



Title	Interploid and intraploid hybridizations to produce polyploid Haskap ( <i>Lonicera caerulea</i> var. <i>emphyllocalyx</i> ) plants
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1 **Title:**

2 **Interploid and intraploid hybridizations to produce polyploid Haskap (*Lonicera caerulea* var.**  
3 ***emphyllocalyx*) plants**

4 **Interploid and intraploid hybridization in Haskap (*Lonicera caerulea* var. *emphyllocalyx*)**

5

6 A concise title: Interploid and intraploid hybridizations in *Lonicera caerulea*

7

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9

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21

22 **Abstract**

23 We produced polyploid Haskap (*Lonicera caerulea* var. *emphylocalyx*) plants by performing interploidy  
24 and intraploidy crosses of wild accessions. Embryo rescue in a tetraploid ( $4x$ )  $\times$  diploid ( $2x$ ) cross  
25 produced triploid plants; reciprocal  $2x \times 4x$  cross failed to produce viable seeds. Intraploidy crosses of  $4x$   
26  $\times 4x$  produced mostly tetraploids but also several hexaploid ( $6x$ ) and octoploid ( $8x$ ) plants. Using  
27 hexaploids obtained from this cross, we examined reciprocal  $4x$ – $6x$  crosses and found that both produced  
28 pentaploid plants. An octoploid was produced by applying colchicine to a tetraploid; a  $4x \times 8x$  cross using  
29 this plant and aided by embryo rescue culture produced three hexaploid plants, with an aneuploid number  
30 of chromosomes. Several plants obtained in this study flowered and set fruits. We discuss the overall  
31 efficiency of producing polyploid plants in interploidy and intraploidy crosses.

32  
33 **Keywords:** Caprifoliaceae, Interploidy cross, Intraploidy cross, *Lonicera caerulea*, Haskap, Polyploid

34  
35 **1. Introduction**

36 More than 200 species comprise the genus *Lonicera*, which belongs to the family Caprifoliaceae  
37 (Poyarkova 2000). Blue honeysuckle (*Lonicera caerulea* L.) belongs to the section *Isika*, subsection  
38 *Caeruleae* (Rehder 1903). It is a deciduous shrub with edible fruits that is found in the northern regions of  
39 Eurasia and North America (Rehder 1903). In Japan, blue honeysuckle grows in cold regions, from the  
40 alpine areas of the middle island to the entire Hokkaido (Hara 1983; Sato 1985). Japanese blue  
41 honeysuckle is known as Haskap in the Ainu language used by the indigenous Ainu people of Hokkaido.  
42 The fruits of blue honeysuckle are sour to sweet or bitter in taste, and are known as functional food  
43 because they are high in nutritional value, containing anthocyanins, polyphenolics, minerals, vitamins,  
44 and loganin (Anikina et al. 1989; Terahara et al. 1993; Machida et al. 1995; Anetai et al. 1996; Tanaka and  
45 Tanaka 1998; Plekhanova 2000; Thompson and Chaovanalikit 2003; Chaovanalikit et al. 2004; Svarcova  
46 et al. 2007). Haskap has been cultivated as a berry crop in Hokkaido; Haskap juice, wine, and jam are  
47 popular. For breeding, interspecific hybrids have been produced between *L. caerulea* and *L. gracilipes*  
48 (Miyashita and Hoshino 2010). Recently, Haskap was introduced to North America as a new berry crop  
49 (Thompson 2006).

50 The size and taste of the wild Haskap fruit vary. To increase commercial production, the

51 Hokkaido Research Organization evaluated the botanical and agricultural traits of wild Haskap. Some  
52 elite strains were selected and the first cultivar, 'Yufutsu,' was released in 1992 (Tanaka et al. 1994).  
53 Takada et al. (2003) evaluated the eating qualities and some horticultural characteristics of wild Haskap  
54 species, and made some elite selections. Local agricultural cooperatives also selected several elite strains  
55 from wild plants. Thompson and Barney (2007) performed evaluation and breeding of Haskap in North  
56 America. However, further improvement of the plant is needed to increase its commercial production. A  
57 major issue for Haskap growers is that wild plants have small fruits with thin pericarp. Harvest is  
58 laborious, and the harvest volume barely meets market demand. Therefore, fruit yield and other traits  
59 must be improved.

60 In general, polyploid plants have larger plant parts and greater adaptability than do diploid  
61 plants (Lewis 1980). Polyploids have larger fruits (Notsuka et al. 2000; Wakana et al. 2005; Sasnauskas et  
62 al. 2007; Zhang et al. 2010), many polyploid ornamentals have larger flowers, and others have higher  
63 quantities of effective components (Gao et al. 1996; Zhang et al. 2010). Triploid plants are usually sterile  
64 and in such species as banana, grape, watermelon, and many others triploidy is used for seedless fruit  
65 production. Polyploid variation is important because of the phenotypic variation it introduces and because  
66 it may enhance the effectiveness of plant breeding.

67 Diploid ( $2n = 2x = 18$ ) and tetraploid ( $2n = 4x = 36$ ) varieties of *L. caerulea* have been found in  
68 Eurasia and North America (Ammal and Saunders 1952; Plekhanova et al. 1992; Solovyeva and  
69 Plekhanova 2003; Plekhanova 2007). Both diploids and tetraploids were found in wild populations of  
70 Haskap (Miyashita et al. 2011). In order to prepare for ploidy breeding in this plant species, we examined  
71 the production of octoploids from tetraploids by colchicine, oryzalin, and trifluralin treatments (data not  
72 published). Suzuki et al. (2007) developed a method of *in vitro* colchicine treatment using the nodal  
73 segment. Miyashita et al. (2009) examined the production of hexaploid plants by endosperm culture.

74 The aim of the present study was to produce polyploid Haskap plants to increase the genetic  
75 variation of the species. Intraploid and interploid crosses using diploid, tetraploid, hexaploid, and  
76 octoploid variants were investigated. Lateral, compatibility or incompatibility in interploid hybridization  
77 is discussed from the viewpoints of paternal and maternal genome ratios in the embryo and endosperm.

78

## 79 **2. Materials and Methods**

80

### 81 2.1. Plant materials

82 Haskap (*L. caerulea* var. *emphylocalyx*) plants grown at the Experiment Farms of Hokkaido University  
83 were used. Two diploid ( $2n = 2x = 18$ ) strains, Di-K8 and Di-BeY, were collected from Kiritappu mire  
84 and Betsukai in Hokkaido, respectively, and six tetraploid ( $2n = 4x = 36$ ) strains, Tet-Y2, Tet-Y16,  
85 Tet-Y27, Tet-Y37, Tet-Y0049, and Tet-Has1 were collected from Yufutsu mire in Hokkaido. One  
86 octoploid strain ( $2n = 8x = 72$ ). Oct-T1, was produced by colchicine treatment of a tetraploid plant in our  
87 laboratory.

88

### 89 2.2. Hybridization patterns

90 Intraploid or interpid hybridization was performed using diploid, tetraploid, hexaploid, and octoploid  
91 plants. The cross combinations tested were  $2x \times 2x$ ,  $4x \times 4x$ ,  $2x \times 4x$ ,  $4x \times 2x$ ,  $4x \times 6x$ ,  $6x \times 4x$ , and  $4x \times$   
92  $8x$ . Flowers were emasculated prior to anthesis and hand-pollinated. To prevent cross pollination all  
93 pollinated flowers were covered with paper bags.

94 Fruits were harvested at 40–45 days after pollination (DAP), when mature seeds are generally  
95 present in Haskap. Seeds germinate and develop into seedlings more quickly when cultured on a artificial  
96 media and so seed media culture was utilized in intraploid and interpid crosses. All seeds were  
97 disinfected with 1% sodium hypochlorite solution containing 1–2 drops of polyoxyethylene sorbitan  
98 monolaurate (Tween 20) for 20 min, rinsed three times with sterile-distilled water and cultured on a  
99 half-strength Murashige and Skoog (1962) basal medium containing  $30 \text{ g L}^{-1}$  sucrose and  $2 \text{ g L}^{-1}$  gellan  
100 gum (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). The pH of the medium was adjusted to 5.8 and  
101 it was autoclaved at  $121 \text{ }^\circ\text{C}$  for 20 min. The seeds cultures in  $90 \times 20$ -mm petri dishes were maintained in  
102 a growth chamber at  $20 \text{ }^\circ\text{C}$  under continuous illumination 24 h photoperiod ( $35 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) provided by  
103 40W fluorescent tubes.

104 Immature seed culture was also performed in  $4x-4x$ ,  $2x-4x$  and  $4x-8x$  hybridization. Immature  
105 fruits were harvested at 14–28 DAP, disinfected with 1% sodium hypochlorite solution containing 1–2  
106 drops of Tween 20 for 10 min and rinsed, immature seeds were collected and cultured under the same  
107 conditions as above.

108

109 2.3. *Ploidy analysis using flow cytometry*

110 The relative DNA contents of hybrids from intraploid or interploid crosses were determined using flow  
111 cytometry (Partec PA; Partec GmbH, Münster, Germany). Fresh leaves (ca. 0.5 cm × 0.5 cm) were  
112 chopped in 0.2 mL of nuclei extraction buffer (CyStain UV precise P; Partec, Münster, Germany). After  
113 filtration through a 30-µm nylon mesh, crude nuclear samples were stained with 0.8 mL 4',  
114 6-diamidino-2-phenylindole (DAPI) solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM  
115 MgCl<sub>2</sub>, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100, and 2 mg L<sup>-1</sup> DAPI (pH 7.5) (Mishiba et al. 2000),  
116 incubated for 5 minutes at room temperature and relative DNA contents were measured with fresh leaves  
117 of *Capsicum annuum* L. (cv. 'Kyonami') were used as the internal standard.

118

119 2.4. *Chromosome analysis*

120 Chromosome numbers were counted in the actively growing root tips. The root tips were pretreated in ice  
121 water for 24 h and fixed in a mixture of acetic acid:ethanol (1:3) at 4 °C overnight. Fixed root tips were  
122 treated with a mixture of 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Co., Ltd., Japan) and  
123 0.5% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan) (Shibata and Hizume, 2002) in the  
124 citrate buffer (0.01 M citric acid and 0.01 M trisodium citrate dehydrate) at pH 4.5, at 37 °C, for 20 min,  
125 rinsed in distilled water and squashed with forceps in a drop of 45% acetic acid on a glass slide, covered  
126 with another slide, and squashed again ( I do not understand this procedure? Double Squashing? Two  
127 slides?). Covers slips were removed by freezing in liquid nitrogen, and slides were dried at 37 °C. A drop  
128 of DAPI solution [0.233 g 1,4-diazabicyclo(2.2.2)-octane, 1 mL 0.2 M Tris-HCl, pH 8.0, 9 mL glycerol,  
129 0.5 µg ml<sup>-1</sup> of DAPI] (Sahara et al. 2003) was added for staining and preparations were observed under a  
130 fluorescence microscope at ×1000 magnification (Axio Imager M1; Carl Zeiss, Oberkochen, Germany).  
131 For each sample, 10 measurements were recorded.

132

133 2.5. *Characterization of polyploid plants*

134 Corolla length, pollen diameter, pollen germination rate, the size of guard cells, and fresh fruit weight  
135 were measured in polyploids and their parents. The corolla length is an average of seven flowers. Pollen  
136 diameter ( $n = 20$  per plant) was measured following staining of fresh pollen in aceto-carmin. The pollen  
137 germination ability was tested in a pollen culture medium, following Brewbaker and Kwack (1963), with

138 minor modifications. The culture medium was liquid, containing 100 g L<sup>-1</sup> sucrose, 100 mg L<sup>-1</sup> boric acid,  
139 300 mg L<sup>-1</sup> calcium nitrate, 200 mg L<sup>-1</sup> magnesium sulfate, and 100 mg L<sup>-1</sup> potassium nitrate in water. For  
140 each plant, three replicates were performed. The size of the guard cells ( $n = 30$  per plant) was measured  
141 using the replica method. Microscopic observations were performed using Primo Star (Carl Zeiss,  
142 Oberkochen, Germany) at  $\times 40$  magnification.

143 Statistical tests were performed using the SPSS 16.0 J program. The differences were analyzed  
144 using one-way analysis of variance (ANOVA) followed by Bonferroni's test, with  $p < 0.05$  as the level of  
145 statistical significance.

146

## 147 2. 6. *Evaluation of intraploid and interploid hybridization*

148 To evaluate interploid hybridization, seed development in  $4x \times 2x$  and  $2x \times 4x$  was observed. In total, the  
149 relationship between the embryo/endosperm genome ration and germination efficiency (the number of  
150 seed germination / the number of seeds obtained) was analyzed.

151

## 152 3. Results

153

### 154 3.1. $2x \times 2x$ crosses

155 The results are shown in Table 1. The seed germination rate of  $2x \times 2x$  (Di-K8  $\times$  Di-BeY) crosses was  
156 100%. Flow cytometry showed that all progeny obtained from  $2x \times 2x$  crosses were diploid.

157

### 158 3.2. $2x-4x$ crosses

159 The results of reciprocal  $2x-4x$  crosses are shown in Table 2. The  $2x-4x$  crosses produced many shriveled  
160 seeds compared with the  $2x-2x$ ,  $4x-4x$ , and  $4x-6x$  crosses. The seeds from the  $2x \times 2x$  and  $4x \times 4x$  crosses  
161 contained embryos at the torpedo-shape stage, and the endosperm almost filled the seed at 40 DAP. In  
162 contrast, in  $2x \times 4x$  crosses at 40 DAP, the embryos were at the globular stage, and the endosperm  
163 degenerated. In  $4x \times 2x$  crosses, most of the embryo and endosperm were underdeveloped.

164 In reciprocal crosses between the diploids and tetraploids, only  $4x \times 2x$  crosses were successful.  
165 The reciprocals failed to produce viable seeds and no germination was observed. Only a small percentage  
166 of the  $4x \times 2x$  crosses succeeded in setting fruit. Nearly all of these produced shriveled seeds (97–100%).

167 However, several shriveled seeds germinated in seed culture and grew into plants. The germination rate  
168 varied from 0% to 14.7%. A total of 9 (7 + 2) plants survived out of 13 (10 + 3) germinated seeds. Flow  
169 cytometric analysis showed that they were triploid (Fig. 1a) and in five individual seedlings the  
170 chromosome number was confirmed at  $2n = 3x = 27$  (Fig. 2a). These triploid seedlings were acclimated  
171 and grown in pots (Fig. 3a) and were still growing normally three months after acclimation.

172 The effect of the immature seed culture was confirmed in reciprocal  $2x-4x$  crosses using the  
173 strains Di-K8 and Tet-Y27. The results of immature seed culture are shown in Table 3. A total of 107 (31  
174 + 49 + 27) immature seeds from  $2x \times 4x$  crosses were cultured but no germination was observed at any of  
175 the harvested stages. In contrast, triploids were obtained by immature seed culture in  $4x \times 2x$  crosses. The  
176 germination rate (19% to 37.6%) of immature seeds in culture was higher than that of seed culture in  $4x \times$   
177  $2x$  crosses. The highest germination rate was observed in immature seeds harvested at 21 DAP. A total of  
178 69 (18 + 27 + 24) plants survived out of 93 (24 + 38 + 31) germinated immature seeds. Progeny obtained  
179 from immature seed culture were triploid.

180

### 181 3.3. $4x - 4x$ crosses

182 The results of  $4x \times 4x$  crosses are shown in Table 4. The germination rate of seeds from  $4x \times 4x$  crosses  
183 (Tet-Y27  $\times$  Tet-Y37 and Tet-Y37  $\times$  Tet-Y27) ranged from 84.5% to 94.4%. The germination rate in  
184 immature seed culture (Tet-Y27  $\times$  Tet-Y37, Tet-Y37  $\times$  Tet-Y27, and Tet-Has1  $\times$  Tet-Y16) was 13.6% to  
185 29.2%. A total of 382 progeny were obtained from  $4x \times 4x$  crosses. Flow cytometry analysis showed that  
186 almost all the progeny (378 seedlings) were tetraploid. The exceptions were two hexaploids and two  
187 octoploid (Fig. 3e, f, j). The chromosome number of both hexaploids was  $2n = 6x = 54$  (Fig. 2b). One  
188 hexaploid plant (strain: Hex-1,  $2n = 6x = 54$ ) grew normally and set fruit. This individual was used for  
189 hybridization listed below. Vigor of the octoploids was strongly suppressed; they were very small  
190 compared to the plants of other ploidy levels.

191

### 192 3.4. $4x-6x$ crosses

193 A hexaploid plant (strain: Hex-1) obtained from the  $4x \times 4x$  cross was used in reciprocal  $4x-6x$  crosses  
194 (Table 5). Similar levels of seed shriveling was observed in both crosses, 52% for  $4x \times 6x$  58% for  $6x \times 4x$ .  
195 However, these seeds were able to germinate in culture and grew into plants. The germination rates were



196 75.4% (for  $4x \times 6x$  crosses) and 89.5% (for  $6x \times 4x$  crosses). Flow cytometry showed that progeny  
197 obtained from  $4x \times 6x$  crosses and  $6x \times 4x$  crosses were pentaploids (Fig. 1b). The chromosome number  
198 was confirmed for 17 plants at  $2n = 5x = 45$  (Fig. 2c) from 17. These pentaploid plants were acclimated  
199 and grown in pots (Fig. 3b, c, d). Three of them bloomed and set fruit at the age of two.

200

### 201 3.5. $4x \times 8x$ crosses

202 An octoploid strain (Oct-T1) produced by colchicine treatment of a tetraploid was used for  $4x \times 8x$   
203 crosses. In  $4x \times 8x$  direction all crosses were successful and set fruits (Table 6). The total 131 ( $37 + 84 +$   
204 10) immature seeds were cultured and three seedlings were obtained. The germination rates were 2.4% to  
205 10%. Flow cytometry analysis showed that these progeny were hexaploid (Fig. 1c) but chromosome  
206 counts revealed aneuploid chromosome numbers of chromosomes at  $2n = 6x-4 = 50$  (Fig. 2d),  $2n = 6x-3$   
207  $= 51$  (Fig. 2e), and  $2n = 6x-2 = 52$  (Fig. 2f). Aneuploid plants obtained from  $4x \times 8x$  crosses grew  
208 normally (Fig. 3g, h), except for one plant with  $2n = 6x-2 = 52$  form (Fig. 3i) showing growth  
209 suppression.

210

### 211 3.6. Characterization of polyploids hybrids

212 Triploid, tetraploid, pentaploid, hexaploid, and octoploid hybrids were obtained from intraploidy and  
213 interploidy crosses. Flowering was observed in three pentaploid plants and one hexaploid plant (strain:  
214 Hex-1).

215 Corolla length, pollen diameter, pollen germination, the size of guard cells, and the fresh  
216 weight of fruit were investigated in several individuals and their parents (Table 7). The corolla lengths of  
217 tetraploid, hexaploid, and octoploid plants were larger than those of diploids. Octoploid plant Oct-T1 had  
218 the largest flowers of all plants evaluated (larger by 43% compared to diploid), but the octoploids  
219 showed reduced growth and set no fruit. Pollen diameter tended to increase with the ploidy level (Fig. 4).  
220 Octoploid pollen diameter was 1.6× larger than that of diploid. A pollen germination rate of more than  
221 70% was observed in diploid (75.5%), tetraploid (72.7% to 87.3%), and hexaploid (81.4%) plants. In  
222 contrast, the pollen germination rate of pentaploid (15.4% to 24%) and octoploid plants (3.5%) was low.  
223 The size of guard cells tended to increase with the ploidy level (Fig. 5) being 1.63× larger in the  
224 octoploid than in the diploid. In external appearance, thick leaves were observed in pentaploid, hexaploid,

225 and particularly octoploid plants. Three pentaploid and one hexaploid plant set fruit (Fig. 6a, b). The fresh  
226 weight of the fruit was 1.4 g in the pentaploid (strain: Pen-No.12) and 0.8 g in the hexaploid (strain:  
227 Hex-1) plant.

228

### 229 3. 7. Evaluation of intraploid and interploid hybridization

230 Seeds from the  $4x \times 2x$  (seed parent:  $4x$ , pollen parent:  $2x$ ) cross contained developed endosperm (Fig. 7a).  
231 In contrast, the  $2x \times 4x$  cross (seed parent:  $2x$ , pollen parent:  $4x$ ) contained undeveloped endosperm (Fig.  
232 7b) and failed to produce viable seeds.

233 In the present study, the  $2x \times 2x$  and  $4x \times 4x$  crosses that produced with  $2m:1p$  ( $m$ : maternal  
234 genome;  $p$ : paternal genome) endosperm resulted in successful seed development. On the other hand, the  
235  $2x \times 4x$ ,  $4x \times 2x$ ,  $4x \times 6x$ , and  $6x \times 4x$  crosses that produced  $1m$ ,  $4m:1p$ ,  $4m:3p$ , and  $3m:1p$  endosperm,  
236 respectively, showed abnormal seed formation. The relationship between the embryo:endosperm genome  
237 ratio and germination efficiency in interploidy crosses is shown in Figure 8.

238

## 239 4. Discussion

240

241 Various interploidy cross combinations were tested and progeny was obtained from all except for the  $2x \times$   
242  $4x$ , which produced no viable progeny. The reciprocal cross with the tetraploid as female was successful.  
243 The  $2x$  by  $4x$  combination set few seeds and the seeds exhibited abnormal growth. Crosses between  
244 diploid and tetraploid plants often fail in other plant species because seeds develop abnormally and/or are  
245 nonviable (Haig and Westoby 1991; Ramsey and Schemske 1998). Low rates of seed set from crosses  
246 between diploid and tetraploid species of *L. caerulea* were also reported by Plekhanova (2000). In the  
247 present study, although the germination rate was substantially lower than that of other cross combinations,  
248 several triploid seedlings were obtained from the  $4x \times 2x$  cross. The seeds had endosperm (Fig. 7a),  
249 however, the  $2x \times 4x$  cross could not produce viable seeds and contained undeveloped endosperm (Fig.  
250 7b). The direction of the cross affects both the endosperm development and viability of progeny. Embryo  
251 rescue by immature seed culture produced triploid seedlings only in the  $4x \times 2x$  combination. In other  
252 words, production of triploid plants in reciprocal  $2x-4x$  crosses appeared to be successful only in one  
253 direction in Haskap, when the seed parent is  $4x$ .

254 Interploidy crosses often lead to abnormal seed development, followed by seed abortion (Scott  
255 et al. 1998). In *Solanum*, a 2:1 ratio of maternal to paternal genomes in the endosperm is necessary for  
256 normal endosperm development in intraspecific interploidy crosses (Johnston et al. 1980). In maize, Lin  
257 (1984) observed that normal seed development required a 2:1 maternal to paternal genome ratio in the  
258 endosperm, and suggested that the involvement of parentally imprinted genes is required in a 2m (m;  
259 maternal genome) : 1p (p; paternal genome) ratio. The results of interploidy crosses in *Arabidopsis*  
260 *thaliana* also suggested that different ratios of maternally and paternally expressed imprinted loci affect  
261 endosperm development (Scott et al. 1998).

262 In the present study, the  $2x \times 2x$  and  $4x \times 4x$  crosses that produced 2m:1p endosperm resulted  
263 in successful seed development. On the other hand, combinations  $2x \times 4x$ ,  $4x \times 2x$ ,  $4x \times 6x$ , and  $6x \times 4x$   
264 crosses that resulted in 1m:1p, 4m:1p, 4m:3p, and 3m:1p endosperm genome ratios, respectively, showed  
265 abnormal seed formation. Haskap, similarly to other species also appears to require a 2m:1p ratio in the  
266 endosperm for normal seed development. In particular, the  $2x \times 4x$  cross might be highly influenced by  
267 genetic regulation because germination was not observed despite embryo rescue by immature seed culture.  
268 Tiwari et al. (2010) reported that a paternal genomic imbalance particularly affects proliferation of the  
269 endosperm. The results obtained in the present study might indicate genomic imbalance in interploidy  
270 crosses as shown in Figure 8 The aberration from 2:3 (embryo:endosperm genome ratio) reduced  
271 germination frequency, confirming previous results.

272 In our study, the majority of plants (99%) obtained from the  $4x \times 4x$  cross were tetraploid, but a  
273 small proportion of hexaploid (0.5%) and octoploid (0.5%) plants were obtained. Similar results were  
274 previously reported in other plant species. For example, production of tetraploid (98.7–99.0%), hexaploid  
275 (0.3–0.7%), and octoploid (0.2–0.7%) progeny from a  $4x \times 4x$  cross were reported in intersectional  
276 crosses of *Primula* (Hayashi et al. 2007) due to the fertilization of unreduced gametes. In *Allium*  
277 *tuberosum*, hexaploid plants naturally occurred in open-pollinated seedlings of tetraploid plants (Sharma  
278 and Gohil 2013). These authors suggested that the hexaploid plant was produced from the fusion of  
279 reduced male and unreduced female gametes. Production of unreduced gametes is one mechanism of  
280 spontaneous polyploid formation, which has been identified in many plant species (Bretagnolle and  
281 Thompson 1995; Ramsey and Schemske 1998). Other mechanisms of spontaneous polyploid formation in  
282 plants are somatic doubling of meristem tissue, zygotes, or young embryos; and polyembryonic seeds

283 (Ramsey and Schemske 1998). The hexaploid and octoploid plants from the  $4x \times 4x$  cross in the present  
284 study may have resulted from one of these mechanisms.

285 The  $4x \times 8x$  cross produced hexaploid plants with aneuploid chromosome numbers ( $6x-2$ ,  $6x-3$ ,  
286 and  $6x-3$ ). These aneuploids might have originated via aneuploid pollen from the octoploid parent. The  
287 octoploid plant used in this study was produced from a colchicine-treated tetraploid (Takada 2001).  
288 Generally, autopolyploid plants have the potential to produce aneuploid gametes through irregular meiosis  
289 (Ramsey and Schemske 2002; Comai 2005).

290 In the present study, triploid, tetraploid, pentaploid, hexaploid, and octoploid plants were  
291 obtained from  $2x-4x$ ,  $4x \times 4x$ ,  $4x-6x$ , and  $4x \times 8x$  crosses. Polyploid seedlings were acclimated and  
292 grown in pots. Several pentaploid and hexaploid plants flowered and set fruits. Polyploid variation can  
293 provide phenotypic variation and introduce new perspectives in the breeding of Haskap. These different  
294 ploidy progenies will be utilized for analyses of sexual events during interploidy hybridization and  
295 provide new material for breeding.

296

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304

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412 **Table 1** Seed production, germination, and ploidy level of progeny in  $2x \times 2x$  cross

Cross combination (seed parent $\times$ pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds obtained	No. of seeds germinated	% of seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
$2x \times 2x$							
Di-K8 $\times$ Di-BeY	3	3	39	39	100.0	38	$2x$ (38)

413 Seeds harvested at 40–45 days after pollination were cultured.

414 **Table 2** Seed production, germination, and ploidy level of progeny in reciprocal 2x–4x crosses

Cross combination (seed parent × pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds obtained	No. of seeds germinated	% of seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
<i>2x × 4x</i>							
Di-K8 × Tet-Y16	10	9	84	0	0.0	–	–
Di-K8 × Tet-Y27	5	5	35	0	0.0	–	–
Di-K8 × Tet-Y37	10	9	81	0	0.0	–	–
<i>4x × 2x</i>							
Tet-Y16 × Di-K8	10	6	68	10	14.7	7	3x (5)
Tet-Y27 × Di-K8	5	3	45	0	0.0	–	–
Tet-Y37 × Di-K8	10	3	36	3	8.3	2	3x (2)

415 Seeds harvested at 40–45 days after pollination were cultured.

416 Dashes indicate no data.

417

418 **Table 3** Immature seed culture in reciprocal 2x–4x crosses

Cross combination (seed parent × pollen parent)	Days after pollination (DAP)	No. of fruits used	No. of immature seeds obtained	No. of immature seeds that germinated	% of immature seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
<i>2x × 4x</i>							
Di-K8 × Tet-Y27	14 DAP	5	31	0	0.0	–	–
	21 DAP	4	49	0	0.0	–	–
	28 DAP	4	27	0	0.0	–	–
<i>4x × 2x</i>							
Tet-Y27 × Di-K8	14 DAP	5	126	24	19.0	18	3x (11)
	21 DAP	4	101	38	37.6	27	3x (18)
	28 DAP	4	103	31	30.1	24	3x (12)

419 Dashes indicate no data.

420

**Table 4** Seed production, germination, and ploidy level of progeny in  $4x \times 4x$  cross

Cross combination (seed parent $\times$ pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds (immature seeds) obtained	No. of seeds (immature seeds) that germinated	% of immature seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
$4x \times 4x$							
Seed culture <sup>a</sup>							
Tet-Y27 $\times$ Tet-Y37	5	5	148	125	84.5	125	4x (125)
Tet-Y37 $\times$ Tet-Y27	5	5	71	67	94.4	67	4x (67)
Immature seed culture <sup>b</sup>							
Tet-Y27 $\times$ Tet-Y37	21	21	497	74	14.9	74	4x (72), 8x (2)
Tet-Y37 $\times$ Tet-Y27	15	15	250	73	29.2	73	4x (73)
Tet-Has1 $\times$ Tet-Y16	14	14	317	43	13.6	43	4x (41), 6x (2)

421 <sup>a</sup> Seeds harvested at 40–45 days after pollination (DAP) were cultured.422 <sup>b</sup> Immature seeds harvested at 14 to 28 DAP were cultured.

423 **Table 5** Seed production, germination, and ploidy level of progeny in reciprocal 4x–6x crosses

Cross combination (Seed parent × Pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds obtained	No. of seeds that germinated	% of seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
<i>4x × 6x</i>							
Tet-Y27 × Hex-1	4	4	65	49	75.4	43	5x (30)
<i>6x × 4x</i>							
Hex-1 × Tet-Y27	2	2	19	17	89.5	13	5x (13)

424 Seeds harvested at 40–45 DAP were cultured.

425 Dashes indicate no data.

426 **Table 6** Seed production, germination, and ploidy level of progenies in 4x–8x cross by immature seed culture

Cross combination (seed parent × pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of immature seeds obtained	No. of immature seeds that germinated	% of germination	No. of seedlings that survived	Ploidy level
<i>4x × 8x</i>							
Tet-Y2 × Oct-T1	5	5	37	0	0.0	–	–
Tet-Y27 × Oct-T1	8	8	84	2	2.4	2	$2n = 6x-3 = 51$ , $2n = 6x-2 = 52$
Tet-Y0049 × Oct-T1	1	1	10	1	10.0	1	$2n = 6x-4 = 50$

427 Dashes indicate no data.

428 Immature seeds harvested at 14 to 21 days after pollination were cultured.

429 **Table 7** Characterization of diploid, triploid, tetraploid, pentaploid, hexaploid, and octoploid plants

Strain	Corolla length (mm)	Pollen diameter ( $\mu\text{m}$ )	Pollen germination (%)	Length of guard cell ( $\mu\text{m}$ )	Fresh weight of fruit (g)
Diploid (2x)					
Di-K8	14.4 $\pm$ 0.3 c	55.4 $\pm$ 1.5 e	75.5 $\pm$ 8.8 a	21.0 $\pm$ 1.0 e	0.7 $\pm$ 0.1 (n = 10)
Triploid (3x)					
Tri-No.2	–	–	–	21.9 $\pm$ 1.3 e	–
Tri-No.3	–	–	–	21.7 $\pm$ 1.8 e	–
Tetraploid (4x)					
Tet-Y16	18.7 $\pm$ 0.5 b	67.0 $\pm$ 3.1 c	83.7 $\pm$ 2.3 a	23.8 $\pm$ 1.4 d	0.8 $\pm$ 0.1 (n = 10)
Tet-Y27	18.2 $\pm$ 0.7 b	60.1 $\pm$ 2.7 d	72.7 $\pm$ 12.5 a	23.7 $\pm$ 1.3 d	1.1 $\pm$ 0.1 (n = 10)
Tet-Has1	17.6 $\pm$ 0.4 b	61.4 $\pm$ 3.6 d	87.3 $\pm$ 7.7 a	23.3 $\pm$ 0.8 d	0.7 $\pm$ 0.2 (n = 10)
Pentaploid (5x)					
Pen-No.12	–	67.9 $\pm$ 4.5 c	24.0 $\pm$ 5.9 b	27.2 $\pm$ 1.1 c	1.4 $\pm$ 0.1 (n = 3)
Pen-No.21	–	66.5 $\pm$ 2.6 c	15.4 $\pm$ 4.1 bc	28.6 $\pm$ 1.4 b	–
Hexaploid (6x)					
Hex-1	17.7 $\pm$ 1.6 b	75.8 $\pm$ 4.1 b	81.4 $\pm$ 1.9 a	28.7 $\pm$ 1.9 b	0.8 $\pm$ 0.2 (n = 6)
Octoploid (8x)					
Oct-T1	20.6 $\pm$ 1.0 a	89.0 $\pm$ 9.2 a	3.5 $\pm$ 1.9 c	34.3 $\pm$ 2.5 a	–

430 Triploid plants were from the 4x  $\times$  2x cross (Tet-Y27  $\times$  Di-K8). Pentaploid plants were from the 4x  $\times$  6x cross (Tet-Y27  $\times$  Hex-1). Hexaploid plant was from the 4x  $\times$  4x cross  
 431 (Tet-Has1  $\times$  Tet-Y16).

432 Means  $\pm$  SD followed by the same letter are not significantly different (Bonferroni's test,  $p < 0.05$ ). Dashes indicate no data.

433 **Figure captions**

434 Fig. 1. Histograms of the relative fluorescence intensity of nuclei isolated from seedlings of interploidy crosses.  
435 (a) Seedling (3Cx) obtained from  $4x \times 2x$  cross. (b) Seedling (5Cx) obtained from  $4x \times 6x$  cross. (c) Seedling  
436 (6Cx) obtained from  $4x \times 8x$  cross.

437  
438 Fig. 2. Chromosomes in root-tip cells of plants derived from interploidy crosses. (a) Triploid ( $2n = 3x = 27$ ). (b)  
439 Hexaploid ( $2n = 6x = 54$ ). (c) Pentaploid ( $2n = 5x = 45$ ). (d) Aneuploid ( $2n = 6x - 4 = 50$ ). (e) Aneuploid ( $2n =$   
440  $6x - 3 = 51$ ). (f) Aneuploid ( $2n = 6x - 2 = 52$ ). Bar = 5  $\mu$ m.

441  
442 Fig. 3. Triploid, pentaploid, hexaploid, and octoploid plants of *Lonicera caerulea*. (a) Triploid plants obtained  
443 from  $4x \times 2x$  cross, 3 months after acclimation. (b–d) Pentaploid plants from  $4x \times 6x$  cross (strain: Pen-No.9,  
444 Pen-No.12, and Pen-No.21), 2 years old. (e) Hexaploid plant (strain: Hex-1), 4 years old. (f) Hexaploid plant  
445 (strain: Hex-2), 4 years old. (g) Aneuploid ( $2n = 6x - 4 = 50$ ) plant from  $4x \times 8x$  cross. (h) Aneuploid ( $2n = 6x -$   
446  $3 = 51$ ) plant from  $4x \times 8x$  cross. (i) Aneuploid ( $2n = 6x - 2 = 52$ ) plant from  $4x \times 8x$  cross showing growth  
447 suppression. All aneuploid plants are 3 years old. (j) Octoploid plant from  $4x \times 4x$  cross, 1 year old. Bar = 10 cm.

448  
449 Fig. 4. Pollen grains of diploid, tetraploid, pentaploid, hexaploid, and octoploid plants. (a) Diploid (strain:  
450 Di-K8). (b) Tetraploid (strain: Tet-Y27). (c) Pentaploid (strain: Pen-No.12) from  $4x \times 6x$  cross (Tet-Y27  $\times$   
451 Hex-1). (d) Hexaploid (strain: Hex-1). (e) Octoploid (strain: Oct-T1). Bar = 50  $\mu$ m.

452  
453 Fig. 5. Guard cells of diploid, triploid, tetraploid, pentaploid, hexaploid, and octoploid plants. (a) Diploid (strain:  
454 Di-K8). (b) Triploid (strain: Tri-No. 3) from  $4x \times 2x$  cross (Tet-Y27  $\times$  Di-K8). (c) Tetraploid (strain: Tet-Y27).  
455 (d) Pentaploid (strain: Pen-No.12) from  $4x \times 6x$  cross (Tet-Y27  $\times$  Hex-1). (e) Hexaploid (strain: Hex-1). (f)  
456 Octoploid (strain: Oct-T1). Bar = 20  $\mu$ m.

457  
458 Fig. 6. Fruits of pentaploid and hexaploid plants. (a) Pentaploid (strain: Pen-No.9). (b) Hexaploid (strain: Hex-1).  
459 Bar = 1cm.

460  
461 Fig. 7. Seed development in  $4x \times 2x$  and  $2x \times 4x$ . (a) A seed from Tet-Y27 ( $4x$ )  $\times$  Di-K8 ( $2x$ ) 40 days after  
462 pollination. A seed coat is removed for observation. Developing endosperm (arrow) is exposed. (b) A seed from



463 Di-K8 (2x) × Tet-Y27 (4x) 40 days after pollination. No endosperm development is observed. Bars = 1 mm.

464

465 Fig. 8. The relationship between the embryo/endosperm genome ratio and germination efficiency in interploidy

466 crosses.

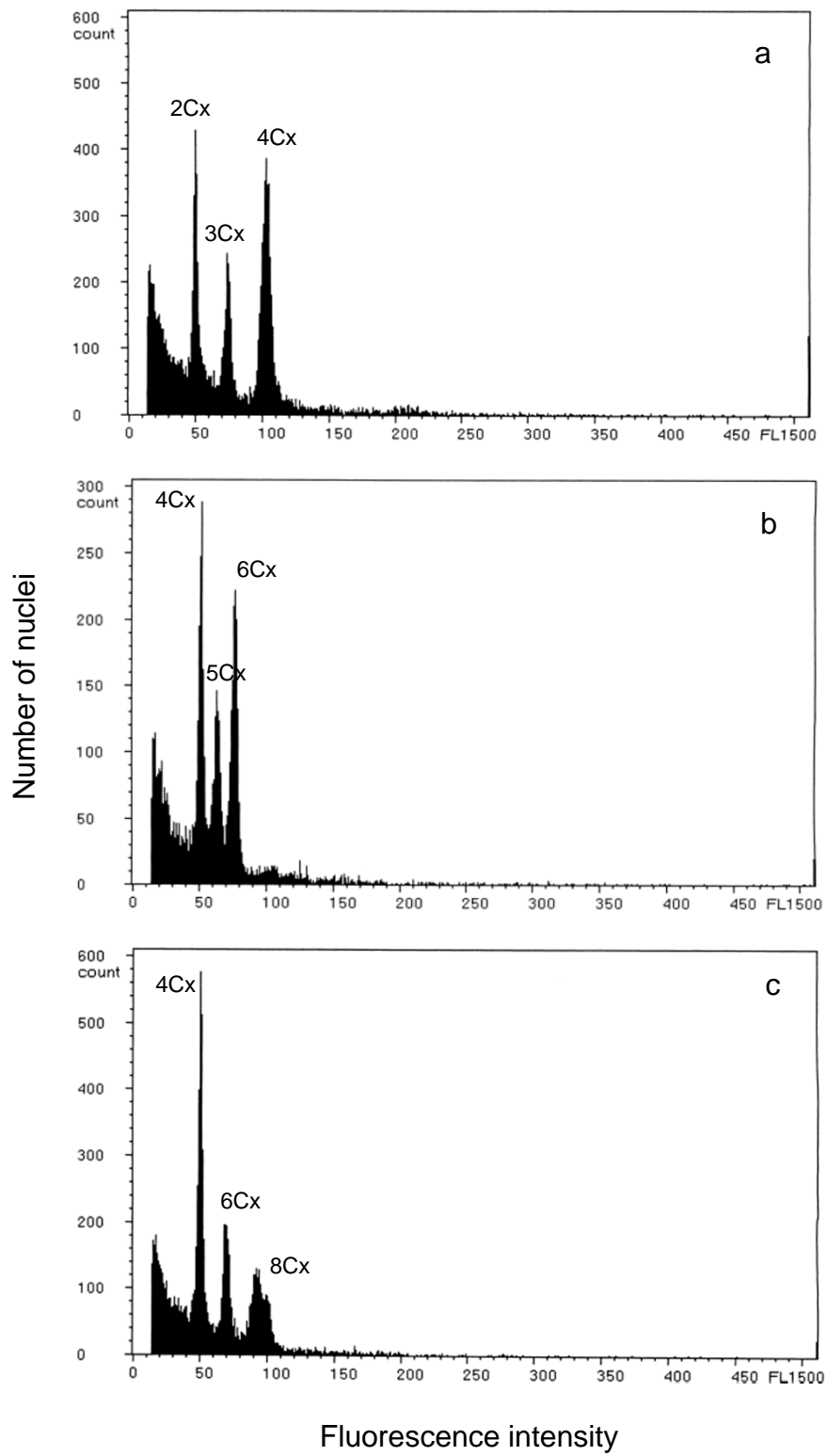


Figure 1

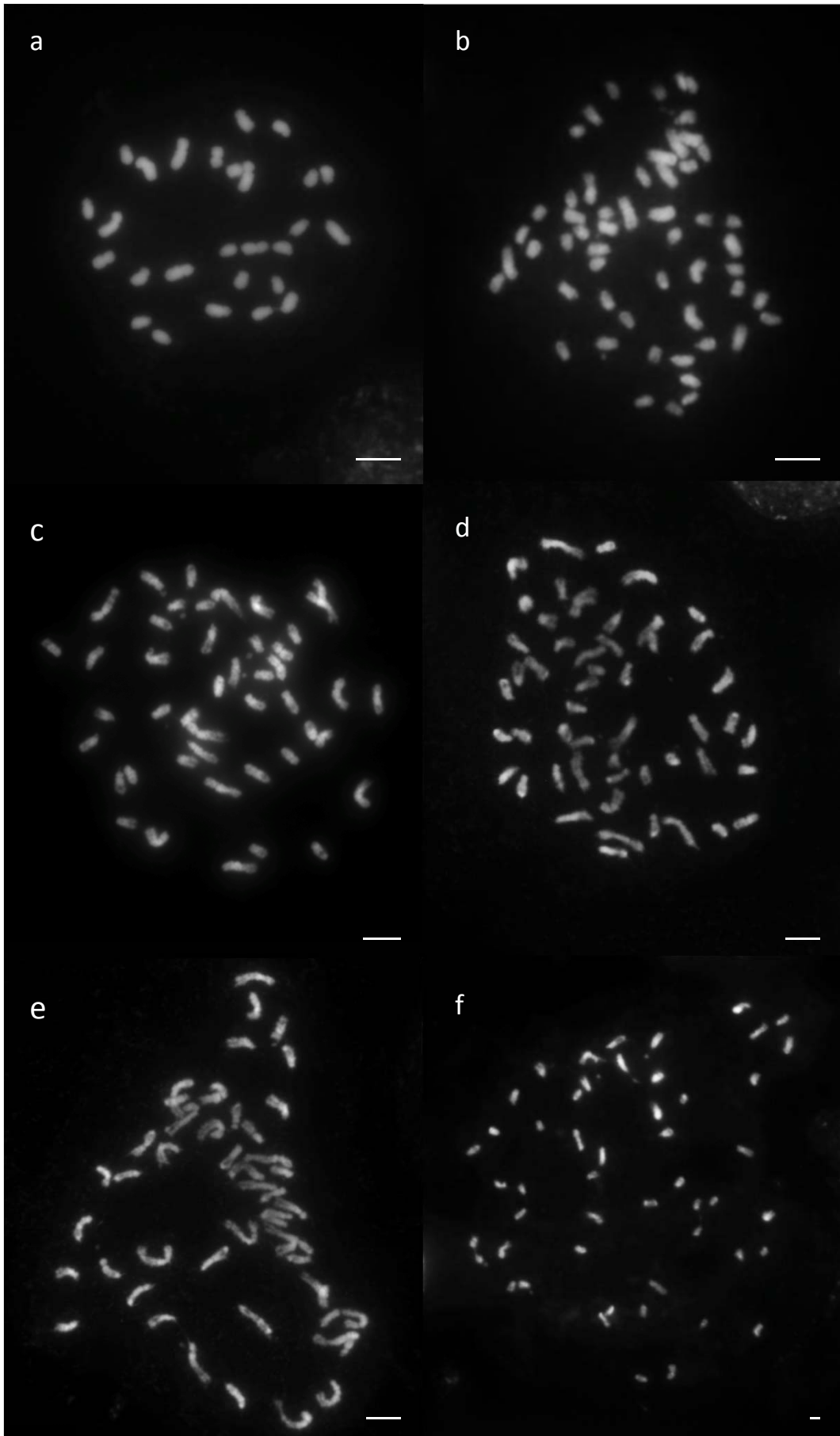


Figure 2



Figure 3

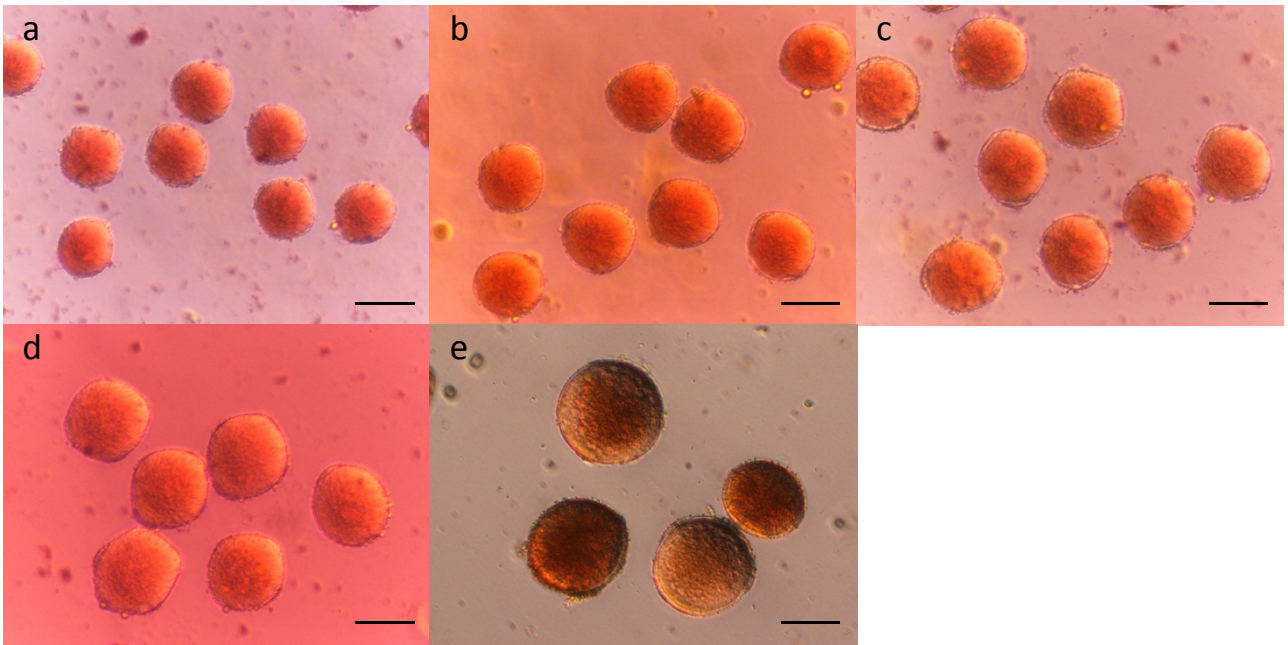


Figure 4



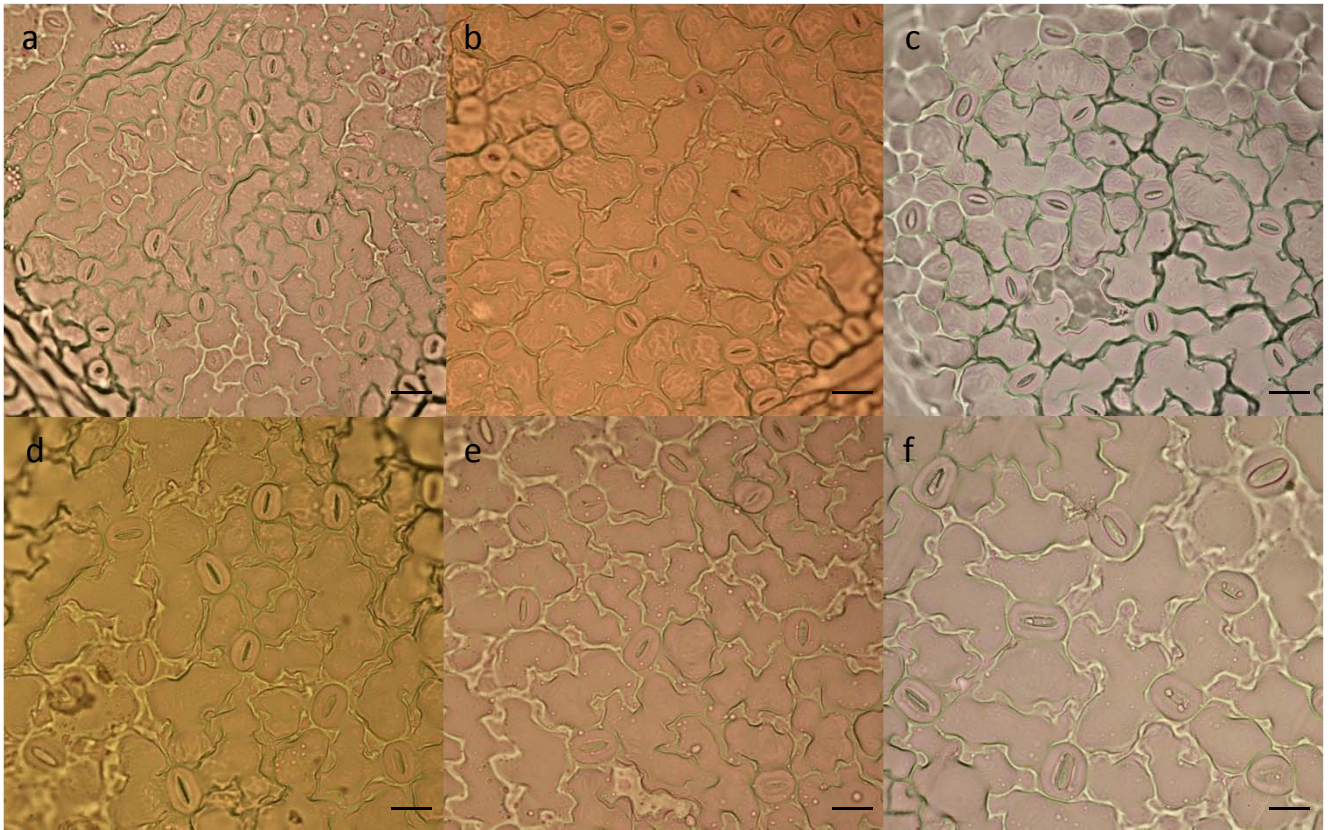


Figure 5



Figure 6

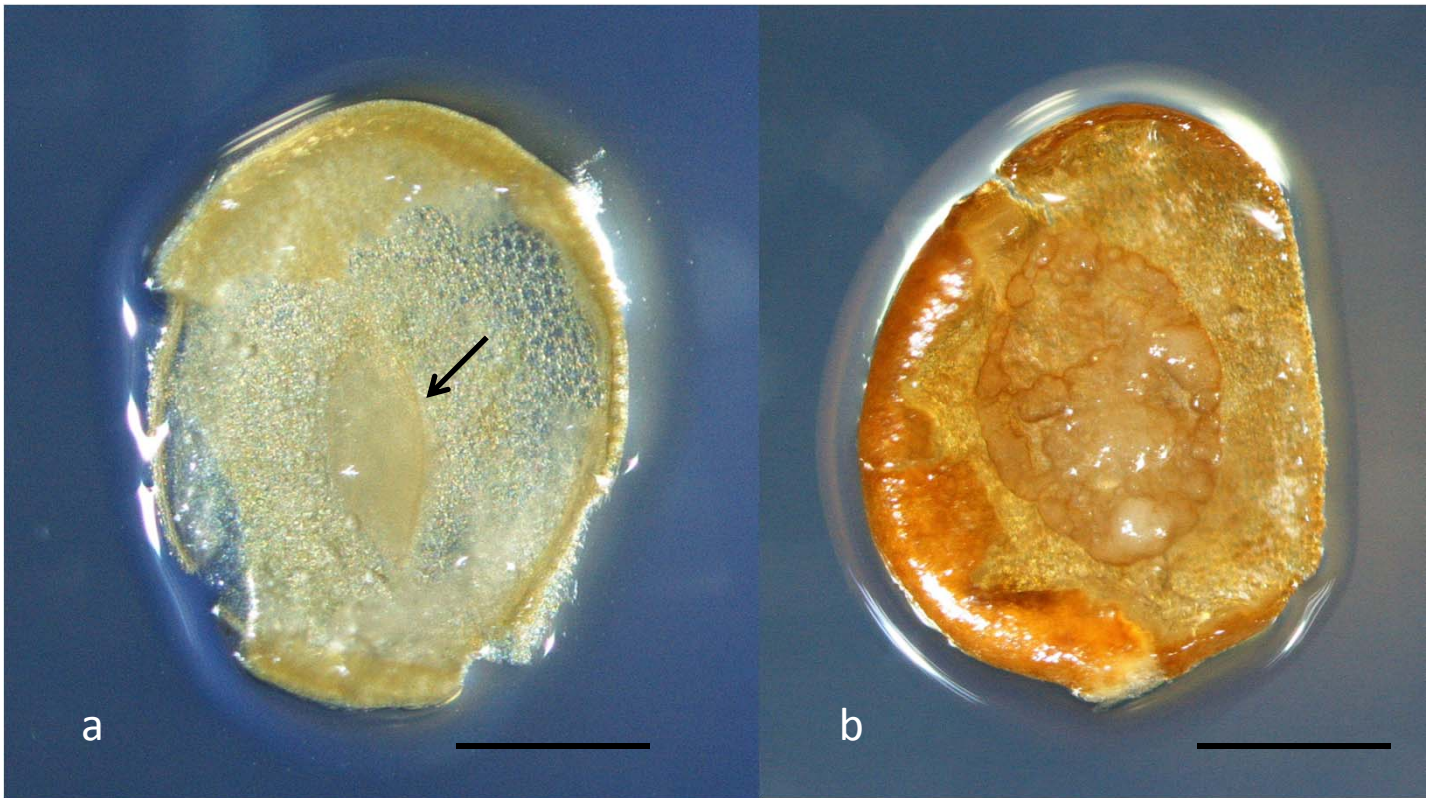


Figure 7



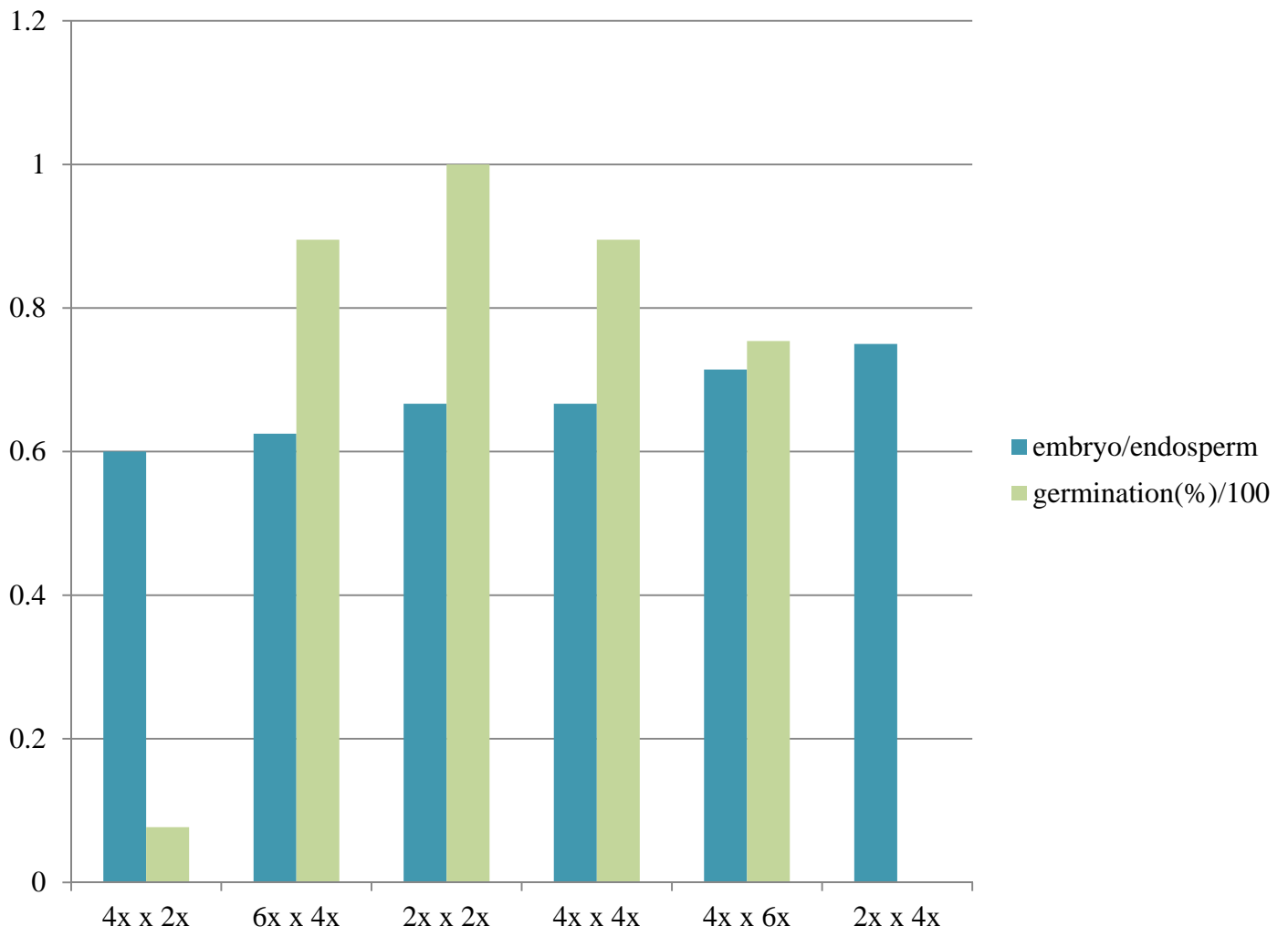


Figure 8