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Characteristics of the staminate flower and pollen from autotetraploid *Betula platyphylla*

Received: 5 April 2012; Accepted 10 June 2012

Abstract: Comparison of the staminate flower and pollen of *Betula platyphylla* between autotetraploid and diploid individuals may reveal the reason for cross sterility when an autotetraploid male parent is crossed with a diploid female parent. Staminate flowers and pollen from seven autotetraploid individual trees were examined. The morphologies of the staminate flower and pollen and pollen germination of these autotetraploid individuals were compared with diploid trees. The structure of the staminate flower did not differ between autotetraploids and diploids, whereas the size of the staminate flower was larger in the autotetraploids compared with the diploids. The mean diameter and pore depth of the pollen were significantly larger in autotetraploid individuals compared with diploids, and several abnormalities in pollen morphology were found commonly amongst pollen from the autotetraploid trees. The germination rate of pollen from autotetraploids was significantly inferior to that of diploids and there was a significant negative correlation between germination rate and the percentage of abnormal pollen. These results demonstrate that the autotetraploid should not be chosen as the male parent to obtain hybrid triploid progenies because the pollen is abnormal and results in low fertility despite its large size. The results provide important information for selecting and distributing parent strains with the aim of building seed orchards of triploid birch trees.

Additional key words: birch, germination rate, pollen morphology, polyploidy

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Introduction

The natural European aspen (*Populus tremula*) triploid was discovered in Sweden by Nilsson-Ehle (1936) and Müntzing (1936). Since then, plant-breeding scientists have given extensive attention to creating triploids in forest tree species due to the huge growth of forestry. One possible approach to breed triploids is to undertake artificial pollination using unreduced $2n$ pollen that exists in natural pollen populations to pistillate flowers of diploids. Nevertheless, this is not a simple because unreduced $2n$ pollen is scarce in nature and is unable to complete germination

with the typical n pollen, meaning that such ordinary pollination often results in just a small quantity of triploids. After Blakeslee and Avery (1937) discovered that colchicine can loosen the spindle fibers during cell division and induce chromosome doubling, a new method for plant breeding was developed, and this is known as triploid breeding. Since then, this technology has been used extensively in many fields of forestry breeding, especially for *Populus* spp. Johnsson and Eklundh (1940) obtained triploids using artificial pollination with $2n$ pollen (induced by colchicine treatment) to pistillate flowers of diploid strains of *P. tremula* and *P. tremuloides*. Since this first report, artifi-

cial pollination with $2n$ pollen to pistillate diploid flowers has been used to produce triploids in *P. canescens*, *P. balsamifera*, *P. tremula*, *P. tremuloides* and *P. tomentosa* (Einspahr 1984; Zhu et al. 1997; Kang 2003).

Members of the genus *Betula* form a particularly significant group of broadleaved trees that grow naturally in Eurasia and North America. Certain birch species, such as *B. platyphylla*, *B. pendula*, *B. pubescens* and *B. papyrifera*, are valuable sources of wood, and breeding work aimed at their economic improvement is of great importance (Valanne 1972; Eriksson and Jonsson 1986). The natural triploid of the European birch (*Betula verrucosa*) was discovered by Löve (1944). This triploid shows “gigantism” with respect to the morphology of its breast-height diameter, leaf and fruit. Furthermore, Johnsson (1956) used two different tetraploids (*B. verrucosa* and *Betula japonica* × *B. verrucosa*) as female parents with a diploid (*B. verrucosa*) as the male parent to obtain triploid progenies from open pollination. In theory, a certain quantity of $2n$ pollen exists in the pollen population produced by autotetraploids. Consequently, triploids could be obtained through hybridization of an autotetraploid male parent with a diploid female parent. However, there have been no reports of triploids obtained through this approach in *Betula*. Previous research has reported that there is morphological variation in the pollen produced by *Betula* spp. Nevertheless, there are no reports concerning the staminate flower or the relationship between germination and the morphology of pollen produced by autotetraploids. In 2004, 234 autotetraploids (*B. platyphylla*) were obtained after colchicine treatment. Attempts were made to obtain a triploid through hybridization between the autotetraploids and diploid lines in 2009. The progeny saplings were all triploid when the autotetraploid was the female parent and a diploid was the male parent ($\text{♀}4x \times \text{♂}2x$), whereas the seeds generated from crosses using a diploid as the female parent and the autotetraploid as the male parent ($\text{♀}2x \times \text{♂}4x$) were all sterile (Mu et al. 2012). In order to reveal the reason for the cross sterility of $\text{♀}2x \times \text{♂}4x$, the staminate flowers and pollen from seven autotetraploid individuals were examined, and the morphology of these structures and pollen germination rates were compared between autotetraploid and diploid trees. The results provide important information for selecting and distributing parent strains with the aim of building seed orchards of triploid birch trees.

Methods

Plant materials

Mature staminate flowers and pollen samples were collected in April 2010 from seven autotetraploid individual trees (*B. platyphylla*, named Q12, Q13, Q14,

Q19, Q33, Q34 and Q103). Staminate flowers and pollen samples from 30 diploid individuals served as controls. The trees were located in an intensive seed orchard in Harbin, China.

Staminate flower morphology measurements

The lengths of mature male catkins were measured using a pair of vernier calipers. At least 30 healthy, undamaged and fully expanded catkins from each individual tree were measured, and these were selected at random. The structure of the cymule was examined and the length of the primary bract from the cymule was measured with a stereomicroscope (SteREO Lumar. V12, ZEISS, Germany). Cymules were chosen at the top, middle and bottom of each catkin, and 90 cymules and primary bracts were examined in total.

Pollen morphology measurements

Pollen samples were prepared and mounted in silicon oil as described by Karlisdóttir et al. (2007; 2008). This method includes sequential treatment at 40°C in the following solutions: 10% (w/v) NaOH for 15 min, 10% (v/v) HCl for 10 min, 40% (v/v) HF for 5 min and 10% HCl for 10 min. Then the samples underwent acetolysis and dehydration in ethanol and TBA buffer. At least 1000 pollen grains from each individual tree were photographed and measured using a microscope (Axio Imager A1, ZEISS, Germany). According to the method of Perveen and Qaisek (1999), only triporate pollen grains (normal grains with three pores) lying in polar view were used, and pollen diameter and pore depth were determined for each pollen grain. The pollen diameter was defined as the distance from the outside tip of the pore to the outer margin of the facing wall, whereas pore depth was defined as the distance from the outside tip of the pore to the inner margin of the nexine through the vestibulum. When the pollen shape was asymmetric, the largest diameter and pore depth measurements were used. Moreover, from the same preparations any abnormal pollen grains were examined and noted. At least 30 fields of pollen grains per individual tree were counted and all types of abnormalities were recorded.

Pollen germination measurements

Pollen germination was performed *in vitro* in agar culture medium. Briefly, molten culture medium (all w/v: 0.015% H_3BO_3 , 0.015% CaCl_2 , 0.5% agar, 20% sucrose; pH 6.0) was spread evenly on to microscope slides. Once solidified, pollen samples were blown evenly on to the culture medium surface using an auralave until the color of the culture medium was slightly yellow. The slides covered in pollen grains were put into petri dishes between layers of moist filter paper and incubated at 25°C for 24 h. After that, at least 30 fields of pollen grains per individual tree were

examined using the microscope and the rate of germination was determined. A germinating pollen grain was defined to have a pollen tube of a length greater than the diameter of the grain itself.

Statistical analysis

All data were analyzed by analysis of variance (ANOVA), the Duncan test and Pearson's correlation coefficient. These tests were performed using the SPSS v16.0 software (SPSS Inc.).

Results

Morphological variations of the staminate flower versus tree ploidy

The structure of the staminate flower from autotetraploid trees did not differ from those from diploid trees; both were catkins comprised of several cymules arranged helically along an inflorescence axis. Each cymule consisted of one primary bract, two secondary bracts and three flowers. Furthermore, each flower consisted of two tepals and two stamens, and each stamen consisted of one filament and two anthers. Nevertheless, the staminate flower from autotetraploids was larger than measured for diploids (Fig. 1). The length of the catkin and primary bracts were significantly greater in autotetraploid individuals compared with diploids (Table 1). Mean catkin length for

each autotetraploid individual was significantly greater than the lengths observed in the diploids, except for Q12 and Q33. The largest catkin (mean length of 107.80 mm) was from Q19, and this was 30.22% greater in length than the mean catkin length observed for the diploids. The primary bract length of each autotetraploid individual was significantly greater than each of the diploids, except for Q13 and Q34. The largest primary bract (mean length of 3.21 mm) was observed for Q14, and this was 20.68% greater than the primary bract lengths of the diploids (Table 2). However, there was no correlation between catkin length and primary bract length ($r = -0.415$; $P = 0.335$; $n = 7$) in different autotetraploid individuals. These results suggest that autotetraploids showed "gigantism" with respect to their staminate flower organs versus those observed in the diploid individuals.

Germination and morphological variation of pollen versus ploidy

Pollen germination rates differed significantly between the autotetraploids and diploids (Table 1). The pollen germination rate of each autotetraploid individual was significantly lower than each diploid individual (Fig. 2 and Table 2). The mean pollen germination rate of the seven autotetraploid individuals was only 12.51%, which was 65.48% lower than the rate observed for the diploids. The maximal pollen germi-

Table 1. ANOVA of different traits from individual trees: catkin length, primary bract length, pollen diameter, pore depth, abnormal pollen percentage and pollen germination rate.

Dependent variable	SS	df	MS	F	P
Catkin length	18776.401	7	2682.343	22.422	0.000
Primary bract length	36.286	7	5.184	119.783	0.000
Pollen diameter	49100.163	7	7014.309	940.440	0.000
Pore depth	903.983	7	129.140	848.714	0.000
Abnormal pollen percentage	14.802	7	2.115	172.728	0.000
Pollen germination rate	2.358	7	0.337	191.088	0.000

Table 2. Multiple comparisons of different individual trees with respect to catkin length, primary bract length, pollen diameter, pore depth, abnormal pollen percentage and pollen germination rate.

Individual trees	Catkin length/mm	Primary bract length/mm	Pollen diameter/ μ m	Pore depth/ μ m	Abnormal pollen percentage/%	Pollen germination rate/%
Q12	88.39 \pm 5.89c	2.88 \pm 0.20c	26.78 \pm 3.11g	2.94 \pm 0.36g	69.20 \pm 12.37b	9.69 \pm 1.83 d
Q13	104.83 \pm 12.45a	2.65 \pm 0.18d	30.21 \pm 2.97c	3.67 \pm 0.47a	58.29 \pm 13.28c	12.23 \pm 0.84c
Q14	95.06 \pm 6.26b	3.21 \pm 0.19a	27.35 \pm 3.26f	3.21 \pm 0.41f	56.19 \pm 11.13c	13.47 \pm 1.74bc
Q19	107.80 \pm 13.61a	3.04 \pm 0.20b	28.21 \pm 2.68e	3.31 \pm 0.41e	43.51 \pm 10.88d	14.90 \pm 3.64b
Q33	83.05 \pm 7.03c	3.17 \pm 0.18a	29.27 \pm 2.68d	3.48 \pm 0.40c	46.18 \pm 11.01d	13.75 \pm 2.22bc
Q34	99.21 \pm 12.29b	2.58 \pm 0.11e	31.59 \pm 2.11a	3.35 \pm 0.33d	44.41 \pm 8.01d	14.39 \pm 2.01b
Q103	98.45 \pm 13.96b	2.93 \pm 0.31c	30.63 \pm 2.99b	3.56 \pm 0.41b	77.36 \pm 9.28a	9.16 \pm 2.13d
Control	82.78 \pm 12.15c	2.66 \pm 0.24d	23.32 \pm 1.68h	2.58 \pm 0.31h	4.02 \pm 1.28e	36.24 \pm 8.01a

Columns containing different lower-case letters differ significantly ($P < 0.05$).

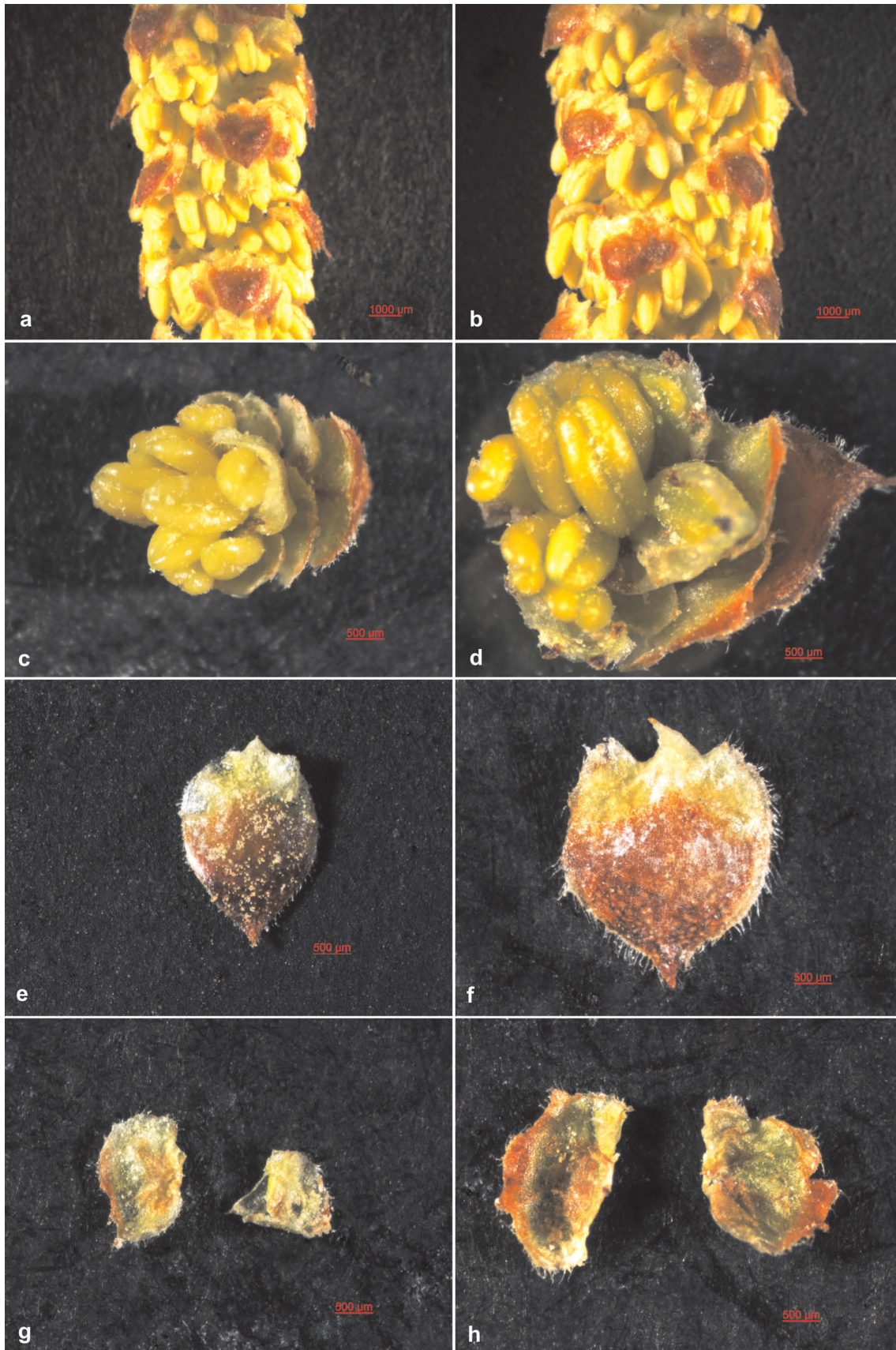


Fig. 1. Staminate flowers from diploid and autotetraploid individuals

a – part of a mature catkin from the diploid, b – part of a mature catkin from the autotetraploid, c – cymule of the diploid, d – cymule of the autotetraploid, e – primary bract of the diploid, f – primary bract of the autotetraploid, g – secondary bracts of the diploid, h – secondary bracts of the autotetraploid

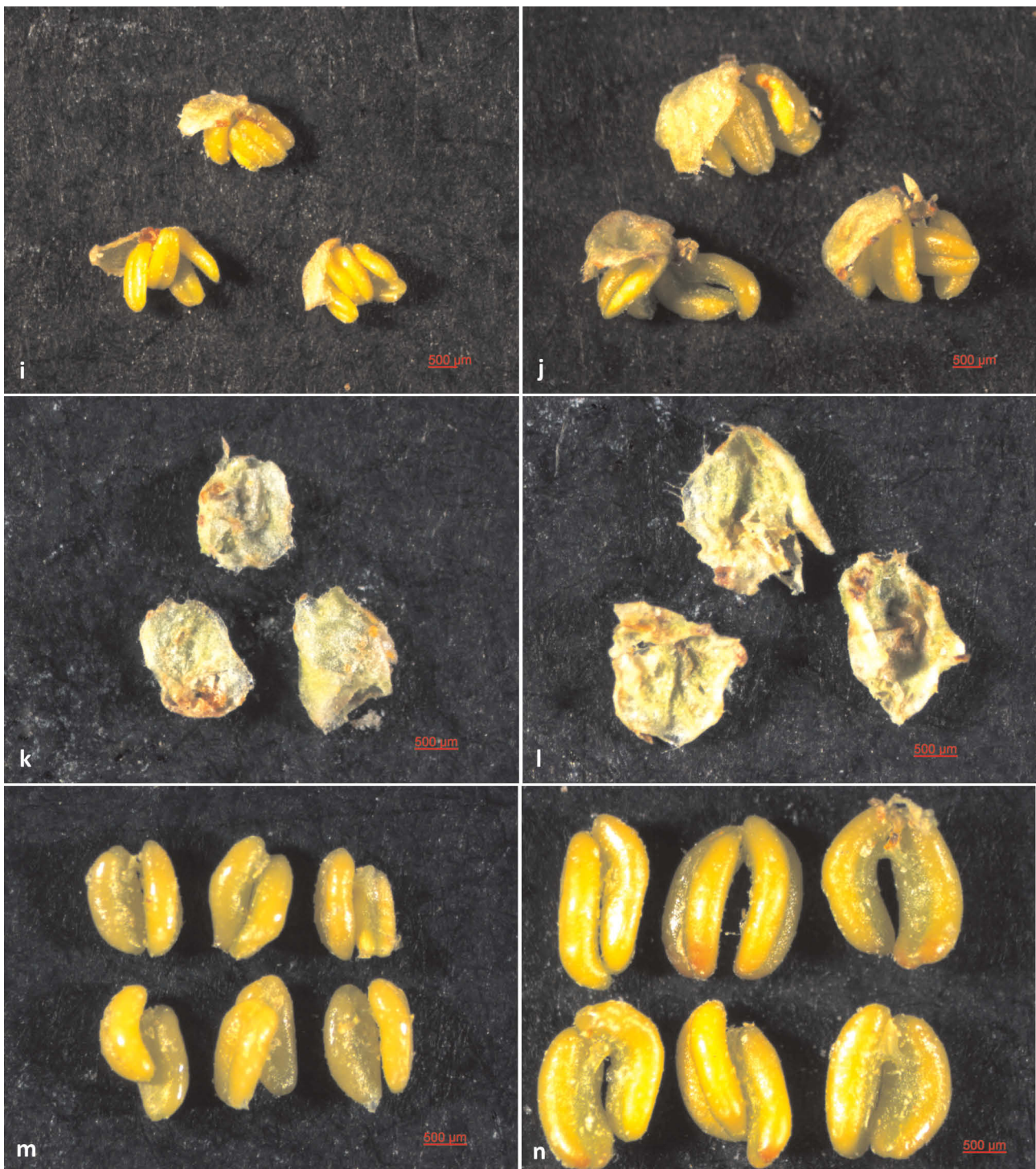


Fig. 1. Staminate flowers from diploid and autotetraploid individuals
 i – flowers of the diploid, j – flowers of the autotetraploid, k – tepals of the diploid, l – tepals of the autotetraploid, m – anthers of the diploid, n – anthers of the autotetraploid. Please note that all filaments were removed

nation rate of autotetraploid Q19 was only 14.90%, which was 58.89% lower than the mean rate observed for the diploids. These results indicate that pollen fertility is inferior in autotetraploids compared with the diploids.

ANOVA confirmed that there were significant variations in pollen diameters and pore depths between individual trees (Table 1). Pollen diameter and pore

depth of each autotetraploid individual was significantly larger than observed for the diploids (Fig. 3 and Table 2). The largest pollen (mean diameter of $31.59 \mu\text{m}$) was from Q34, which was 35.46% larger than the mean diameter observed for the diploids, while the deepest pore depth (mean value of $3.67 \mu\text{m}$) was measured in Q13, which was 42.25% deeper than the mean pore depth recorded for the diploid individuals.

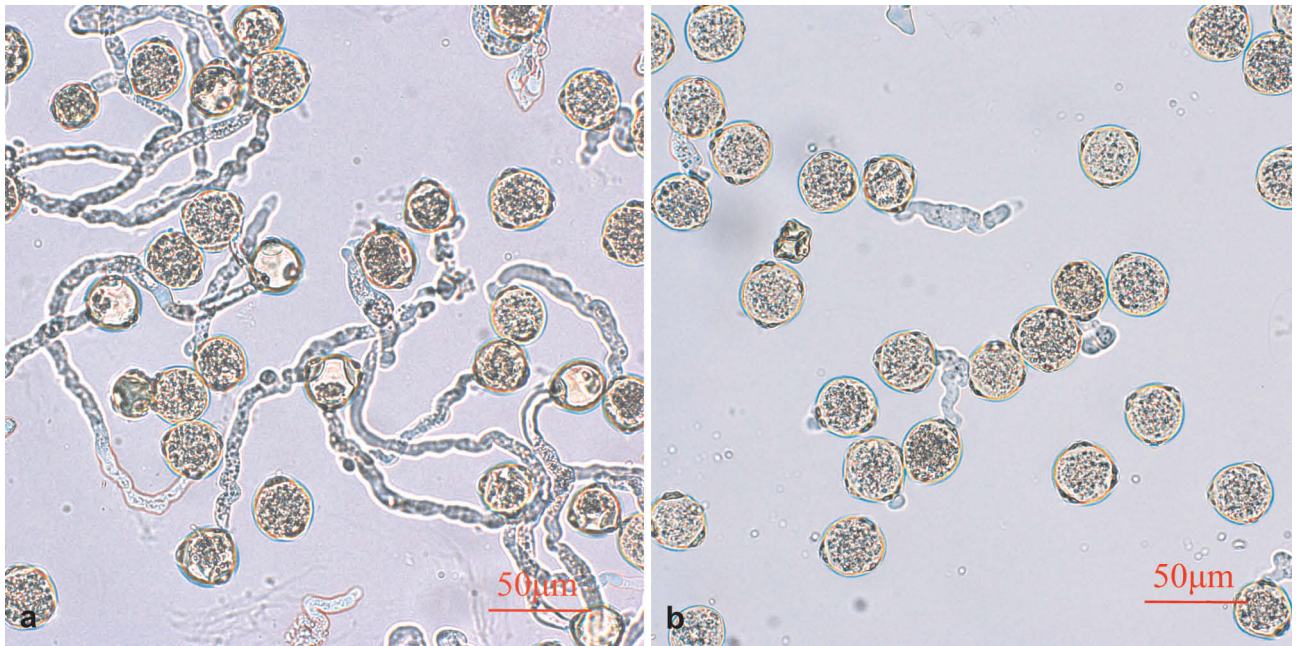


Fig. 2. Germination of pollen grains from diploid (a) and autotetraploid (b) trees

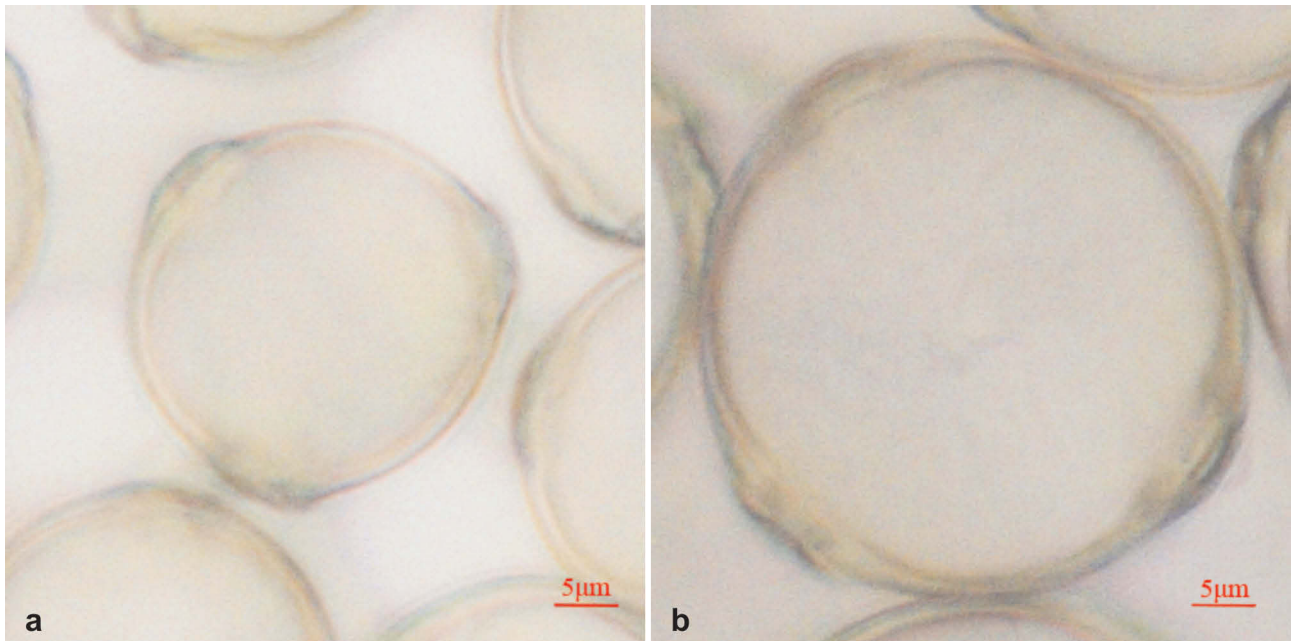


Fig. 3. Normal triporate pollen from diploid (a) and autotetraploid (b) trees

Seven classes of abnormal pollen grains were recorded: shrunken, rhombic, damaged, dwarf, uneven exine, four pores and five pores (Fig. 4). Abnormal pollen morphologies were significantly more frequent in samples from autotetraploid individuals than diploid samples (Table 1 and Table 2). Abnormal pollen in autotetraploid individuals ranged from 43.51% to 77.36%, while the lowest percentage of abnormal pollen in an autotetraploid (Q19) was 982.34% greater than the mean rate calculated for diploid trees. Furthermore, there was a significant negative correlation ($r = -0.973$; $P < 0.01$; $n = 7$) between the percentage of abnormal pollen and the ger-

mination rate in autotetraploid individuals, which indicates that lower proportions of abnormal pollen are associated with greater pollen fertility.

Discussion

Variations exist in between diploid and autotetraploid plants in terms of vegetative growth, morphology, fertility and organogenesis of reproductive structures (Hao et al. 2006; Diao et al. 2010). Autotetraploids can show “gigantism” in their reproductive organs compared with diploids, but abnormal meiosis in pollen mother cells can lead to low pollen fertility in

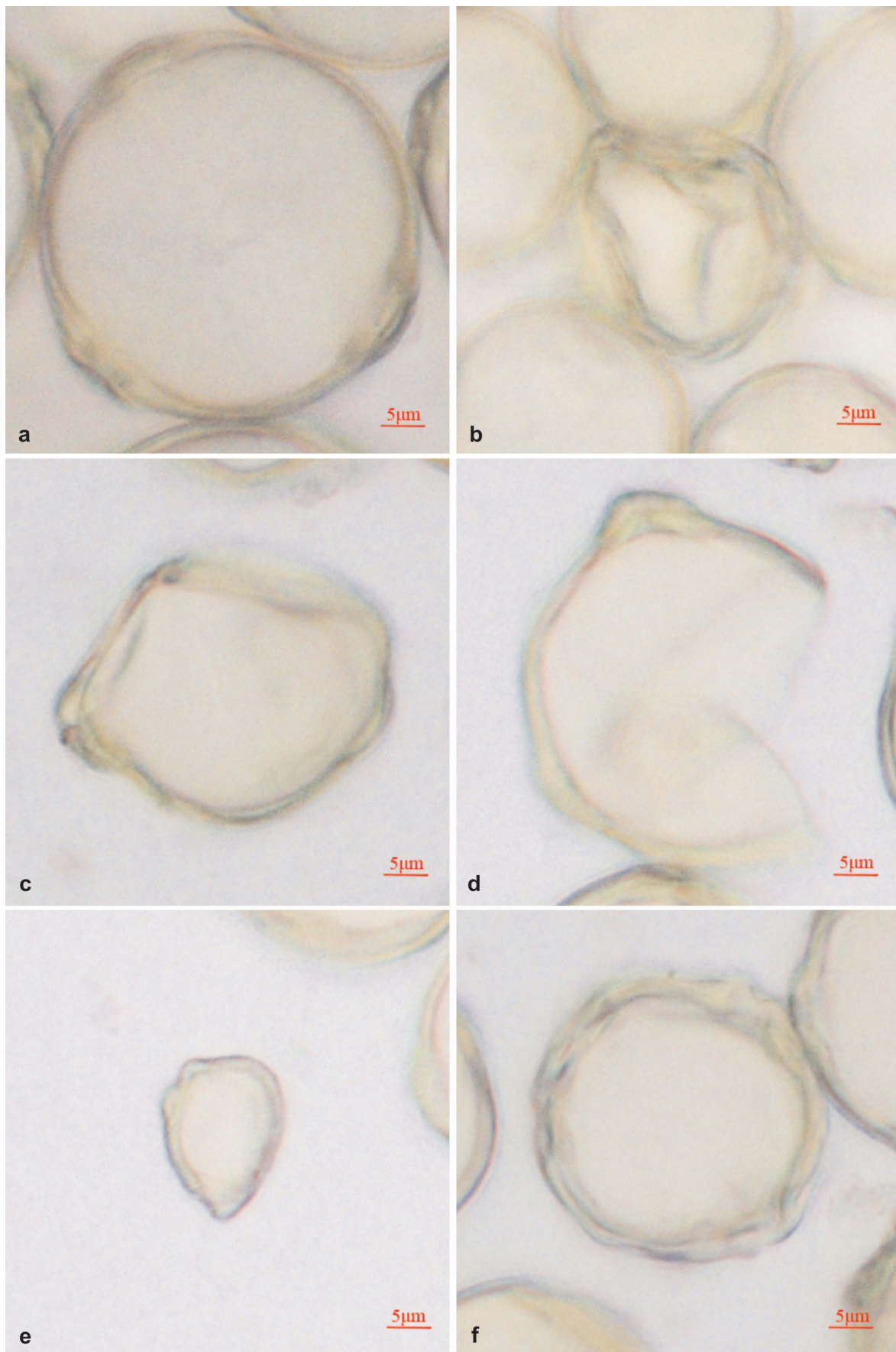


Fig. 4. Examples of abnormal pollen found in pollen samples from different trees
 a – normal triporate type, b – shrunken, c – rhombic, d – damaged, e – dwarf, f – uneven exine

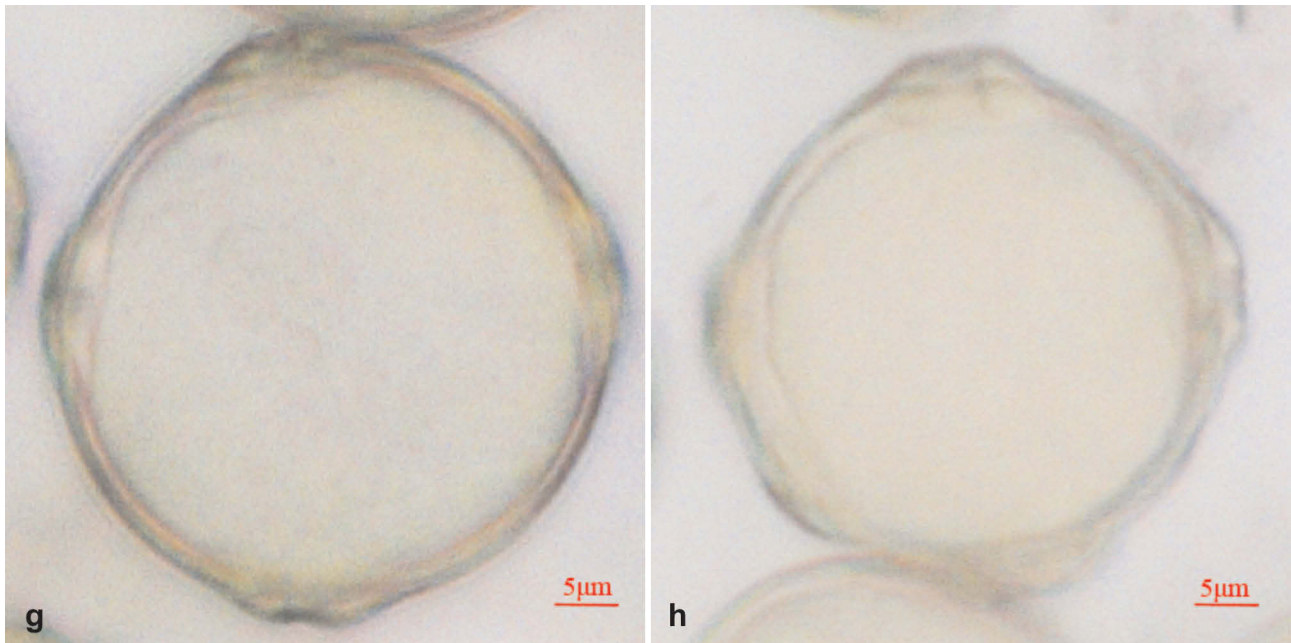


Fig. 4. Examples of abnormal pollen found in pollen samples from different trees
g – four pores, h – five pores

autotetraploids. For example, the proportion of sterile pollen was up to 86.65% in autotetraploid *Oryza sativa* (Huang et al. 1999), while pollen fertility of autotetraploid *Citrus sinensis* was 36.62% lower than the diploid (Deng et al. 1995). This present study discovered that the structure of the staminate flower of autotetraploid *B. platyphylla* did not differ from the diploid, but that the overall size of staminate flower in the autotetraploids was larger than observed for the diploids. Moreover, the pollen from autotetraploid *B. platyphylla* individuals was significantly larger than from diploid trees. Meanwhile, there was significant variation in the size of the staminate flowers and pollen between different autotetraploid individuals. Furthermore, the germination rates and pollen tube lengths from autotetraploid individuals were lower than those from diploids, and for autotetraploids a significant negative correlation was found between the proportion of abnormal pollen and the germination rate. These phenomena may result from abnormal meiosis in pollen mother cells in the autotetraploids.

Polyloid breeding in plants has been developed using artificial methods such as colchicine and high temperature treatments (Zhu et al. 1998). Though polyloid plants sometimes exhibit certain defects such as slow growth and low fertility, others can possess excellent economic traits (Ahuja 2005; Ewald et al. 2009). In a previous study, low fertility of the male gamete but high fertility of the female gamete from autotetraploid *B. platyphylla* meant that seeds from $\text{♀}2x \times \text{♂}4x$ crosses were all sterile, while progeny saplings from $\text{♀}4x \times \text{♂}2x$ crosses were all triploids. These results provide an important reference for building a seed orchard of triploid birch, which is an

autotetraploid tree surrounded by superior diploids. This knowledge could be used to obtain triploid seeds from abundantly available high fertility pollen, and to determine the most optimum distribution of different diploid trees to obtain superior diploid hybrids. As a result, the seed orchard of triploid birch could bring greater economic benefits via the production of superior triploid and diploid hybrid seeds.

Acknowledgements

The research was financially supported by National Forestry Department Public Benefit Research Foundation of China (201204302).

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