Original Research Production of Haploids and Doubled Haploids from Unfertilized Ovule Culture of Various Wild Species of Gentians (Gentiana spp.)

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Original Article

Keywords: Gentiana, Wild species, Unfertilized ovule culture, Bud stage, Doubled haploid, Molecular marker

Posted Date: February 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-181197/v1

Original research

Production of haploids and doubled haploids from unfertilized ovule culture of various wild species of gentians (*Gentiana* spp.)

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Abstract

The production of haploids and doubled haploids (DHs) on unfertilized ovule culture was examined in 19 wild species of gentians (*Gentiana* ssp.) classified into eight sections. Of the 19 species including 35 strains, embryo-like structures (ELSs) were obtained in 15 species, and regenerated plants were produced in 11 species. ELS production has varied greatly among the15 species, i.e., 0.5%-79.2% frequency of responding flower buds and 0.01-1.99 ELSs per flower bud. Of the ELS-producing species, almost all were classified into the sects. *Pneumonanthe* or *Cruciata*. Species in sect. *Pneumonanthe* showed higher responses than those in sect. *Cruciata*. In examining the effect of flower bud stage on ELS production, more than twice as many ELSs were observed at the anther-dehiscent stage than that at the anther-indehiscent stage. Ploidy level was determined in 117 randomly selected regenerated plantlets, which suggests that most were haploid (32.5%) and diploid (46.2%). When 12 diploid plants were examined using simple sequence repeat (SSR) markers, 8 (66.7%) were DH. This study revealed that unfertilized ovule culture can be applied not only on cultivated gentian species but also on a number of wild species. The production of haploids and DHs in wild gentians provides novel prospects for ornamental and/or medicinal gentian breeding.

Key Message

This is the first report of a successful haploid and doubled haploid production by unfertilized ovule

35 culture of various wild species of gentians (*Gentiana* spp.).

36

37 **Key Words:** *Gentiana*, Wild species, Unfertilized ovule culture, Bud stage, Doubled haploid,

38 Molecular marker

39

40

41 **Introduction**

42 Species of the genus *Gentiana* have long been used as traditional medicine in Eurasia and South

43 America. Currently, they are more widely used as ornamental plants such as garden plants, cut flower

44 and pot plants. In Japan, the commercial cultivation of gentians for use as cut flowers began in the

45 1950s from two Japanese-endemic species, that is, *G. scabra* and *G. triflora*. Since the first F1

46 hybrid cultivar was developed in 1977, more than 300 F1 and clonal cultivars have been developed

47 (Nishihara et al. 2017). In the 1980s, these gentian cut flowers were introduced from Japan into

48 Europe as new ornamental plants. However, compared with the major ornamental plants worldwide

49 such as chrysanthemum, rose and carnation, gentians had limited variation in several traits of flower

50 and plant morphology, which could be a result of their short breeding history and narrow genetic

51 resources based on two cultivated species of *G. scabra* and *G. triflora*.

52 Wild plant species have been useful for developing novel varieties and broadening the
53 genetic base of crops. The genus *Gentiana* has been comprised of 15 sections and about 360 species
54 (Ho and Liu 2001), and a number of species exhibit wide variation in traits such as flower color,
55 flower shape, flowering time, plant architecture, etc. (Kohlein 1991). Wide hybridization between
56 cultivated crops and their wild relatives has been widely utilized to introduce genetic variation and
57 develop new varieties in commercial crops including ornamental plants (Van Tuyl and De Jeu 1997,
58 Kuligowska et al. 2016). In gentians, a limited number of interspecific hybrids between cultivated
59 gentians and wild species have been reported (Morgan 2004, Tamagake et al. 2014). Recently,
60 however, Takamura et al. (2019) were able to successfully produce many hybrids with wild species,
61 indicating the great potential of wild gentians in terms of breeding. In addition, homozygous inbred
62 lines developed from original heterogenous wild relatives are suggested to be more valuable than
63 their original plant population for crop improvement and genome research such as genetic mapping
64 and understanding the inheritance of useful traits (Melchinger et al. 2017). In gentians, production of
65 homozygous lines of wild species is also considered important in order to develop not only excellent
66 new ornamental varieties but also high-value medicinal plants, the breeding of which has thus far
67 been neglected (Ferrie 2007, Foster et al. 2007).
68 In cultivated gentians, uniform inbred lines have been difficult to obtain through recurrent

69 selfing of conventional methods because of severe inbreeding depression, however doubled haploid
70 (DH) technology has been developed using in vitro culture of anther (androgenesis) and unfertilized
71 ovule (gynogenesis) to address this issue (Doi et al. 2010, 2011, 2013, Pathirana et al. 2011). Further,
72 the latter has more advantages than the former in terms of the high frequencies of embryogenesis and
73 haploid/DH production and small influence on genotypes (Doi and Takahata 2015). Although
74 unfertilized ovule culture is an effective tool for obtaining DHs of cultivated gentians (*G. scabra* and
75 *G. triflora*), it is not yet known whether this technique can be used with wild gentians.
76 In this study, we are the first to report a successful production of embryo-like structures
77 (ELSs) and plant regeneration (haploid and DH plants) from unfertilized ovule culture of various
78 wild gentian species. In addition, the influence of ovule developmental stage on gynogenesis was
79 examined. Moreover, regenerated plants were characterized using flow cytometry and molecular
80 genetic markers.
81
82 **Materials and Methods**
83 **Plant materials**
84 Nineteen species including 35 strains of gentians (*Gentiana* spp.), which are classified into eight
85 sections, were used in this study (Table 1). All materials were grown in an experimental field and a

86 greenhouse at Hachimantai City Floricultural Research and Development Center, Hachimantai, Iwate,
87 Japan.
88
89 **Unfertilized ovule culture**
90 Unfertilized ovule culture was performed as described by Doi et al. (2011) with minor modifications.
91 Flower buds, in two developmental stages, i.e., anther-indehiscent and anther-dehiscent stages, were
92 used for culture, except in the case of *G. lutea* (Fig. 1ab). In both stages, anthers extend above the
93 stigma, and though anthers are dehiscent, fertilization is not successful because the top of the stigma
94 is closed (immature pistil). In *G. lutea*, on the other hand, the top of the stigmas is observed to be
95 open (mature pistil) when the anthers are dehisced, therefore, only flower buds with indehiscent
96 anthers were used in this species.
97 The flower buds were harvested from donor plants and immediately stored at 4°C in the
98 dark until they were used for culture. After the petals and stamens were removed, the pistils were
99 surface-sterilized in 70% ethanol for 30 sec followed by sodium hypochlorite solution containing
100 2.0% active chlorite for 15 min, and then rinsed three times with sterile distilled water for 5 min each
101 time. Ovules were excised from a pistil and cultured on a 60-mm plastic Petri dish containing 0.8%
102 agar-solidified 1/2 NLN medium (Takahata and Keller 1991) supplemented with 10% sucrose (Fig.

1c). The Petri dishes were maintained in an incubator at 25°C under dark conditions. The ELSs developed from the ovules were then transferred to plant regeneration medium containing modified 0.8% agar-solidified MS medium (Murashige and Skoog 1962) with the concentration of major salts reduced by 50% (1/2MS) supplemented with 3% sucrose and 1.0 mg/l GA_3. They were then incubated at 20°C with a 16-h day photoperiod. After acclimatization, the regenerated plants were transferred to 2:2:1 akadama-peat moss-soil and grown in a greenhouse.

For statistical analysis, data were subjected to analysis of variance followed by the Tukey–Kramer HSD test and the Student's t-test using the computer program JMP 8.0 (SAS Institute Inc., USA).

Determination of ploidy levels by flow cytometry and molecular marker analysis

The ploidy level of regenerated plantlets was analyzed using CyFlow Ploidy Analyser (Sysmex Partec GmbH, Germany). Nuclear DNA was extracted from fresh young leaf tissue (Approx. 25 mm^2). The leaves were chopped using a razor blade in a plastic Petri dish, which contained the nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Sysmex Partec GmbH). After filtration through a 30-μm nylon sieve, a staining solution of 4',6-diamidino-2-phenylindole (DAPI) (CyStain UV Precise P Staining Buffer) was added. The nuclei mixture was then

120 analyzed using CyFlow Ploidy Analyser.

121 The homozygosity of the obtained diploid plants obtained was examined using simple
122 sequence repeat (SSR) markers (Sato-Ushiku et al. 2011). Total DNA was extracted from leaves
123 using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Polymerase
124 chain reaction (PCR) of SSR markers was carried out using a PCR Thermal Cycler Dice (Takara Bio
125 Inc., Shiga, Japan) in a 20 μl volume containing 50 ng template DNA, 200 μM dNTP, 0.25 U Ex *Taq*
126 DNA polymerase (Takara Bio Inc.), 0.5 μM primer, and 1×Ex *Taq* Buffer. The sequences of primers
127 (8 SSRs) used in this study, which were selected from 14 SSR markers, are listed in Supple. Table 1.
128 The PCR conditions included an initial denaturation step at 94°C for 2 min followed by 35 cycles of
129 95°C for 20 sec, 60°C for 40 sec, 72°C for 1 min, and finally an extension at 72°C for 5 min. The
130 amplified SSR marker products were electrophoresed on a 4% agarose gel and stained with ethidium
131 bromide.

132

133 **Results**

134 **ELS production and plant regeneration in wild gentians**

135 After 1-2 months of culture, spherical ELSs were observed emerging from the ovules (Fig. 1d).
136 These ELSs regenerated to plantlets after being transferred to regeneration medium (Fig. 1e),

137 however, some ELSs have stopped growing and failed to develop to plantlets. After acclimatization,
138 regenerants grew normally and flowered into soil (Fig. 1f).
139 The ELS production and plant regeneration from unfertilized ovule culture of 19 wild
140 gentian species containing 35 strains is shown in Table 2. Two kinds of developmental stages of
141 flower buds (anther-indehiscent and anther-dehiscent stages) were used together in this experiment.
142 Of these 19 species, ELSs were obtained from 15 species, excluding *G. squarrosa*, *G. tibetica*, *G.*
143 *algida* and *G. cachemirica*. Almost all species that produced ELSs were classified into the sects.
144 *Cruciata* or *Pneumonanthe.* Especially*, G. parry* and two strains of *G. septemfida* classified in sect.
145 *Pneumonanthe* showed the highest frequencies of responding flower buds (65.4% and 60.4-79.2%,
146 respectively) and number of ELSs per flower bud (1.50 and 1.67-1.99, respectively). Except for *G.*
147 *tibetica*, all species classified in sect. *Cruciata* were able to produce ELSs, ranging from 0.09-0.40
148 ELSs per flower bud. Genotypic variation within a species was also observed in ELS production, that
149 is, *G. cruciata*, *G. pneumonanthe* and *G. septemfida* showing 0.06-0.36, 0.00-0.78 and 0.07-1.99
150 ESLs per flower bud, respectively. Although *G. oschtenica* (sect. *Calathianae*), *G. lutea* (sect.
151 *Gentiana*), *G. sino-ornata* (sect. *Kudoa*) and *G. purpurea* (sect. *Microsperma*) produced small
152 numbers of ELSs, they could not produce plantlets.
153 When a total of 561 ELSs obtained were transplanted to the regeneration medium, 212

154 plants were regenerated (Table 2). Although ELSs were obtained from 15 species, regenerated plants
155 were obtained from 11 species, all of which were classified in two sects. *Cruciata* and
156 *Pneumonanthe*.
157
158 **Effect of flower bud stage**
159 The effect of flower bud stage (anther-indehiscent vs. anther-dehiscent stage) on ELS production and
160 plant regeneration was examined over two years using three species (*G. gracilipes*, *G. asclepiadea*,
161 and *G. septemfida*) including 6 strains (Fig. 2). Generally, the ovules excised from the buds in the
162 anther-dehiscent stage exhibited more than twice as many ELSs than those in the anther-indehiscent
163 stage. Significantly more ELSs were produced in *G. septemfida* '7-131-1' in 2017 and in *G.*
164 *septemfida* '7-131-2' in 2018. In *G. asclepiadea*, ELSs and regenerated plants were obtained only
165 when flower buds with dehiscent anthers were used for over two years. Within-species genotypic
166 variation on ELS production was observed in *G. septemfida*.
167
168 **Ploidy levels of regenerated plants and molecular marker analysis**
169 The ploidy levels of 117 regenerated plants, which were randomly chosen from 333 regenerants
170 obtained in two experiments (Table 2, Fig. 2), were examined using flow cytometry (Fig. 1gh, Table

171 3). Of these 117 plants, 38 (32.5%) were determined to be haploid, and 54 (46.2%) were diploid
172 (Table 3). Haploids were obtained in all species except *G. olivieri* and *G. paradoxa*. In addition to
173 haploids and diploids, a small number of triploids (5.1%), tetraploids (5.1%), and chimera (11.1%)
174 were also confirmed in several species. To determine whether the diploid plants obtained were DHs,
175 molecular marker analysis was carried out using two strains of *G. septemfida*. When six diploid
176 plants of strain '7-131-2' were examined using six SSR markers, which amplified two codominant
177 bands in the donor plant, all diploid plants showed a single band (Fig. 3b, Table 4). On the other hand,
178 when six diploids of '7-131-1' were examined using five SSRs, two diploid plants (29-3098,
179 30-3479) showed a single band in all SSRs, whereas four plants had the same two bands as the donor
180 plant in one or two SSRs (Fig. 3a, Table 4).
181
182 **Discussion**
183 In this study, we were able to succeed in obtaining ELSs in 15 species of *Gentiana* and regenerated
184 plants in 11 species. These results demonstrate that unfertilized ovule culture can be widely applied
185 not only on cultivated gentians but also on wild species. However, culture responses varied among
186 species. High production of ELSs was shown in species classified in two sects. *Pneumonanthe* and
187 *Cruciata*, especially, species in the former sect. exhibited more ELSs than those in the latter. These

188 results may have been due to the culture methods used and species relationships. The culture
189 protocol used in this study was developed for cultivated gentians (*G. scabra* and *G. triflora*) (Doi et
190 al. 2011, 2013), which are classified in sect. *Pneumonanthe*. Furthermore, the two sects.
191 *Pneumonanthe* and *Cruciata* have been determined to be closely related according to molecular
192 phylogeny (Yuan et al. 1996, Mishiba et al. 2009) and interspecific hybridization (Takamura et al.
193 2019). Species classified in other sections produced small numbers of ELSs or none at all, however,
194 recovery of plants could not be achieved. In other crop species, including onion, sugar beet, and
195 cucurbit, successful production of regenerants in unfertilized ovule/ovary culture was reported to be
196 dependent on several factors such as genotype, culture media, culture conditions, stress pretreatment,
197 the developmental stage of the embryo sac, etc. (Gurel et al. 2000, Bohanec 2009, Chen et al. 2011,
198 Dong et al. 2016). Further research will be necessary to identify the optimum conditions for the
199 production of ELSs and regenerants in the unsuccessful *Gentiana* species.

200 Within-species genotypic variation in ELS production was observed in several wild species
201 such as *G. cruciata*, *G. dahurica*, *G. pneumonanthe*, and *G. septemfida*, which was consistent with
202 the results reported for cultivated gentians (Doi et al. 2011, 2013). Donor plant genotype has been
203 reported to significantly influence haploid and DH production via gynogynesis in many other crops
204 (reviewed by Mukhambetzhanov 1997, Keller and Korzun 1996, Bohanec 2009, Chen et al. 2011).

205 Although genotypic variation and selected responsive genotypes have frequently been reported, very
206 few studies have examined the genetics and/or mechanism of gynogenesis. In an ongoing study, we
207 are examining the mode of inheritance of ELS production from unfertilized ovule culture, wherein
208 we have found that recessive genes have positive effects on ELS production. These detail results will
209 be reported elsewhere.

210 Although both developmental stages of flower buds showed gynogenic responses, the later
211 stage of flower buds (anther-dehiscent) was more responsive. In this study, the anther-dehiscent stage
212 corresponded to the day of anthesis or just before, whereas the anther-indehiscent stage corresponded
213 to a few days before anthesis. Our results have been consistent with those reported by Doi et al.
214 (2011), who examined the gynogenetic responses of six developmental stages of flowers and found
215 that the later stages (just before anthesis or flower anthesis) were more responsive than bud stages in
216 cultivated gentians. These results for gentians are also observed for other crops, of which the
217 explants from just before anthesis or at anthesis (embryo sac is mature) show the highest gynogenic
218 response (Chen et al. 2011, Dong et al. 2016, Khan et al. 2020, Mukhambetzhanov 1997).

219 The ploidy level of regenerated plants differed among species and/or strains, however, the
220 117 regenerants tested were dominated by haploids (32.5%) and diploids (46.2%). Similar results
221 have been reported in cultivated gentians, in which more than 80% of regenerants were haploid and

222 diploid (Doi et al. 2011, 2013, Pathirana et al. 2011). By determining the homozygousity of 12
223 diploid plants derived from two strains of *G. septemfida* using SSR markers, we found that 8 (66.7%)
224 were DHs. However, the DH frequency varied, depending on the genotype; 100% and 33.3% for the
225 '7-131-2' and '7-131-1' strains, respectively. In the six diploids of '7-131-1', two plants showed
226 homozygosity in all SSR loci examined, whereas four had some SSR loci showing homozygosity
227 and some identical to donor plants. The reasons why homozygous and heterozygous SSR loci are
228 present in a diploid remains to be unknown. However, we speculate that such phenomenon may be
229 due to somaclonal variation during the culture period and the presence of multiple amplification sites
230 of equal size using identical SSR primers. In the present study, the diploids showing both
231 homozygous and heterozygous SSR loci were not identified as DH, thus further examination of S1
232 progenies obtained by self-pollinating are needed. In our analysis, 5.1% of tetraploids were obtained,
233 and tetraploid plants have been considered to be essential for breeding new cultivars in gentians such
234 as triploid hybrid cultivars.

235 The present study demonstrated that unfertilized ovule culture can be applied to not only
236 cultivated gentians but also various wild species. These results are expected to contribute not only to
237 the breeding of ornamental and/or medicinal gentians but also to genomics research. Unfertilized
238 ovule culture using other wild species and strains, the establishment of an effective culture method,

239 and breeding using DHs obtained from this study, are currently being carried out.

240

241 **Acknowledgements**

242 We wish to thank Ms Motoko Tsukazaki, Iwate University for assistance with flow cytometry and
243 tissue culture. This work was partially supported by grants from the Project of the NARO
244 Bio-oriented Technology Research Advancement Institution (Research program on development of
245 innovative technology).

246

247 **Author contributions**

248 YT and YT conceived and designed this research. YT performed the experiments. RT and TH
249 collected and maintained plant materials. KH provided advice on experimental implementation and
250 manuscript. YT and YT wrote the manuscript.

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322
323

Figure Legends

Fig. 1 Embryo like structure (ELS) induction and plant regeneration from unfertilized ovule culture of wild gentian (*G. septemfida*). **a** Gentian flower at the time of harvest. **b** Two stages of flower buds; anther-indehiscent stage (left) and anther-dehiscent stage (right). **c** Ovules cultured on 1/2 NLN solid medium. **d** A ELS emerged from ovules; **e** A plantlet regenerated from ELS. **f** A regenerated plant. **g**, **h** Flow cytometoric analysis; **g** showing a haploid, **h** showing a diploid. Bars in **a** = 50 mm, **b**, **c**, **e** = 10 mm, **d** = 1 mm, **f** = 30 mm.

Fig. 2 Effect of flower bud stage (anther-indehiscence vs. anther-dehiscence) on ELS production and plant regeneration in unfertilized ovule culture of wild gentians over two years (2017 and 2018) examinations. **a, c** No. of ELSs per flower bud. **b, d** No. of regenerated plants per flower bud. **a, b** 2017 and **c, d** 2018. Error bars present the standard error (SE). Asterisks indicate statistically significant differences at the 0.05 level according to Student's *t*-test.

Fig. 3 Analysis of genetic constitution of diploid plants derived from unfertilized ovule culture in *G. septemfida*. **a** *G. septemfida* '7-131-1' analyzed using Gtm12 SSR marker. **b** *G. septemfida* '7-131-2' nalyzed using Gtm68 SSR marker. M: 100bp molecular marker.

Figures

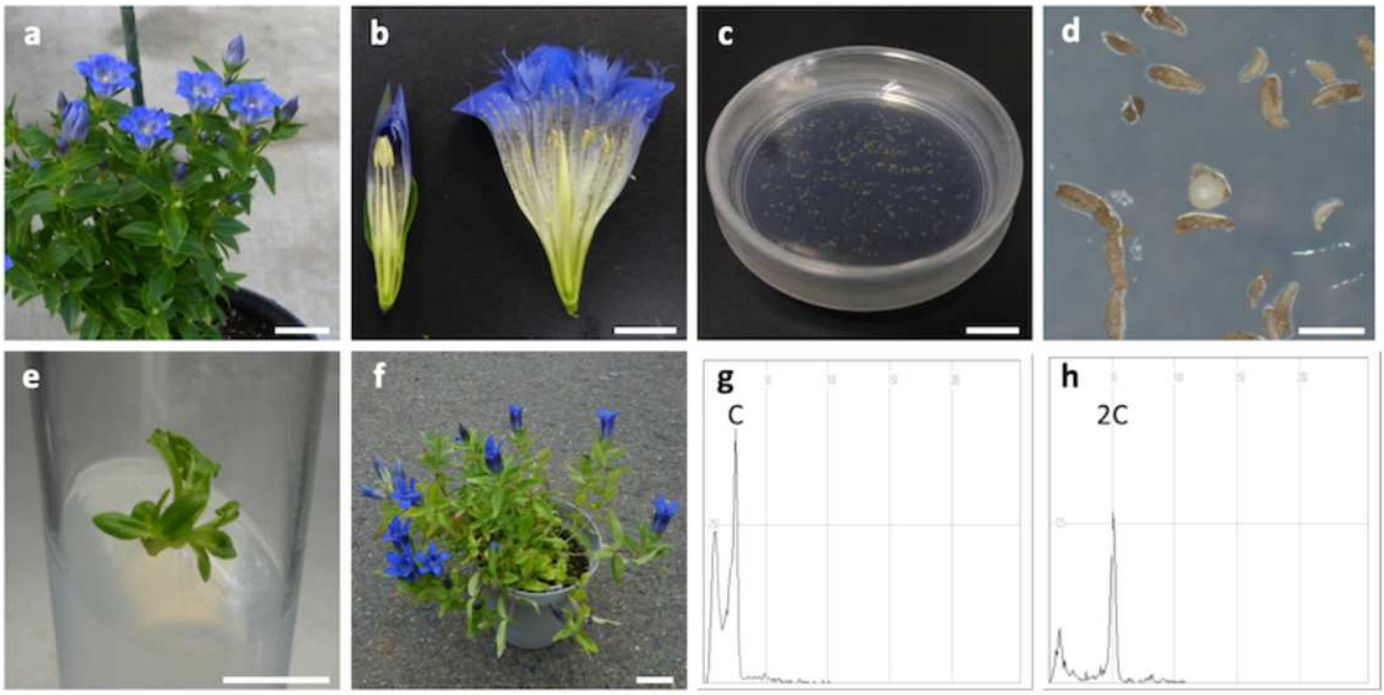

Figure 1

Embryo like structure (ELS) induction and plant regeneration from unfertilized ovule culture of wild gentian (G. septemfida). a Gentian flower at the time of harvest. b Two stages of flower buds; anther-indehiscent stage (left) and anther-dehiscent stage (right). c Ovules cultured on 1/2 NLN solid medium. d A ELS emerged from ovules; e A plantlet regenerated from ELS. f A regenerated plant. g, h Flow cytometoric analysis; g showing a haploid, h showing a diploid. Bars in a = 50 mm, b, c, e = 10 mm, d = 1 mm, f = 30 mm.

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Figure 2

Effect of flower bud stage (anther-indehiscence vs. anther-dehiscence) on ELS production and plant regeneration in unfertilized ovule culture of wild gentians over two years (2017 and 2018) examinations. a, c No. of ELSs per flower bud. b, d No. of regenerated plants per flower bud. a, b 2017 and c, d 2018. Error bars present the standard error (SE). Asterisks indicate statistically significant differences at the 0.05 level according to Student's t-test.

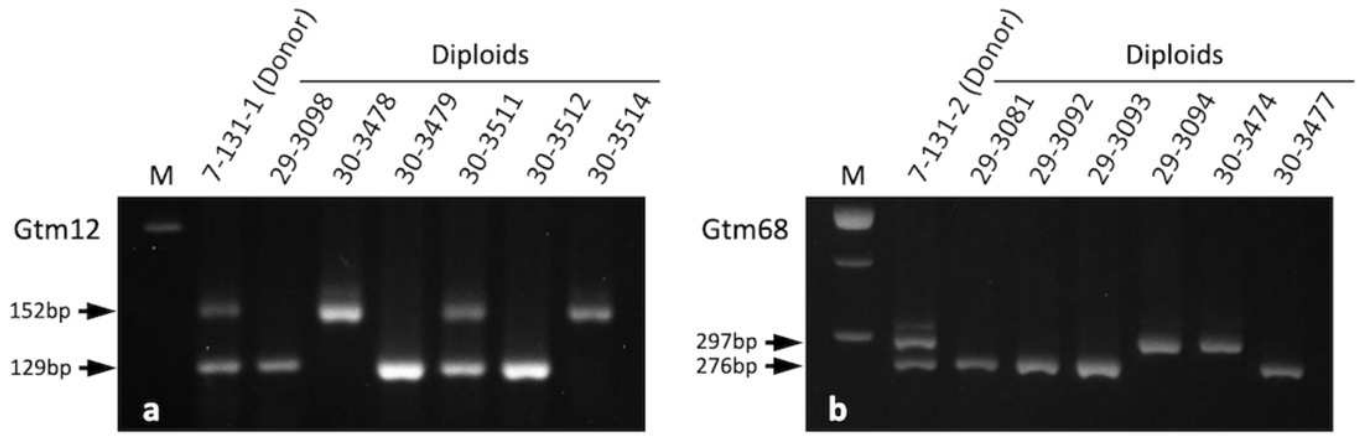

Figure 3

Analysis of genetic constitution of diploid plants derived from unfertilized ovule culture in G. septemfida. a G. septemfida '7-131-1' analyzed using Gtm12 SSR marker. b G. septemfida '7-131-2' nalyzed using Gtm68 SSR marker. M: 100bp molecular marker.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Table4.pdf
- Table3.pdf
- Supple.Table1.pdf