on male catkins. The fresh pollen sample was mounted immediately in a drop of 1% aceto-carmine on a microscope slide. Empty and shrunken pollen grains were scored. The diameter of 1,500 stained spherical pollen grains was measured using an ocular micrometer to establish a histogram of the frequency distribution.

The fresh pollen samples were germinated on agar medium containing 15 g L\(^{-1}\) agar, 120 g L\(^{-1}\) sucrose, 60 mg L\(^{-1}\) boric acid and 80 mg L\(^{-1}\) calcium nitrate to test its viability. After 24 h of culturing, the germination rates were recorded based on five replications.

**Crossing experiment**

When catkins of female parent TB03 acquired stigma receptivity, they were pollinated with fresh ‘Yinzhong’ pollen in a greenhouse. After the catkins matured, seeds were collected and germinated in sterile soil, and the seedlings were transplanted to the field when they reached approximately 30 cm in height.

**Analysis of ploidy levels**

Determination of the ploidy level of ‘Yinzhong’ was conducted by somatic chromosome counting. Stem tips were removed from seedlings and pretreated with a saturated solution of paradichlorobenzene for 4 h at 25˚C. The materials were then fixed in fresh Farmer’s solution (ethanol/acetic acid, 3:1) for 24 h at 4˚C and then hydrolyzed in 38% HCl/ethanol (1:1) for 25 min at room temperature. After washing in distilled water three times for 15 min, the hydrolyzed materials were squashed in a carbol fuchsin solution on a microscope slide. The preparation was observed using an Olympus BX51 microscope.

For the progeny, when seedlings had more than three leaves, a young leaf was used to analyze the ploidy level by flow cytometry. Flow cytometric analysis was conducted according to Galbraith et al. [46]. Briefly, young leaves of the seedlings were chopped in modified Galbraith’s buffer (45 mM MgCl\(_2\)-6H\(_2\)O, 20 mM MOPS, 30 mM sodium citrate, 0.5% Triton X-100, 1% PVP-10, pH 7.0) using a sharp razor blade on ice. Subsequently, the nuclear suspension was filtered through a 40-μm nylon mesh to remove large debris. Nuclei were stained with 50 μg mL\(^{-1}\) propidium iodide with RNase at 50 μg mL\(^{-1}\). After incubation on ice for 30 min in the dark, samples were analyzed with a BD FACSCalibur flow cytometer. TB03 and ‘Yinzhong’ were used as diploid and triploid criteria, respectively. The somatic chromosome numbers of some seedlings were also checked using the above method.

**Results**

**Chromosome counting of triploid ‘Yinzhong’**

Although ‘Yinzhong’ was previously determined to be a triploid by Chen et al. [26], the ploidy level of the male clone used in this study was detected to avoid errors. Chromosome counting showed that ‘Yinzhong’ had 57 chromosomes with 3 large ones corresponding to chromosome I of Populus (Fig 1); this indicates that the male clone is a triploid.
Abnormal chromosome behaviors during microsporogenesis

Chromosome pairing was examined at early metaphase I, showing the existence of univalents (I), bivalents (II), and trivalents (III) (Table 1, Fig 2A). The average meiotic configuration was 15.2I + 14.0II + 4.6III. The univalents ranged from 6 to 30, indicating partial failure of uniparental chromosomal pairing and high genomic heterozygosity of ‘Yinzhong’. The trivalents ranged from 1 to 7, suggesting some degree of homology between the two parents of ‘Yinzhong’, although the parents belong to two different sections.

A large number of meiotic abnormalities were recorded in this allotriploid ‘Yinzhong’ (Table 2), showing unbalanced chromosome segregation. At diakinesis, several univalents and multivalents were found (Fig 2A), suggesting aberrant synapsis in this allotriploid. At metaphase I, abnormal chromosome disposition was observed (Fig 2B), and as a result, the spindle formed an abnormal structure (Fig 2C). Moreover, some chromosomes migrated precociously to the poles in almost all cells (Fig 2D). In anaphase I, lagging chromosomes (Fig 2E) and chromosome bridges were found with frequencies of 59.07% and 6.56%, respectively. Some lagging

![Image](https://doi.org/10.1371/journal.pone.0181767.g001)

**Fig 1.** Somatic chromosome counting (2n = 3x = 57) of *P. alba × P. berolinensis ‘Yinzhong’*. Arrows indicate the chromosome I. Bar is equal to 10 μm.

![Table 1](https://doi.org/10.1371/journal.pone.0181767.t001)

**Table 1.** Meiotic pairing configuration of the allotriploid *P. alba × P. berolinensis ‘Yinzhong’*

| Clone  | Chromosome number | No. of early metaphase I cells | Pairing configuration |
|--------|------------------|-------------------------------|-----------------------|
|        |                  |                               | Univalents            |
|        |                  |                               | Bivalents             |
|        |                  |                               | Trivalents            |
| Yinzhong | 57               | 21                            | 15.2 (6–30)           |
|         |                  |                               | 14.0 (10–18)          |
|         |                  |                               | 4.6 (1–7)             |

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chromosomes could form micronuclei at telophase I (Fig 2F) with a frequency of 68.87%. During the second meiotic division, micronuclei located in the cytoplasm still were observed (Fig 2G). In anaphase II and telophase II, lagging chromosomes and chromosome bridges were also found (Fig 2H and 2I).

**Misorientation of spindles and aberrant cytokinesis**

Precocious cytokinesis was observed in some microsporocytes after the first meiotic division because the cell plate formed in advance to separate the two daughter nuclei into different cytoplasm domains (Fig 3A). During the second meiotic division, the chromosomes in different domains could be positioned in the equatorial region and separated to two different poles to form daughter nuclei (Fig 3B–3D). The frequency of precocious cytokinesis increased from 21.03% at prophase II to 37.12% at telophase II, suggesting that precocious cytokinesis also occurred during the second meiotic division. In some cells with precocious cytokinesis,
secondary cell plates formed between sister nuclei to achieve second cytokinesis at telophase II (Fig 3E). The formation of phragmoplasts between sister nuclei defined the position of the cell plate and predicted the occurrence of cytokinesis (Fig 3F). However, fusion of sister nuclei located in the same cytoplasm domain was also observed in some microsporocytes when secondary cell plates failed to form (Fig 3F and 3G), resulting in the formation of dyads and triads in meiotic products (Fig 3H and 3I).

In some microsporocytes without precocious cytokinesis during the second meiotic division, misoriented spindles, including parallel, fused, and tripolar spindles, were observed (Fig 4A–4C). Fluorescence location analysis showed that there was an organelle layer positioned between two spindles of parallel type (Fig 4D), which separated the spindle domains. In metaphase II, the micronucleus formed isolated chromosomes located beyond the primary chromosome sets (Fig 4E and 4G). The isolated chromosomes led to the formation of minispindles in the cytoplasm (Fig 4F and 4H), which gave rise to microcytes in the meiotic products (Fig 4I). The chromosomes in microcytes were eliminated from daughter nuclei. Among the meiotic products, 1.96% dyads, 3.67% triads and 7.95% polyads were recorded. The meiotic index was 86.42%.

Table 2. Meiotic abnormalities in the allotriploid P. alba × P. berolinensis ‘Yinzhong’.

| Phase          | No. of analyzed cells | No. of abnormal cells (%) | Abnormalities                          | No. of each abnormality |
|----------------|-----------------------|---------------------------|----------------------------------------|-------------------------|
| Metaphase I    | 1,026                 | 1,003 (97.76)             | Precocious chromosome migration        | 1,003                   |
| Anaphase I     | 259                   | 162 (62.55)               | Lagging chromosomes                    | 153                     |
|                |                       |                           | Chromosome bridge                      | 17                      |
| Telophase I    | 379                   | 261 (68.87)               | Micronuclei                            | 261                     |
| Prophase II    | 233                   | 201 (86.27)               | Micronuclei                            | 174                     |
|                |                       |                           | Precocious cytokinesis                 | 49                      |
| Anaphase II    | 468                   | 209 (44.66)               | Parallel spindles                      | 5                       |
|                |                       |                           | Fused spindles                         | 11                      |
|                |                       |                           | Tripolar spindles                      | 8                       |
|                |                       |                           | Precocious cytokinesis                 | 185                     |
| Telophase II   | 590                   | 468 (79.32)               | Micronuclei                            | 328                     |
|                |                       |                           | Precocious cytokinesis                 | 219                     |
| Meiotic product| 1,936                 | 263 (13.58)               | Dyad                                   | 38                      |
|                |                       |                           | Triad                                  | 71                      |
|                |                       |                           | Polyad                                 | 154                     |

Meiotic index 86.42

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Size variation and viability of pollen grains

In newly collected pollen samples, approximately 11.3% of the pollen grains were empty and shrunken (Fig 5A). The diameter of the stainable pollen grains ranged from 23.9 to 61.3 μm with an average of 39.6 μm (±4.2). The pollen diameters approximately followed a Gaussian frequency distribution (Fig 5B), suggesting that most ‘Yinzhong’ pollen grains are aneuploid owing to completion of triploid meiosis, although a few large pollen grains may be unreduced. A germination test showed that just 2.78% (±0.38) of pollen grains germinated for this allotriploid, indicating that the viability of ‘Yinzhong’ pollen is poor. The tubes of the germinated pollen grains underwent normal growth on medium (Fig 5C).
Segregation of ploidy levels among progeny

Fresh pollen from the allotriploid 'Yinzhong' was used to pollinate a highly fertile TB03 female parent. Only 691 seeds were collected from 22 catkins, indicating the low pollen fertility of the allotriploid. After seed sowing, 99 seedlings were obtained. The progeny exhibited extensive segregation of ploidy levels, including 29 diploids, 18 triploids, 4 tetraploids and 48 aneuploids (Fig 6A), suggesting that some aneuploid pollen grains of 'Yinzhong' are fertile and that unreduced pollen took part in the fertilization to produce seeds. Based on chromosome number, 39 of the aneuploids were classified into “aneuploid I” (Fig 6B–6D) with a chromosome number between 38 and 57, and 9 aneuploids were classified into “aneuploid II” (Fig 6E) with a chromosome number between 57 and 76.

Discussion

Meiosis in polyploids is characterized by complex chromosomal pairing and chromosome segregation. Imbalanced chromosome pairing and segregation result in the production of lagging

Fig 3. Precocious cytokinesis and nuclear fusion. a–d. Precocious cytokinesis in prophase II (a), metaphase II (b), anaphase II (c) and telophase II (d); e. Completion of successive cytokinesis showing formation of secondary cell plates between sister nuclei (arrows); f. Telophase II with precocious cytokinesis showing a fused nucleus in a daughter cell and separate nuclei by phragmoplast (arrow) in the other cell; g. Telophase II with precocious cytokinesis showing nuclear fusion in both daughter cells; h. Triad with a fused nucleus; i. Dyad with two fused nuclei. Bars are equal to 10 μm.

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chromosomes and micronuclei [11,47]. In an allotriploid white poplar, multivalents, lagging chromosomes and micronuclei were recorded at high frequencies [44]. In this study, the allotriploid ‘Yinzhong’ showed partial failure of uniparental chromosomal pairing, with an average meiotic configuration of 15.2I + 14.0II + 4.6III. Meiotic abnormalities such as precocious chromosome migration, lagging chromosomes, and micronuclei, were also found with high frequency in the allotriploid ‘Yinzhong’, indicating that intersectional hybridization and polyploidization may induce abnormal chromosome segregation in Populus. Moreover, chromosome bridges were also observed during meiosis in this study, suggesting that chromosome structural variation occurred in ‘Yinzhong’.

![Spindle misorientations and meiotic products](https://doi.org/10.1371/journal.pone.0181767.g004)

**Fig 4. Spindle misorientations and meiotic products.** a–c. Anaphase II with parallel spindles (a), fused spindles (b) and tripolar spindles (c); d. Metaphase II with an organelle band (arrow) between two spindles; e–f. Metaphase II with a mini-spindle showing chromosome arrangement (e) and microtubule distribution (f); g–h. Anaphase II with a mini-spindle showing chromosome arrangement (g) and microtubule distribution (h); i. Polyad with microcytes (arrow); j–o. Tetrads with different arrangements of daughter cells; p. Tetrad with unbalanced cytokinesis. Bars are equal to 10 μm in (a)–(c), (i)–(p); bars are equal to 5 μm in (d)–(h).

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Microsporogenesis of *P. alba* occurs normally with the sole exception of several laggards in anaphase I and II [48]. In *P. berolinensis*, which is a putative natural hybrid between *P. laurifolia* and *P. nigra* var. *italica*, abnormal meiotic phenomena such as multinuclei, multinucleoli, monads, dyads, and polyads have been observed [49]. Previous studies on diploid *Populus* hybrids also showed that hybridization tends to induce occurrence of meiotic abnormalities [45,50]. However, compared with the diploid species and hybrids of *Populus*, although meiosis in ‘Yinzhong’ showed similar abnormalities, the frequency of abnormalities was high in ‘Yinzhong’, suggesting that more abnormalities might be induced by its distant genomic composition and polyploidization.

Production of unreduced gametes has been reported in many species and hybrids of the genus *Populus* [44,45,50–54]. There are multiple cytological mechanisms of unreduced gamete formation, which have been reviewed frequently [55,56]. In diploid species of *Populus*, formation of unreduced gametes have been attributed to misorientation of spindles and failure of cytokinesis [52–54], while Wang et al. [44] found that fused and tripolar spindles in the second meiotic division and aberrant cytokinesis result from complete or partial lack of nuclear-based radial microtubule systems (RMSs), leading to unreduced pollen formation in an allotriploid
white poplar. In this study, besides fused and tripolar spindles, fusion of sister nuclei within the same cytoplasm domain in microsporocytes with precocious cytokinesis also contributed to nucleus restitution. The fused and tripolar spindles initiated first meiotic division restitution (FDR), and the fusion of sister nuclei induced second meiotic division restitution (SDR). For diploid species, both FDR- and SDR-type unreduced gametes possess complete chromosome sets. For triploids, however, SDR-type gametes are likely to contain incomplete chromosome sets due to unbalanced segregation of homologous chromosomes during the first meiotic division. Therefore, these FDR-type unreduced 3x pollen grains probably resulted in tetraploid production in the progeny, with the triploid functioning as an intermediate in the transition from diploid to tetraploid [18,21].

Occurrence of parallel spindles does not necessarily lead to unreduced gamete formation in some species, including *P. tomentosa* [11,54]. During the second meiotic division, organelles, including mitochondria and plastid, are usually located between the two spindles to form a layer, separating the cytoplasm into dyad domains [57]. In this study, an organelle layer was observed in microsporocytes with normal and parallel spindle orientations, which likely prevented the fusion of spindles [58].

Generally, gametes of triploid plants are expected to be highly sterile because most are aneuploid with an incomplete or unbalanced chromosome composition [59]. However, in previous studies on blueberry [13], melon [14], *Musa* [15], apple [16], and *Populus* [12], aneuploid progeny were produced by crossing triploids with diploids, suggesting that some aneuploid gametes may be fertile. In the allotriploid studied here, the wide range of pollen diameter may represent the various chromosome numbers of pollen grains. Nevertheless, the germination test showed that the viability of the pollen grains was low, indicating that some special chromosomal combinations are more likely to be fertile than others [60].
Crossing with polyploids is an effective way to induce variation in ploidy levels. In *Actinidia chinensis* Planch., a cross between a hexaploid and a diploid produced hybrids with extensive ploidy level segregation, including triploids, tetraploids, pentaploids, heptaploids, and octaploids [61]. In aspen, pollen grains from triploid plants have been used to pollinate diploids, resulting in the production of triploids, tetraploids, and aneuploids [12]. In this study, inter-ploidy hybridization between the diploid TB03 and the allotriploid ‘Yinzhong’ also resulted in segregation of ploidy levels among progeny, with diploids, triploids, tetraploids and aneuploids being produced. This diversity in ploidy levels can be used in polyploidy breeding programs and genetic research into *Populus*.

In the evolution of polyploid plants, compensated aneuploids can be synthesized such that the loss of one chromosome is compensated for by the gain of a chromosome from another type despite having a “euploid” chromosome number [62–64]. Such compensated aneuploids play important roles in maintaining genome balance and chromosome stability [65,66]. In this study, 18 triploids were produced. Although these triploids had the “euploid” 57 chromosomes, they were unlikely to contain three integrated chromosome sets owing to unbalanced segregation of meiotic chromosomes in triploids. It is inferred that these triploids are likely compensated aneuploids.

Aneuploids are an important bridge for chromosomal introgression and substitution in crop breeding. Aneuploids have been systematically studied in wheat to reveal the origin and function of different chromosomes and to locate important genes on chromosomes [20]. Furthermore, chromosome engineering based on aneuploids has produced a series of wheat-alien chromosome addition or substitution lines [67]. However, chromosomal introgression and substitution do not occur in trees owing to a lack of aneuploidy series. In this study, *Populus* aneuploids were produced by crossing with the allotriploid ‘Yinzhong’. Aneuploids are valuable for analysis of chromosomal function and chromosome engineering breeding in *Populus*; thus, it is important to clarify the chromosomal composition of each aneuploid in the progeny.

Karyotypic analysis based on chromosome morphology is difficult for poplar trees because their chromosomes are all small, with the exception of chromosome I. Multicolor fluorescent *in situ* hybridization (McFISH) is a feasible method for analyzing the chromosomal karyotype [68]. Hu et al. [69] located ribosomal DNA and telomere repeat sequence probes in *Populus* chromosomes using McFISH; however, the development of chromosome-specific probes is necessary to identify each chromosome of the *Populus* species. With ongoing advances in molecular biology, array comparative genome hybridization (aCGH), single nucleotide polymorphism (SNP) genotyping, quantitative fluorescent PCR (QF-PCR), and genome sequencing have been used to conduct karyotyping [70–73]. Henry et al. [74] developed a system of dosage-based functional genomics to analyze variations in chromosomal number and structure in poplar, and they precisely detected abundant deletions and insertions of chromosomal segments in poplar progeny derived from crossing with gamma-irradiated pollen. These molecular karyotyping techniques can also be used to investigate features of the chromosomal composition of the aneuploids in this investigation.

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References
1. Van de Peer Y, Maere S, Meyer A. The evolutionary significance of ancient genome duplications. Nat Rev Genet. 2009; 10:725–732. https://doi.org/10.1038/nrg2600 PMID: 19652647
2. De Storme N, Mason A. Plant speciation through chromosome instability and ploidy change: Cellular mechanisms, molecular factors and evolutionary relevance. Curr Plant Biol. 2014; 1:10–33.
3. Reif JC, Zhang P, Dreisigacker S, Warburton ML, van Ginkel M, Hoisington D, et al. Wheat genetic diversity trends during domestication and breeding. Theor Appl Genet. 2005; 110(5):859–864. https://doi.org/10.1007/s00122-004-1881-8 PMID: 15690175
4. Jørgensen MH, Ehrich D, Schmickl R, Koch MA, Brysting AK. Interspecific and interploidal gene flow in Central European Arabidopsis (Brassicaceae). BMC Evol Biol. 2011; 11(1):346.
5. Li DW, Liu YF, Li XW, Rao JY, Yao XH, Zhong CH. Genetic diversity in kiwifruit polyploid complexes: insights into cultivar evaluation, conservation, and utilization. Tree Genet Genom. 2014; 10(5):1451–1463.
6. Den Nijs TPM, Peloquin SJ. 2n gametes in potato species and their function in sexual polyploidization. Euphytica. 1977; 26:955–600.
7. Lupton FGH. Wheat breeding: its scientific basis. London: Chapman and Hall; 1987.
8. Comai L. The advantages and disadvantages of being polyploid. Nat Rev Genet. 2005; 6:836–846. https://doi.org/10.1038/nrg1711 PMID: 16304599
9. Köhler C, Scheid OM, Erilova A. The impact of the triploid block on the origin and evolution of polyploid plants. Trends Genet. 2010; 26(3):142–148. https://doi.org/10.1016/j.tig.2009.12.006 PMID: 20089326
10. Schatlowski N, Köhler C. Tearing down barriers: understanding the molecular mechanisms of interploidal hybridizations. J Exp Bot. 2012; 63(17):6059–6067. https://doi.org/10.1093/jxb/ers288 PMID: 23105129
11. Tel-Zur N, Abbo S, Mizrahi Y. Cytogenetics of semi-fertile triploid and aneuploid intergeneric vine cacti hybrids. J Heredity. 2005; 96(2):124–131.
12. Johnsson H. Cytological studies of diploid and triploid Populus tremula and of crosses between them. Hereditas. 1940; 26(3–4):321–352.
13. Dweikat IM, Lyrene PM. Production and viability of unreduced gametes in triploid interspecific blueberry hybrids. Theor Appl Genet. 1988; 76:555–559. https://doi.org/10.1007/BF00260907 PMID: 24232275
14. Ezura H, Kikuta I, Oosawa K. Production of aneuploid melon plants following in vitro culture of seeds from a triploid × diploid cross. Plant Cell Tissue Organ. 1994; 38:61–63.
15. Osuji JO, Vuylsteke D, Ortiz R. Ploidy variation in hybrids from interploid 3x × 2x crosses in Musa. Tropicult. 1997; 15(1):37–39.
16. Zhang CH, Park SM. Aneuploid production form crosses with diploid and triploid in apple tree. Hort Environ Biotechnol. 2009; 50(3):203–207.
17. Mason AS, Pires JC. Unreduced gametes: meiotic mishap or evolutionary mechanism? Trends Genet. 2015; 31:5–10. https://doi.org/10.1016/j.tig.2014.09.011 PMID: 25445549

18. Jackson RC. Evolution and systematic significance of polyploidy. Annu Rev Ecol Syst. 1976; 7:209–234.

19. Husband BC. The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biol J Linn Soc. 2004; 82:537–546.

20. Law CN, Snape JW and Worland AJ. Aneuploidy in wheat and its uses in genetic analysis. In: Lupton FGH, editor. Wheat Breeding: Its Scientific Basis. London: Chapman and Hall; 1987. pp. 71–108.

21. Henry IM, Dilkes BP, Young K, Watson B, Wu H, Comai L. Aneuploidy and genetic variation in the Arabidopsis thaliana triploid response. Genetics. 2005; 170:1979–1988. https://doi.org/10.1534/genetics.104.037788 PMID: 15944363

22. Henry IM, Dilkes BP, Miller ES, Burkart-Waco D, Comai L. Phenotypic consequences of aneuploidy in Arabidopsis thaliana. Genetics. 2010; 186:1231–1245. https://doi.org/10.1534/genetics.110.121079 PMID: 20876566

23. Rae AM, Street NR, Rodrı́guez-Acosta M. Populus trees. In: Kole C, editor. Genome mapping and molecular breeding in plants, vol 7, Forest trees. Berlin Heidelberg: Springer; 2007. pp. 1–28.

24. Jansson S, Douglas CJ. Populus: a model system for plant biology. Annu Rev Plant Biol. 2007; 58:435–458. https://doi.org/10.1146/annurev.arpplant.58.032806.103956 PMID: 17280524

25. Baumeister G. Beispiele der polyploidie-Züchtung. Allg For. 1980; 35:697–699.

26. Chen CB, Qi LW, Zhang SG, Han SY, Li XL, Song WQ, et al. The karyotype analysis of triploid poplar. J Wuhan Bot Res. 2004; 22(6):565–567.

27. Zhang S, Qi L, Chen C, Li X, Song W, Chen R, et al. A report of triploid Populus of the section Algeiros. Silvae Genet. 2004; 53:69–75.

28. Zhang SG, Chen CB, Han SY, Li XL, Ren JZ, Zhou YQ, et al. Chromosome numbers of some Populus taxa from China. Acta Phytotaxon Sin. 2005; 43:539–544.

29. Weigserber H, Rau HM, Gartner EJ, Baumeister G, Kohnert H, Karner L. 25 years of forest tree breeding in Hessen. Allg For. 1980; 26:665–712.

30. Zhu ZT, Kang XY, Zhang ZY. Studies on selection of natural triploid of Populus tomentosa. Sci Silvae Sinicae. 1998; 34:22–31.

31. Einspahr DW. Production and utilization of triploid hybrid aspen. Iowa State J Res. 1984; 58(4):401–409.

32. Mashkina OS, Burdaeva LM, Belozerova MM, Vyuntova LN. Method of obtaining diploid pollen of woody species. Lsoovedenie. 1989; 1:19–25.

33. Wang J, Kang XY, Li DL, Chen HW. Induction of diploid eggs with colchicine during embryo sac development in Populus. Silvae Genet. 2010; 59(1):40–48.

34. Wang J, Kang XY, Li DL. High temperature-induced triploid production during embryo sac development in Populus. Silvae Genet. 2012; 61(3):85–93.

35. Wang J, Li DL, Kang XY. Induction of unreduced megaspores with high temperature during megasporogenesis in Populus. Ann For Sci. 2012; 69(1):59–67.

36. Wang J, Shi L, Song SY, Tian J, Kang XY. Tetraploid production through zygotic chromosome doubling in Populus. Silva Fenn. 2013; 47(2):id 932.

37. Dong CB, Suo YJ, Wang J, Kang XY. Analysis of transmission of heterozygosity by 2n gametes in Populus (Salicaceae). Tree Genet Genom. 2015; 11:799.

38. Zhou LJ, Kang ZX, Geng LY, Wen BY. Breeding and utilization of Populus alba × P. berolinensis. Protect For Sci Technol. 1992; 1:23–27.

39. Stanton BJ, Neale DB, Li SW. Populus breeding: from the classical to the genomic approach. In: Jansson S, Bhalerao R, Groover A, editors. Genetics and Genomics of Populus. New York: Springer; 2010. pp. 309–348.

40. Shi QL, Zhuge Q, Huang MR, Wang MX. The characteristic of molecular evolution in Populus based on ITS sequence analysis. Mol Plant Breed. 2006; 4:255–261.

41. Lin HB, Zhu ZT. Studies on breeding strategies of Populus tomentosa. J Beijing For Univ. 1988; 10:97–101.

42. Kang XY, Zhu ZT, Zhang ZY. Breeding of triploids by the reciprocal crossing of Populus alba × P. glandulosa and P. tomentosa × P. bollleana. J Beijing For Univ. 2000; 22:8–11.

43. Farco GE, Dematteis M. Meiotic behavior and pollen fertility in triploid and tetraploid natural populations of Campuloclinium macrocephalum (Eupatorium, Asteraceae). Plant Syst Evol. 2014; 300(8):1843–1852.
44. Wang J, Kang X, Zhu Q. Variation in pollen formation and its cytological mechanism in an allotriploid white poplar. Tree Genet Genom. 2010; 6(2):281–290.
45. Wang J, You HL, Tian J, Wang YF, Liu MH, Duan WL. Abnormal meiotic chromosome behavior and gametic variation induced by intersectional hybridization in *Populus* L. Tree Genet Genom. 2015; 11:61.  
46. Galbraith DW, Harker KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science. 1983; 220:1049–1051. https://doi.org/10.1126/science.220.4601.1049 PMID: 17754551  
47. Del Bosco SF, Tusa N, Conicella C. Microsporogenesis in a Citrus interspecific tetraploid somatic hybrid and its fusion parents. Heredity. 1999; 83:373–377. PMID: 10583538  
48. Zhang L, Wang J, Luo YJ, Kang XY. Pollen chromosome doubling under high temperature in *Populus* alba L. J Nucl Agricul Sci. 2010; 24:1158–1165.  
49. Liu YH, Chang HF, Lu ZH. Microsporogenesis of *Populus berolinesis*. J North-East For Inst. 1979; 2:1–4.  
50. Tian J, Wang JL, Dong L, Dai F, Wang J. Pollen variation as a response to hybridisation in *Populus* L. section Algeiros Duby. Euphytica. 2015; 206:433–443.  
51. Selitz FW. The occurrence of triploids after self-pollination of anomalous androgynous flowers of a grey poplar. Z Forstgenet. 1954; 1:1–6.  
52. Kang XY. Mechanism of 2n pollen occurring in Chinese white poplar. J Beijing For Univ. 2002; 24(5/6):67–70.  
53. Wang J, Kang X. Distribution of microtubular cytoskeletons and organelle nucleoids during microsporogenesis in a 2n pollen producer of hybrid *Populus*. Silvae Genet. 2009; 58(5/6):220–226.  
54. Zhang Z, Kang X. Cytological characteristics of numerically unreduced pollen production in *Populus tomentosa* Carr. Euphytica. 2010; 173(2):151–159.  
55. Bretagnolle F, Thompson JD. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of polyploid plants. New Phytol. 1995; 129:1–22.  
56. De Storme N, Geelen D. Sexual polyploidization in plants–cytological mechanisms and molecular regulation. New Phytol. 2013; 198:670–684. https://doi.org/10.1111/nph.12184 PMID: 23421646  
57. Brown RC, Lemmon BE. Nuclear cytoplasmic domains, microtubules and organelles in microsporocytes of the slipper orchid *Cyripedium califor nicum* A. Gray dividing by simultaneous cytokinesis. Sex Plant Reprod. 1996; 9:145–152.  
58. Bednara J, Gielwanowska I, Rodkiewicz B. Regular arrangements of mitochondria and plastids during sporogenesis in *Equisetum*. Protoplasm. 1986; 130:145–152.  
59. Ramsay J, Schemske DW. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst. 1998; 29:467–501.  
60. Henry IM, Dilkes BP, Tyagi AP, Lin HY, Comai L. Dosage and parent-of-origin effects shaping aneuploid swarms in *A. thaliana*. Heredity. 2009; 103(6):458–468. https://doi.org/10.1038/hdy.2009.81 PMID: 19603060  
61. Rao JY, Liu YF, Huang HW. Analysis of ploidy segregation and genetic variation of progenies of different interploidy crosses in *Actinidia chinensis*. Acta Horticult Sin. 2012; 39(8):1447–1456.  
62. Ising G. Cytogenetic studies in *Cyrtanthus*, I: Segregation in an allotetraploid. Hereditas. 1966; 56:27–53.  
63. Xiong Z, Gaeta RT, Pires JC. Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus*. Proc Natl Acad Sci USA. 2011; 108(19):7909–7913. https://doi.org/10.1073/pnas.1014381108 PMID: 21512129  
64. Chester M, Gallagher JP, Symonds VV, Cruz da Silva AV, Mavadriev EV, Letch AR, et al. Extensive chromosomal variation in a recently formed natural allopolyploid species, *Tragopogon miscellus* (Asteraceae). Proc Natl Acad Sci USA. 2012; 109(4):1176–1181. https://doi.org/10.1073/pnas.1112041109 PMID: 22228301  
65. Birchler JA, Riddle NC, Auger DL, Veitia RA. Dosage balance in gene regulation: biological implications. Trends Genet. 2005; 21(4):219–226. https://doi.org/10.1016/j.tig.2005.02.010 PMID: 15797817  
66. Birchler JA, Veitia RA. Gene balance hypothesis: Connecting issues of dosage sensitivity across biological disciplines. Proc Natl Acad Sci USA. 2012; 109(37):14746–14753. https://doi.org/10.1073/pnas.1207726109 PMID: 22909297  
67. Gale MD, Miller TE. The introduction of alien genetic variation into wheat. In: Lupton FGH, editor. Wheat Breeding: Its Scientific Basis. London: Chapman and Hall; 1987. pp. 173–210.  
68. Lühr T, Starke H, Heller A, Kosyakova N, Mrasek K, Gross M, et al. Multicolor fluorescence in situ hybridization (FISH) applied to FISH-banding. Cytogenet Genome Res. 2006; 114:240–244. https://doi.org/10.1159/000094207 PMID: 16954660
69. Hu BQ, Dong FP, Wang CG, Qi LW, Song WQ, Chen CB. Multicolor fluorescence in situ hybridization of seven *Populus* species–ribosomal DNA and telomere repeat sequence. Acta Sci Nat Univ Nankai. 2012; 45(1):58–64.

70. Hughes TR, Roberts CJ, Dai H, Jones AR, Meyer MR, Slade D, et al. Widespread aneuploidy revealed by DNA microarray expression profiling. Nature Genet. 2000; 25(3):333–337. https://doi.org/10.1038/77116 PMID: 10888885

71. Rauch A, Rüschendorf F, Huang J, Trautmann U, Becker C, Thiel C, et al. Molecular karyotyping using an SNP array for genomewide genotyping. J. Med. Genet. 2004; 41(12):916–922. https://doi.org/10.1136/jmg.2004.022855 PMID: 15591277

72. Ogilvie CM, Donaghue C, Fox SP, Docherty Z, Mann K. Rapid prenatal diagnosis of aneuploidy using quantitative fluorescence-PCR (QF-PCR). J Histoch. Cytochem. 2005; 53(3):285–288. https://doi.org/10.1369/jhc.4B6409.2005 PMID: 15750003

73. Henry IM, Dilkes BP, Comai L. Molecular karyotyping and aneuploidy detection in *Arabidopsis thaliana* using quantitative fluorescent polymerase chain reaction. Plant J. 2006; 48(2):307–319. https://doi.org/10.1111/j.1365-313X.2006.02871.x PMID: 16995901

74. Henry IM, Zinkgraf MS, Groover AT, Comai L. A system for dosage-based functional genomics in poplar. Plant Cell. 2015; 27(9):2370–2383. https://doi.org/10.1105/tpc.15.00349 PMID: 26320226