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Research Paper

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.

Introduction

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 F_1 hybrid cultivar 'Iwate' (*G. triflora*) was first developed in 1977. Since then, more than 300 F_1 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 crossbreeding, 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

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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. andrewsii*), 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 chlorite for

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

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

[a] 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 GA_3. 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 CACCCAACACACACAT, reverse; ATCCATGTCCTCTC CGTGTAGCTC), Gtm 77 (forward; CTGGTATGCTCACA CACACAA, reverse; GCAAGTGTTCAGATGGTTGAT), *FHT* (flavanone 3β-hydroxylase) first intron 2 (forward; TTACACAAAAATAGGGTCAGTTCC, reverse; TCGTTA TAAATAGATGTGGTCCTC), *FHT* first intron 3 (forward; TTGCACCTGAAGTAGAATTTTACA, reverse; TTCTGA CAGAACTTCAAGCAATTT), *bHLH1* (basic helix loop helix 1) intron (forward; AAGGTGATCGTTGTGAAAA TGTCT, reverse; GGCCGTCTAGTTTGGTGGTTGGTT) and *ANS* (anthocyanidin synthase) intron (forward; TGTA TTTACCCTGAAAGGAAAAGG, reverse; TCTAAACCA AGCCCAACAGAGAGC). 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

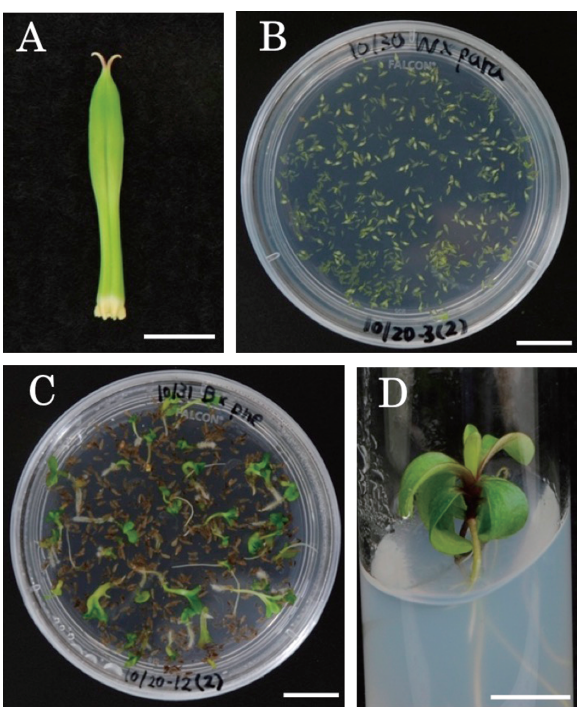

Fig. 1. Ovule culture and plant regeneration in interspecific hybridization of gentian. (A) Swollen pistil of *G. triflora* '18-424' × *G. gracilipes* (10 days after cross), (B) Ovule culture of *G. septemfida* '5-39-3' × *G. triflora* '18-424' on 1/2MS solid medium supplemented with 1.0 mg/l GA_3, (C) 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.

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[a]	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 '22-1176' × *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. pardoxa*	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

[a] 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[a]	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. pneumonanthe × *G. triflora*'18-424'	3	696	685 (98.4)	228.3 ± 55.3	57	10	10
G. septemfida '5-39-3' × *G. triflora*'18-424'	3	17	11 (64.7)	3.7 ± 3.2	11	2	1
G. septemfida '27-1079' × *G. triflora*'18-424'	2	16	8 (50.0)	4.0 ± 1.0	8	1	1
Total							166

[a] 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 seedings 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%)

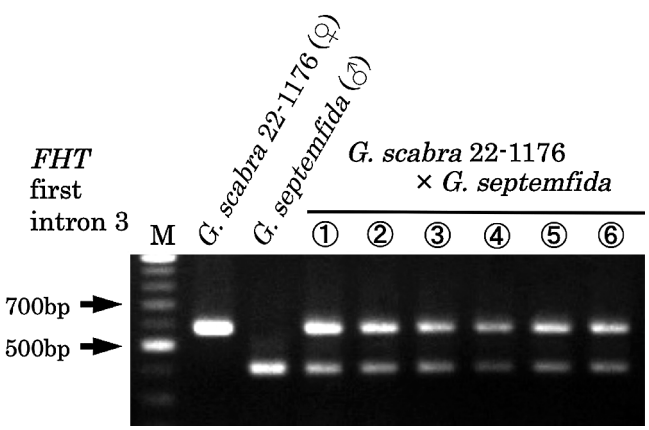

Fig. 2. Confirmation of hybrid plants derived from interspecific crosses between *G. scabra* '22-1176' and *G. septemfida* '27-1079' using SCAR marker of *FHT* first intron 3. M: 100 bp molecular marker.

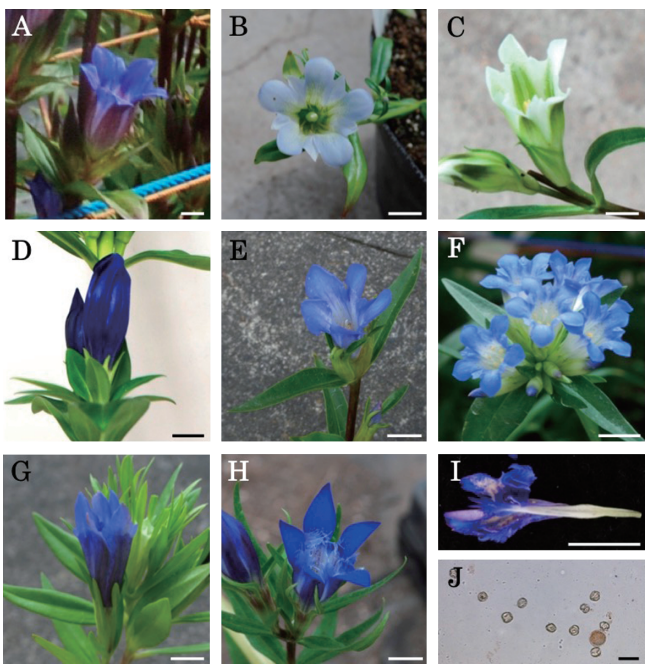

Fig. 3. Flower morphology of hybrid plants. (A) *G. scabra* 'Ashiro no Sawakaze', (B) Hybrid of *G. scabra* 'Ashiro no Sawakaze' × *G. straminea* No. 72, (C) *G. straminea*, (D) *G. triflora* '18-424', (E) Hybrid of *G. triflora* '18-424' × *G. gracilipes* No. 60, (F) *G. gracilipe*, (G) Hybrid of *G. triflora* '18-424' × *G. paradoxa* No. 75, (H) *G. paradoxa*. (I) Petalization of stamens in hybrid of *G. scabra* 'Ashiro no Sawakaze' × *G. paradoxa* No. 39, (J) Pollen fertility of hybrid of *G. triflora* '18-424' × *G. gracilipes* No. 60. Bars = 1 cm for (A–I) and 50 μm for (J).

Table 4. Identification of interspecific hybrids using DNA marker

Maternal parents	Paternal parents						
	G. gracilipes	*G. straminea*	*G. paradoxa*	*G. pneumonanthe*	*G. septemfida*		
					5-39-3	7-131-1	27-1079
G. scabra 'Ashiro no Sawakaze'	18/18[a] (G10)[b]	11/11 (G10)	9/22 (G77)				
G. scabra '22-1176'				6/6 (F3, H)	6/6 (H)		
G. scabra '22-1326'			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)	

[a] No. of interspecific hybrids showing combined bands of the both parental species / No. of examined plants.
[b] 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. *Chondropyllae* and *Frigidae*, though sect. *Chondropyllae* 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 Jeu 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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