Gentian is one of the important ornamental plants for cut flowers and pot plants in Japan. The cultivation of gentian (Gentiana spp.) in Japan began in the 1950s through transplanting native wild plants of G. scabra and G. triflora in the field (Yoshiike 1992). The F1 hybrid cultivar ‘Iwate’ (G. triflora) was first developed in 1977. Since then, more than 300 F1 and clonal cultivars have been produced mainly by conventional crossbreeding using intra-specific and inter-specific crosses of two cultivated species of G. scabra and G. triflora (Nishihara et al. 2018). In Europe, other endemic gentian species have been ornamentally used in rock gardens and garden borders. The cultivation for cut flowers of gentian was introduced from Japan in the 1980s. Because of such a short breeding history and narrow genetic resources, ornamental gentian has limited variations in terms of several traits of flower and plant morphology compared with the major ornamental crops such as chrysanthemum, rose and carnation.

To promote gentian breeding, in addition to conventional crossing, several methods have been developed, i.e., mutation, polyploidy, protoplast culture, doubled haploid production, genetic transformation, marker assisted selection, etc. (Doi and Takahata 2015, Hikage 2016, Nishihara et al. 2015, Takahata et al. 1995). On the other hand, though the genus Gentiana is comprised of 15 sections and about 360 species (Ho and Liu 2001), breeding using wide hybridization was only slightly carried out. As mentioned above, almost all cultivars of gentian have been bred using two closely related species of G. scabra and G. triflora, which are classified in sect. Pneumonanthe. G. scabra usually blooms from September to November in Japan and has traits favored by consumers such as an open corolla. G. triflora blooms from July to September and has upright corolla lobes. The flower color of gentian is predominantly blue. In addition, some cultivars with pink or white color have also been bred. On the other hand, a number of species in Gentiana exhibit a wide range of variation in flower color, flower shape, flowering time, plant architecture, etc. (Kohlein 1991), and they could be utilized for the production of interspecific hybrids between Japanese gentians and wild species of Gentiana

Production of interspecific hybrids between Japanese gentians and wild species of Gentiana

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Wide hybridization, which is a powerful tool to broaden genetic variation, has been used in breeding of many crops. However, in ornamental gentian few wide hybridizations have been reported. Interspecific hybridizations between two gentian cultivated species (Gentiana scabra and G. triflora) and 11 wild species, which were classified in five sections, were carried out using ovule culture. When G. scabra was used as a female parent, normal seedlings and hybrid plants were obtained from eight and five interspecific combinations, respectively. The yield of seedling produced from ovule culture depended on interspecific combinations, ranging from 0.3 to 427.7 normal seedling per flower. In the hybridization of G. triflora with five wild species, normal seedlings and plants were produced in five and four interspecific combinations, respectively. The yield of normal seedling ranging from 0.4 to 228.3 was different between not only interspecific combinations but also reciprocal crosses. Two cultivated species are classified in sect. Pneumonanthe, and successful production of hybrids was obtained from the hybridization with species classified in sections Pneumonanthe or Cruciata. The hybrid nature of the produced plants was confirmed by molecular marker and morphology. The production of interspecific hybrids opens a novel prospect in ornamental gentian breeding.

Key Words: Gentiana scabra, G. triflora, interspecific hybridization, molecular marker, ovule culture, wild species.
development of novel varieties. Few interspecific hybridizations between cultivated species and other species have been reported, except for two reports. Morgan (2004) produced the interspecific hybrid between G. triflora and G. lutea through ovule culture. Tamagake et al. (2014) reported that effectiveness of ovule culture on the production of interspecific hybrids between G. triflora and five wild species (G. paradoxa, G. septemfida, G. dahurica, G. tibetica and G. andrewsi), and also obtained progenies by backcrossing on the hybrid between G. triflora and G. paradoxa. Hikage (2016) described that G. pneumonanthe has crossability with G. triflora, and red flower cultivars are developed using undisclosed foreign species in New Zealand. However, these detailed data findings remain unclear.

Wide hybridization, which is one of the conventional breeding techniques, is a powerful tool to broaden the available genetic pool, and using wider genetic variation breeders have developed a number of varieties in many crops. Especially within ornamental breeding, wide hybridization represents a main tool for supplying genetic variations (Van Tuyl and De Jeu 1997). Kuligowska et al. (2016) described that wide hybridization has evolved from a conventional breeding tool into a modern methodology through improvement in various technology; for example, pistil manipulation and in vitro fertilization for overcoming prezygotic barriers, some embryo rescue techniques for overcoming postzygotic barriers, and molecular markers for verification of hybrids and progenies. Of these, embryo rescue techniques such as embryo culture, ovule culture, and ovary culture are frequently used as a means of producing interspecific and intergeneric hybrids in many crops including ornamental ones such as Lilium (Van Tuyl et al. 1991), Gypsophila (Kishi et al. 1994), Alstroemeria (De Jeu and Jacobsen 1995), Sandersonia (Morgan et al. 2001), Chrysanthemum (Deng et al. 2011) and Begonia (Chen and Mii 2012). Cross direction has also been known as an important factor in hybrid production in wide hybridization of many crops (Kagawa 1957).

In the present study, we report the effective production of interspecific hybrids through ovule culture between two cultivated species of gentian and other wild species. Some factors affecting production of hybrids such as interspecific combinations and the direction of crosses were examined. Moreover, hybrid plants were characterized by molecular marker analysis and morphology.

### Materials and Methods

#### Plant materials

Two cultivated species including four strains and 11 wild species including 13 strains of gentians (Gentiana spp.), which are classified into five sections, were used in this study (Table 1). All materials were grown in an experimental field and a greenhouse at Hachimantai City Floricultural Research and Development Center, Hachimantai, Iwate, Japan, except for G. paradoxa, which was grown in a greenhouse at Iwate University, Morioka, Iwate, Japan.

#### Interspecific hybridization

The stem with inflorescence was cut and put in water. Its flower buds were emasculated several days before crossing. After opening of the top of the stigma, pistils were pollinated with pollens, which were stored in a freezer. These materials were maintained in a biotron (Koitotoron, Koito Industries Co., Yokohama, Japan) with daily cycles of 16 h of light at 25°C and 8 h of dark at 15°C, due to the prevention of damage from exposure to field condition such as undesired pollination, bad weather and insect pests. These pollinated flowers were used for ovule culture.

#### Ovule culture

Ovule culture was carried out as described by Morgan (2004) with some modifications. Pistils were excised 10 to 13 days after pollination, and surface-sterilization in 70% ethanol was carried out for 30 sec followed by sodium hypochlorite solution containing 1.4% active chlorine for

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**Table 1. List of Gentian species used in this study**

| Type      | Sections | Species       | Cultivar/line | Distribution* |
|-----------|----------|---------------|---------------|--------------|
| Cultivated| Pneumonanthe | G. scabra     | Ashiro no Sawakaze | Eastern Asia |
|           |          | G. triflora   | 18-424        | Northeastern Asia |
|           |          | G. jamesii    | 22-1176       | Northeastern Asia |
|           |          | G. squarrosa  | 22-1326       | Central, Northern and Eastern Asia |
|           | Cruciata | G. gracilipes | 25-576        | China |
|           |          | G. pneumonanthe | 25-576   | China |
|           |          | G. straminea  | 25-566        | China |
|           | Frigidiae| G. algida     | 25-585        | Central, Northern and Northeastern Asia |
|           | Microsperma| G. purpurea   | 25-566        | Central Europe |
|           | Pneumonanthe | G. asclepiadea| 21-1049       | Europe, Caucasus, Western Asia |
|           |          | G. paradoxa   | 5-39-3        | Caucasus |
|           |          | G. pneumonanthe | 7-131-1      | Europe, Caucasus, Central Asia |
|           |          | G. septemfida | 27-1079       | Caucasus to Western, Central and Northern Asia |

* Referred to Ho and Liu (2001).
15 min. After three times of rinsing with sterile distilled water (5 min each time), ovules isolated from a pistil were cultured on 0.8% agar-solidified MS medium (Murashige and Skoog 1962) with the concentration of major salts reduced by 50% (1/2MS) and supplemented with 3% sucrose and 1.0 mg/l GA3. The ovule culture was performed at 20°C with a 16-h photoperiod. When seedlings, which were developed from ovules, reached approximately 5–10 mm in length, they were transferred to 0.8% agar-solidified 1/2MS medium supplemented with 3% sucrose and incubated at 20°C with a 16-h photoperiod. Regenerated plants were grown in 2:2:1 akadama-peat moss-soil, and then transferred to soil.

Molecular marker analysis

The hybridity of plants regenerated was examined using simple sequence repeat (SSR) markers (Sato-Ushiku et al. 2011) and sequence characterized amplified region (SCAR) markers (Nakatsuka et al. 2012, Shimada et al. 2009, M. Nishihara personal communication) based on the length polymorphisms in introns of flavonoid biosynthetic genes and transcription factor gene. Total DNA was extracted from leaves by CTAB method (Murray and Thompson 1980). Polymerase chain reaction (PCR) of SSR and SCAR markers was carried out using a PCR Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan) in a 20 μl volume containing 50 ng template DNA, 200 μM dNTP, 0.5 U Ex Taq DNA polymerase (Takara Bio Inc.), 0.15 μM primer, and 1 × Ex Taq Buffer. The sequence of primers used in this study was as follows (from 5′ to 3′): Gtm10 (forward; CTGGAAAA CACCCACACACACAT, reverse; ATCCATGTCTCTCT CGTGTAGCTC), Gtm 77 (forward; CTGGTATGCTCACA CACACAA, reverse; GCAAGTGGTACAGTGGTGTAT), FHT (flavanone 3β-hydroxylase) first intron 2 (forward; TTACACAAAATAGGTTGTCTTC, reverse; TCGTGA TAATAGGTGTTGCTTC), FHT first intron 3 (forward; TTGCACCTGAAGTGAATTITACA, reverse; TTCTGA CAGAACTTTAAGCAATTTT), bHLH1 (basic helix loop helix 1) intron (forward; AAGGTGATCGTTGTGAAA TTGTCT, reverse; GGCCTCTAGTGGGGTTGTGTTT) and ANS (anthocyanidin synthase) intron (forward; TGTA TTACACCTGAAGGAAAGG, reverse; TCTAAACCA AGCCCACAGAGAGC). The PCR condition was an initial denaturation step at 94°C for 2 min followed by 35 cycles of 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 amplified products of SSR and SCAR makers were electrophoresed in 3.5% and 1.6% agarose gel, respectively, and stained with ethidium bromide.

Examination of morphology and pollen fertility

The plants obtained from ovule culture were planted in the soil and grown in a greenhouse at Iwate University or in an experimental field at Hachimantai City Floricultural Research and Development Center. The flower and leaf morphology of these plants was compared to that of the parental species. Pollen fertility was determined by counting aceto-carmine stainable pollen grains. About 200 pollen grains were examined for each plant.

Results

Production of interspecific hybrids using ovule culture

Ten to 13 days after pollination, ovules excised from the ovaries swollen were cultured (Fig. 1A, 1B). After one month of culture, embryos developed and germinated to seedlings normally (Fig. 1C). These seedlings developed into plantlets after transfer to regeneration medium (Fig. 1D), whereas some embryos proliferated abnormally such as callus-like proliferation and atypical growth without first leaf development and failed to develop normal seedlings. The results of ovule culture on interspecific hybridization between G. scabra and 11 wild species are presented in Table 2. The yield of normal seedlings was different among cross combinations, and the number of normal seedlings per flower varied from 0 to 427.7. Normal seedlings could be obtained from combinations between G. scabra and eight wild species. All combinations between G. scabra and G. septemfida produced the highest number of seedlings (94.5–427.7 normal seedlings per flower) and showed the highest frequency of normal seedling (81.5–89.5%), though

![Fig. 1. Ovule culture and plant regeneration in interspecific hybridization of gentian. (A) Swollen pistil of G. triflora ‘18-424’ × G. gracilipes (10 days after cross), (B) Ovule culture of G. septemfida ‘5-39-3’ × G. triflora ‘18-424’ on 1/2MS solid medium supplemented with 1.0 mg/l GA3, (C) Seedlings germinated from ovule culture of G. scabra ‘22-1326’ × G. pneumonanthe, (D) Hybrid plant of G. scabra ‘22-1326’ × G. septemfida ‘5-39-3’. Bars = 1 cm.](image-url)
Table 2. Seedling production from ovule culture in interspecific hybridization between G. scabra and 11 wild species

| Cross combination (♀ × ♂) | No. of swollen ovaries cultured | No. of total seedlings obtained | No. of normal seedlings obtained (%) | Normal seedlings/flower | No. of normal seedlings transplanted | No. of plants acclimated | No. of potted plants obtained |
|---------------------------|---------------------------------|---------------------------------|-------------------------------------|-------------------------|-------------------------------------|--------------------------|----------------------------|
| G. scabra ‘Ashiro no Sawakaze’ × G. gracilipes | 6 | 54 | 39 (72.2) | 6.5 ± 4.5 | – | 23 | 18 |
| G. scabra ‘Ashiro no Sawakaze’ × G. straminea | 6 | 33 | 14 (42.4) | 2.3 ± 1.5 | – | 12 | 12 |
| G. scabra ‘Ashiro no Sawakaze’ × G. paradoxa | 4 | 153 | 104 (68.0) | 17.3 ± 8.3 | – | 44 | 26 |
| G. scabra ‘22-1176’ × G. jamesii | 3 | 0 | 0 (0.0) | 0 ± 0 | 0 | 0 | 0 |
| G. scabra ‘22-1176’ × G. squarrosa | 3 | 0 | 0 (0.0) | 0 ± 0 | 0 | 0 | 0 |
| G. scabra ‘22-1176’ × G. gracilipes | 3 | 16 | 1 (6.3) | 0.3 ± 0.3 | 1 | 1 | 1 |
| G. scabra ‘22-1176’ × G. siphonantha | 3 | 7 | 1 (14.3) | 0.3 ± 0.3 | 1 | 0 | 0 |
| G. scabra ‘22-1176’ × G. straminea | 2 | 41 | 19 (46.3) | 9.5 ± 2.5 | 19 | 3 | 1 |
| G. scabra ‘22-1176’ × G. algida | 3 | 0 | 0 (0.0) | 0 ± 0 | 0 | 0 | 0 |
| G. scabra ‘22-1176’ × G. purpurea | 3 | 4 | 1 (25.0) | 0.3 ± 0.3 | 1 | 0 | 0 |
| G. scabra ‘22-1176’ × G. asclepiadea | 3 | 12 | 0 (0.0) | 0 ± 0 | 0 | 0 | 0 |
| G. scabra ‘22-1176’ × G. paradoxa | 4 | 1137 | 924 (81.3) | 231.0 ± 54.6 | 91 | 47 | 23 |
| G. scabra ‘22-1176’ × G. pneumonanthe | 3 | 56 | 35 (62.5) | 11.7 ± 1.2 | 35 | 24 | 17 |
| G. scabra ‘22-1176’ × G. septemfida ‘5-39-3’ | 2 | 608 | 501 (82.4) | 250.5 ± 17.3 | 42 | 25 | 17 |
| G. scabra ‘22-1176’ × G. septemfida ‘7-131-1’ | 3 | 1434 | 1283 (89.5) | 427.7 ± 49.7 | 62 | 44 | 26 |
| G. scabra ‘22-1326’ × G. septemfida ‘27-1079’ | 2 | 896 | 770 (85.9) | 385.0 ± 24.7 | 42 | 23 | 12 |
| G. scabra ‘22-1326’ × G. gracilipes | 4 | 36 | 11 (30.6) | 2.8 ± 1.6 | 11 | 4 | 3 |
| G. scabra ‘22-1326’ × G. asclepiadea | 4 | 277 | 9 (3.2) | 2.3 ± 0.5 | 9 | 0 | 0 |
| G. scabra ‘22-1326’ × G. paradoxa | 4 | 1000 | 769 (76.9) | 192.3 ± 14.6 | 80 | 40 | 17 |
| G. scabra ‘22-1326’ × G. pneumonanthe | 3 | 259 | 65 (25.1) | 21.7 ± 4.8 | 65 | 11 | 10 |
| G. scabra ‘22-1326’ × G. septemfida ‘5-39-3’ | 3 | 882 | 723 (82.0) | 241.0 ± 19.7 | 60 | 20 | 15 |
| G. scabra ‘22-1326’ × G. septemfida ‘7-131-1’ | 5 | 1696 | 1456 (85.8) | 291.2 ± 32.0 | 96 | 36 | 19 |
| G. scabra ‘22-1326’ × G. septemfida ‘27-1079’ | 2 | 232 | 189 (81.5) | 94.5 ± 22.3 | 46 | 23 | 16 |
| Total | | | | | | | 233 |

*Values represent the mean ± SE.

Table 3. Seedling production from ovule culture in interspecific hybridization between G. triflora and 5 wild species (reciprocal cross)

| Cross combination (♀ × ♂) | No. of swollen ovaries cultured | No. of total seedlings obtained | No. of normal seedlings obtained (%) | Normal seedlings/flower | No. of normal seedlings transplanted | No. of plants acclimated | No. of potted plants obtained |
|---------------------------|---------------------------------|---------------------------------|-------------------------------------|-------------------------|-------------------------------------|--------------------------|----------------------------|
| G. triflora ‘18-424’ × G. gracilipes | 7 | 55 | 39 (70.9) | 5.6 ± 2.3 | 29 | 15 | 12 |
| G. triflora ‘18-424’ × G. asclepiadea | 7 | 163 | 3 (1.8) | 0.4 ± 0.3 | 3 | 0 | 0 |
| G. triflora ‘18-424’ × G. paradoxa | 7 | 137 | 135 (98.5) | 19.3 ± 4.9 | 57 | 33 | 31 |
| G. triflora ‘18-424’ × G. pneumonanthe | 7 | 545 | 432 (79.3) | 61.7 ± 28.1 | 116 | 60 | 57 |
| G. triflora ‘18-424’ × G. septemfida ‘5-39-3’ | 2 | 16 | 15 (93.8) | 7.5 ± 1.8 | 12 | 9 | 8 |
| G. triflora ‘18-424’ × G. septemfida ‘7-131-1’ | 6 | 133 | 126 (94.7) | 21.0 ± 6.4 | 74 | 38 | 35 |
| G. triflora ‘18-424’ × G. septemfida ‘27-1079’ | 2 | 87 | 85 (97.7) | 42.5 ± 5.3 | 16 | 11 | 11 |
| G. gracilipes × G. triflora ‘18-424’ | 3 | 0 | 0 (0.0) | 0 ± 0 | 0 | 0 | 0 |
| G. asclepiadea × G. triflora ‘18-424’ | 3 | 4 | 0 (0.0) | 0 ± 0 | 0 | 0 | 0 |
| G. septemfida ‘5-39-3’ × G. triflora ‘18-424’ | 3 | 696 | 685 (98.4) | 228.3 ± 55.3 | 57 | 10 | 10 |
| G. septemfida ‘27-1079’ × G. triflora ‘18-424’ | 2 | 17 | 11 (64.7) | 3.7 ± 3.2 | 11 | 2 | 1 |
| Total | | | | | | | 166 |

*Values represent the mean ± SE.

two and three different lines was used as a female and a male parent, respectively. The crosses with G. paradoxa or G. pneumonanthe also produced many normal seedlings per flower, ranging from 11.7 to 231.0, and those with G. gracilipes or G. straminea produced several seedlings per flower, ranging from 0.3 to 6.5. Plants were obtained from these five cross combinations. Although a few normal seedlings were obtained on the hybridizations with G. siphonantha, G. purpurea and G. asclepiadea, plants were not obtained because of failure of plant regeneration or acclimatization. On the other hand, no normal seedlings were obtained on the hybridizations with G. jamesii, G. squarrosa, and G. algida. As a result, normal seedlings were produced from eight interspecific combinations, and a total of 233 plants could be obtained from five interspecific combinations.

In the interspecific hybridization with G. triflora, five wild species were used, and the reciprocal cross was carried out. When G. triflora was used as a female parent, normal seedlings were obtained in all cross combinations, although the yield of seedlings varied among them (Table 3). Similar to using G. scabra as a female parent, many normal seedlings were produced in the hybridizations with G. paradoxa, G. pneumonanthe and G. septemfida, ranging from 7.5 to 61.7 seedlings per flower. In contrast, a lower number of
normal seedlings were obtained in the hybridization with G. asclepiadea (0.4 seedlings per flower). A difference of seedling production was observed in reciprocal crosses. In the cross between G. triflora and G. gracilipes or G. asclepiadea, seedlings were obtained only when G. triflora was used as a female parent. In crossing with G. septemfida, a higher production of seedling was obtained when G. triflora was used as a female parent than when used as a male. In contrast, in the hybridization with G. pneumonanthe, when G. triflora was used as a male, more than three times as many seedlings were produced in comparison to the reciprocal cross. Finally, in G. triflora, a total of 166 plants were obtained in four interspecific crosses except for crossing with G. asclepiadea.

**Confirmation of hybridity**

A total of 233 and 166 plants were obtained in the interspecific hybridization with G. scabra and G. triflora, respectively. In order to confirm whether these plants are true hybrids, molecular marker analysis was carried out. When 111 plants, which were derived from 14 cross combinations, were examined, almost all of the plants showed combined bands of the both parental species, except 13 plants derived from G. scabra ‘Ashiro no Sawakaze’ × G. paradoxa, which exhibited maternal bands (Fig. 2, Table 4).

These plants also had morphologically intermediate traits between parents. The leaves of the plants were intermediate in size and shape. The flower morphology of these plants also showed both characters of parents (Fig. 3). For example, the plants obtained from the hybridization between blue flower G. scabra and white flower G. straminea had light blue flowers (Fig. 3A–3C). The cross between dark blue flower G. triflora and light blue flower G. gracilipes resulted in a blue flower plant (Fig. 3D–3F). The plants obtained from cross between G. triflora (straight corolla lobes) and G. paradoxa (reflexed corolla lobes) showed slightly reflexed corolla lobes (Fig. 3D, 3G–3H). Plants obtained from interspecific hybridization had shriveled anthers, and some plants exhibited petaloid stamens when using G. paradoxa or G. septemfida as a male parent (Fig. 3I). And also, their pollen grains were highly sterile (0.0–4.2%) normal seedlings were obtained in the hybridization with G. asclepiadea (0.4 seedlings per flower). A difference of seedling production was observed in reciprocal crosses. In the cross between G. triflora and G. gracilipes or G. asclepiadea, seedlings were obtained only when G. triflora was used as a female parent. In crossing with G. septemfida, a higher production of seedling was obtained when G. triflora was used as a female parent than when used as a male. In contrast, in the hybridization with G. pneumonanthe, when G. triflora was used as a male, more than three times as many seedlings were produced in comparison to the reciprocal cross. Finally, in G. triflora, a total of 166 plants were obtained in four interspecific crosses except for crossing with G. asclepiadea.

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| Maternal parents | G. gracilipes | G. scabra '22-1176' | G. straminea | G. paradoxa | G. pneumonanthe | G. septemfida |
|------------------|--------------|---------------------|--------------|-------------|----------------|--------------|
| G. scabra 'Ashiro no Sawakaze' | 18/18* (G10) | 11/11 (G10) | 9/22 (G77) | 5-39-3 | 7-131-1 | 27-1079 |
| G. scabra '22-1326' | 6/6 (F3, H) | 6/6 (F3, H) | 6/6 (F3, H) | 6/6 (F3, H) | 6/6 (F3, H) | 6/6 (F3, H) |
| G. triflora '18-424' | 6/6 (A) | 6/6 (A) | 4/4 (F3) | 6/6 (A) |

*No. of interspecific hybrids showing combined bands of the both parental species / No. of examined plants.

Table 4. Identification of interspecific hybrids using DNA marker

| Maternal parents              | G. gracilipes | G. scabra '22-1176' | G. straminea | G. paradoxa | G. pneumonanthe | G. septemfida |
|------------------------------|---------------|---------------------|--------------|-------------|----------------|--------------|
| G. scabra 'Ashiro no Sawakaze'| 18/18* (G10) | 11/11 (G10)        | 9/22 (G77)  | 5-39-3      | 7-131-1        | 27-1079      |
| G. scabra '22-1326'          | 6/6 (F3, H)  | 6/6 (F3, H)        | 6/6 (F3, H) | 6/6 (F3, H) | 6/6 (F3, H)    | 6/6 (F3, H)  |
| G. triflora '18-424'         | 6/6 (A)      | 6/6 (A)            | 4/4 (F3)    | 6/6 (A)     |                |              |

DNA markers used to examine hybridity. G10; Gtm10, G77; Gtm77, A; ANS intron, F3; FHT first intron 3, H; bHLH1 intron.
in comparison with pollen fertility of the parents (56.3–82.2%) (Fig. 3J).

**Discussion**

In order to develop new varieties in crops, increasing genetic variation is essential. Wide hybridization has been used as an effective method for broadening genetic variation in ornamental crops. In gentian (Gentiana spp.), wild species have useful traits that are not found in two cultivated species; for example, the reddish-brown flower color of *G. purpurea*, the early flowering time of *G. straminea*, the compact leaf and flexible stem of *G. pneumonanthe*, the dwarf plant type of *G. septemfida*, etc. However, interspecific crosses in gentian have been reported only by Morgan (2004) and Tamagake et al. (2014). In this study we succeeded in the production of new interspecific hybrids between two ornamental gentian species (*G. scabra* and *G. triflora*) and wild species through ovule culture. When *G. scabra* was used as a female parent, interspecific hybrids with five wild species (*G. septemfida, G. pneumonanthe, G. paradoxa, G. gracilipes* and *G. straminea*) were obtained. Interspecific hybrids with *G. triflora* were also obtained using the same species except for *G. straminea*, which was not used in this experiment. Two cultivated species are classified in sect. *Pneumonanthe*, and of these five wild species showing crossability, the former three species belong to sect. *Pneumonanthe* and the latter two to sect. *Cruciata*. Interspecific hybrids between *G. triflora* and *G. paradoxa* or *G. septemfida* are also reported by Tamagake et al. (2014). Crossability between *G. triflora* and *G. pneumonanthe* is mentioned by Hikage (2016). Our results support their description, and also show easy hybridization of *G. scabra* with three wild species of sect. *Pneumonanthe*. It is demonstrated here that cultivated gentians have easy crossability not only with species belonging to the same sect. but also with sect. *Cruciata*. Phylogenetic analysis based on internal transcribed spacers (ITSs) of nuclear ribosomal DNA (Yuan et al. 1996) and on chloroplast DNA sequence (Mishiba et al. 2009) reveals that sects. *Pneumonanthe* and *Cruciata* are closely related phylogenetically. Some germinated seeds were produced in the same cross combinations without *in vitro* technique, though their hybridity has not been investigated (data not shown).

On the other hand, although *G. asclepiadea* is classified in the same section as cultivated species by conventional classification (Ho and Liu 2001, Nilsson 1967), normal seedlings produced between cultivated species and *G. asclepiadea* was low (0–2.3 per flower) and hybrid plants were not obtained. Molecular genetic analysis indicated that *G. asclepiadea* is phylogenetically closer to sect. *Gentiana* than to sect. *Pneumonanthe* (Mishiba et al. 2009, Yuan et al. 1996). Our results support that *G. asclepiadea* is not classified in sect. *Pneumonanthe*. In interspecific hybridization with *G. siphonantha, G. purpurea, G. jamesii, G. squarrosa*, and *G. algida*, no hybrids were produced, either. Of these five wild species, in hybridizations of *G. scabra* with *G. siphonantha* (sect. *Cruciata*) and *G. purpurea* (sect. *Microsperma*), each had one normal seedling produced, but they failed to develop plants. No seedlings were produced from ovule culture of hybridization using the remaining three species, which belong to sects. *Chondropyllaeae* and *Frigidae*, though sect. *Chondropyllaeae* is reported to be closely related to sects. *Pneumonanthe* and *Cruciata* (Mishiba et al. 2009, Yuan et al. 1996). One of the possible reasons for failure to produce hybrids in these interspecific hybridizations is considered to be due to used lines. In the present study, only a single cross combination was examined in each interspecific combination which produced no hybrid plants. The degree of reproductive barrier in wide hybridization was reported to differ depending on used lines in many crops (Hadley and Openshaw 1980) such as *Dianthus* (Nimura et al. 2003), *Brassica* (Tonosaki et al. 2013), etc. Genetic loci or QTLs related to interspecific incompatibility and importance of balance of ploidy levels between female and male parents for successful hybrid embryo development were reported (Johnston et al. 1980, Tonosaki et al. 2013, Udagawa et al. 2010). An attempt to use more genotypes will succeed in production of hybrids.

Our study shows that the production rate of hybrid seedlings differed between reciprocal crosses. Especially, the hybridization with *G. gracilipes* was possible only with *G. triflora* as the female parent. Such unilateral incongruity was observed in wide hybridization of many crops such as *Brassica* (Takahata 1990), *Alstroemeria* (De Jou and Jacobsen 1995), *Dianthus* (Nimura et al. 2003), *Hibiscus* (Van Laere et al. 2007), *Streptocarpus* (Afkhami-Sarvestani et al. 2012) and *Capiscum* (Manzur et al. 2015). The exact cause of such a difference is unclear, but is believed to involve prezygotic and postzygotic barriers such as pollen-pistil interaction, pollen tube guidance, influence of genome imprinting of endosperm, etc. (Kinoshita 2007).

Hybridity of obtained plants could be rapidly and easily confirmed by molecular markers. All hybrids showed an intermediate morphology of the parent in leaf and flower. Some of the hybrids shows desirable traits on flowering time, flower shape and plant architecture. Although they exhibited serious pollen sterility, the findings in this study open up new avenues for gentian breeding. The production of amphidiploids and backcrossing of the hybrids, new interspecific hybridization and improvement of its culture technique are currently being carried out.

**Author Contribution Statement**

Y. T. and Y. T. conceived and designed this research. Y. T. and C. A. performed the experiments. T. H. collected and maintained plant materials. T. H. and K. H. provided advice on experimental implementation and manuscript. Y. T. and Y. T. wrote the manuscript.
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Literature Cited

Afkhami-Sarvestani, R., M. Serek and T. Winkelmann (2012) Interspecific crosses within the Streptocarpa subgenus Streptocarpella and interfertile crosses between Streptocarpa and Stipa paula ionantha genotypes. Sci. Hortic. 148: 215–222.

Chen, Y.M. and M. Mii (2012) Inter-sectional hybrids obtained from reciprocal crosses between Begonia semperflorens (section Begonia) and B. ‘Orange Rubra’ (section Gaerdia × section Prizelia). Breed. Sci. 62: 113–123.

DeJeu, M.J. and E. Jacobsen (1995) Early postfertilization ovule culture in Alstroemeria L. and barriers to interspecific hybridization. Euphytica 86: 15–23.

Deng, Y., S. Chen, X. Cheng and F. Zhang (2011) The embryo rescue derived interfertile hybrid between chrysanthemum and Ajania przewalskii shows enhanced cold tolerance. Plant Cell Rep. 30: 2177–2186.

Doi, H. and Y. Takahata (2015) Haploid and doubled haploid plant production in gentian (Gentiana spp.). In: Rybczyński, J.J., R. Michael and D.A. Mikula (eds.) The Gentianaceae—Volume 2: Biotechnology and Applications, Springer, Berlin, Heidelberg, pp. 187–197.

Hadley, H.H. and S.J. Openshaw (1980) Interspecific and interfertile hybridization. In: Fehr, W.R. and H.H. Hadley (eds.) Hybridization in crop plants. Amer. Soc. Agron., Madison, Wisconsin, pp. 133–159.

Hikage, T. (2014) Gentian. In: Shibata, M. (ed.) Hana no hinshukairo no nihonshii. Yushokan, Tokyo, pp. 109–126.

Ho, T.N. and S.W. Liu (2001) A worldwide monograph of Gentiana. Science Press, Beijing, p. 694.

Johnston, S.A., T.P.M. den Nijs, S.J. Peloquin and R.E. Hanneman Jr. (1980) The significance of genetic balance to endosperm development in interspecific crosses. Theor. Appl. Genet. 57: 5–9.

Kagawa, F. (1957) Plant Breeding through interspecific and interfertile hybridization. Sangyotosho, Tokyo, p. 555.

Kinoshita, T. (2007) Reproductive barrier and genomic imprinting in the endosperm of flowering plants. Genes Genet. Syst. 82: 177–186.

Kishi, F., Y. Kagami, M. Shinohara, S. Hatano and H. Tsurushima (1994) Production of interspecific hybrid in Gypsophila by ovule-embryo culture. Euphytica 74: 85–90.

Kohlein, F. (1991) Gentians. Timber Press, Portland, p. 183.

Kuligowska, K., H. Lütkem and R. Müller (2016) Towards development of new ornamental plants: status and progress in wide hybridization. Planta 244: 1–17.

Manzur, J.P., A. Fita, J. Prohens and A. Rodríguez-Burruezo (2015) Successful wide hybridization and introgression breeding in a diverse set of common peppers (Capsicum annuum) using different cultivated Aji (C. baccatum) accessions as donor parents. PLoS ONE 10: e0144142.

Mishiba, K., K. Yamane, T. Nakatsuka, Y. Nakano, S. Yamamura, J. Abe, H. Kawamura, Y. Takahata and M. Nishihara (2009) Genetic relationships in the genus Gentiana based on chloroplast DNA sequence data and nuclear DNA content. Breed. Sci. 59: 119–127.

Morgan, E.R., G.K. Burge, F.J. Seelye, M.E. Hopping, J.E. Grant, A.G.F. Warren and D. Brundell (2001) Wide crosses in the Colchicaceae: Sandersonia aurantiaca (Hook.) × Littonia modesta (Hook.). Euphytica 121: 343–348.

Morgan, E.R. (2004) Use of in ovulo embryo culture to produce interspecific hybrids between Gentiana triflora and Gentiana lutea. N. Z. J. Crop Hortic. Sci. 32: 343–347.

Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Murray, M.G. and W.F. Thompson (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321–4325.

Nakatsuka, T., E. Yamada, M. Saito, T. Hikage, Y. Ushiki and M. Nishihara (2012) Construction of the first genetic linkage map of Japanese gentian (Gentianaceae). BMC Genomics 13: 672.

Nilsson, S. (1967) Pollen morphological studies in the Gentianaceae—Gentianinae. Grana Palynol. 7: 46–145.

Nimura, M., J. Kato, M. Mii and K. Morioka (2003) Unilateral compatibility and genotypic difference in crossability in interspecific hybridization between Dianthus caryophyllus L. and Dianthus japonicus Thunb. Theor. Appl. Genet. 106: 1164–1170.

Nishihara, M., K. Mishiba, T. Ichimura, H. Takahashi and T. Nakatsuka (2015) Molecular breeding of Japanese gentians—Applications of genetic transformation, metabolome analyses, and genetic markers. In: Rybczyński, J.J., R. Michael and D.A. Mikula (eds.) The Gentianaceae—Volume 2: Biotechnology and Applications, Springer, Berlin, Heidelberg, pp. 239–265.

Nishihara, M., K. Tasaki, N. Sasaki and H. Takahashi (2018) Development of basic technologies for improvement of breeding and cultivation of Japanese gentian. Breed. Sci. 68: 14–24.

Sato-Ushiku, Y., N. Shimada, M. Saito, E. Yamada, T. Hikage, T. Nakatsuka and M. Nishihara (2011) Development of simple sequence repeat markers for identification of Japanese gentian cultivars. J. Japan. Soc. Hort. Sci. 80: 475–485.

Shimada, N., T. Nakatsuka, Y. Nakano, Y. Kakizaki, Y. Abe, T. Hikage and M. Nishihara (2009) Identification of gentian cultivars using SCAR markers based on intron-length polymorphisms of flavonoid biosynthetic genes. Sci. Hortic. 119: 292–296.

Takahata, Y. (1990) Production of interfertile hybrids between a C1-C4 intermediate species Moricandia arvensis and a C3 species Brassica oleracea through ovary culture. Euphytica 46: 259–264.

Takahata, Y., J. Komori, S. Miyano, H. Kunitake and M. Mii (1995) Regeneration of plants from protoplasts of Gentiana species (Gentian). In: Bajaj, Y.P.S. (ed.) Biotechnology in Agriculture and Forestry Vol. 34. Plant Protoplasts and Genetic Engineering VI, Springer-Verlag, Berlin Heidelberg, pp. 55–62.

Tamagake, H., A. Itou and M. Mori (2014) Interspecific hybrids of Gentiana by ovule culture. Bull. Hokkaido Res. Organ. Agri. Exp. Sta. 98: 32–42.

Tonosaki, K., K. Michiba, S.W. Bang, H. Kitashiba, Y. Kaneko and T. Nishio (2013) Genetic analysis of hybrid seed formation ability of Brassica rapa in interfertile crossings with Raphanus sativus. Theor. Appl. Genet. 126: 837–846.

Udagawa, H., Y. Ishimaru, F. Li, Y. Sato, H. Kitashiba and T. Nishio (2010) Genetic analysis of interspecific incompatibility in Brassica rapa. Theor. Appl. Genet. 121: 689–696.

Van Laere, K., J.M. Van Huylenbroeck and E. Van Bockstaele (2007) Interspecific hybridisation between Hibiscus syriacus, Hibiscus sinorosaceus and Hibiscus paramutabilis. Euphytica 155: 271–283.

Van Tuyl J.M., M.P. Van Diën, M.G.M. Van Creij, T.C.M. Van Kleinwee,
Interspecific hybrids between Japanese gentian and wild species

J. Franken and R. J. Bino (1991) Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incompatibility barriers in interspecific Lilium crosses. Plant Sci. 74: 115–126.

Van Tuyl, J.M. and M.J. De Jeu (1997) Methods for overcoming interspecific crossing barriers. In: Shivanna, K.R. and V.K. Sawhney (eds.) Pollen biotechnology for crop production and improvement. Cambridge Univ. Press, London, pp. 273–292.

Yoshiike, T. (1992) Rindou (Gentiana). Seibundo Shinkosha, Tokyo, p. 177.

Yuan, Y.M., P. Küpfer and J.J. Doyle (1996) Infrageneric phylogeny of the genus Gentiana (Gentianaceae) inferred from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. Am. J. Bot. 83: 641–652.