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Genetic relationships in the genus *Gentiana* based on chloroplast DNA sequence data and nuclear DNA content

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Genetic relationships among 50 *Gentiana* accessions, comprising 36 wild species and 14 cultivars, were determined based on analysis of sequence data for the chloroplast *trnL*(UAA) intron, the *rpl16* coding region and the *rpl16-rpl14* intergenic spacer (IGS), together with nuclear DNA content as determined by flow cytometric analysis. The combined chloroplast DNA (cpDNA) data set was analyzed using both neighbor-joining (NJ) and maximum parsimony (MP) methods. The NJ and the strict consensus trees were generally congruent with previous phylogenetic and taxonomic studies, whereas *G. cachemirica* and *G. yakushimensis* were classified in different sectional affinities from their prevailing classifications. Three major cpDNA haplotypes (designated A, B and C), comprising 30 accessions in the sections *Pneumonanthe*, *Cruciata*, and *Kudoa* (ser. *Monanthae*), were each distinguished by two single-nucleotide polymorphisms in the *rpl16-rpl14* IGS and the *rpl16* coding region. Nuclear DNA content varied from 6.47 to 11.75 pg among the taxa possessing cpDNA haplotype A. These results provide genetic information that will assist in the development of future *Gentiana* breeding strategies.

Key Words: Flow cytometry, *Gentiana*, nuclear DNA content, *rpl16-rpl14* IGS, *trnL*(UAA) intron.

Introduction

The genus *Gentiana* comprises more than 360 species (Ho and Liu 2001). Among these species, two Japanese-endemic species, *G. triflora* and *G. scabra*, which have beautiful vivid blue flowers, are popular ornamental plants in Japan and are mainly cultivated for cut flowers. Using these two closely-related species, full-scale breeding of Japanese gentian has been undertaken at the Iwate Agricultural Research Center (IARC) since the late 1970s. The IARC produced a series of gentian cultivars possessing novel characters, such as white and pink flower colors and different flowering-time phenotypes for cut-flower production over an extended period. Until now, more than 100 gentian cultivars were bred in Japan. Recently, discrimination of gentian cultivars using SCAR markers have been developed (Shimada *et al*. 2009). However, compared with the major floricultural crops worldwide, such as chrysanthemum, rose and carnation, cultivated gentians show limited variation (e.g. in flower color, morphology and disease resistance), partly reflecting their short breeding history. In addition, limited natural genetic resources make it difficult to increase genetic variation by conventional breeding techniques.

One approach to introduce novel genes into gentian cultivars has been attempted using genetic transformation techniques (reviewed by Nishihara *et al*. 2008). Specifically, various genes contributing to flower color, plant height, early flowering and disease resistance have been characterized, and genetically modified gentians have been produced (Mishiba *et al*. 2005, 2006, Nakatsuka *et al*. 2006, 2008, Nishihara *et al*. 2006, Kiba *et al*. 2005).

Recently, foreign gentian cultivars derived from wild alpine species, such as *G. acaulis* and *G. clusii*, were introduced to the Japanese market to satisfy consumers' diversifying needs, and these are already available for garden cultivation in Japan. Wild species of gentian have the potential to improve Japanese gentians, into which useful traits such as novel flower color and shape and disease resistance could be introduced by *in vitro* culture techniques, such as embryo rescue (Morgan 2004) and polyploidization (Morgan *et al*. 2003). At the IARC, a number of *Gentiana* accessions collected from foreign countries have been accumulated for the gentian breeding program in cooperation with the Iwate Biotechnology Research Center (IBRC) and Iwate University (IU). The *Gentiana* collection at the IARC comprises over 30 accessions of cultivated gentians and about 50 accessions of wild species. These collections contain considerable genetic diversity and possess enormous potential for new cultivar

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development through the planned breeding program.

Chloroplast DNA (cpDNA) sequence variation has been used extensively to infer plant phylogenies (Palmer *et al*. 1988). For phylogenetic reconstruction at lower taxonomic levels, non-coding cpDNA regions have been utilized because of their higher mutation rates compared with coding regions (Curtis and Clegg 1984, Gielly and Taberlet 1994). In the present study, sequence data for two coding and one non-coding cpDNA regions, namely the *trnL*(UAA) intron, the *rpl16* coding region and the *rpl16-rpl14* intergenic spacer (IGS), were analyzed. The *trnL*(UAA) intron has been often utilized for studying intraspecific phylogenetic relationships, including for some *Gentiana* species (Gielly and Taberlet 1994, Gielly *et al.* 1996, Gielly and Taberlet 1996). As Gielly and Taberlet (1994) pointed out, sequence data for the *trnL*(UAA) intron may be insufficiently variable to enable detailed resolution of genetic relationships among *Gentiana* taxa. So, in addition, we analyzed the *rpl16* coding region and the *rpl16-rpl14* IGS, which was proposed as a plastid subtype ID (PS-ID) for discrimination of plastid subtypes in higher plants (Nakamura *et al*. 1997). The PS-ID sequence has practically been used to evaluate genetic variation in different cultivars of rice (Ishikawa *et al*. 2002) and flowering cherries (Ohta *et al*. 2006), due to the variable *rpl16-rpl14* IGS sequence. However, to our knowledge, no detailed study of *Gentiana* species except *G. scabra* (Nakamura *et al*. 1997) using this PS-ID sequence has been determined.

Although many cytological studies have reported the chromosome numbers of *Gentiana* species (summarized by Ho and Liu 2001), nuclear DNA content (C-value) has been determined only for *G. triflora* (Morgan *et al*. 2003). Because plant nuclear DNA content is known to be under strict genotypic control within defined limits (Bennett *et al*. 2000), nuclear DNA content is expected to be a useful guide to hybridization potential in horticulture (Sabharwal and Doležel 1993). Thus, with the objective of identifying potentially compatible interspecific combinations utilizable for gentian breeding, we analyzed the nuclear DNA content in the *Gentiana* accessions to infer genetic relationships with reference to published reports of chromosome numbers.

In this study, to obtain basic genetic information on the IARC *Gentiana* collection to assist in the establishment of future breeding strategies, we investigated genetic relationships among 50 accessions using cpDNA sequence data and nuclear DNA content as determined by flow cytometric analysis.

Materials and Methods

Plant materials

The 50 *Gentiana* accessions, consisting of 36 wild species and 14 cultivars, utilized in this study are listed in Table 1. *Gentianella saxosa*, which is known to be a close relative of *Gentiana* (Glenny 2004), was chosen as the outgroup. All of the accessions were selected from the collection growing at the IARC, IBRC and IU, and were classified into sections according to the taxonomic classification of Ho and Liu (2001).

DNA extraction, amplification and cycle sequencing

Total genomic DNA was extracted from leaves of each accession with the Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St. Louis, MO., USA) according to the manufacturer's instructions. Briefly, leaf disks (0.5–0.7 cm diameter) were incubated in 50 µl Extraction Solution at 95°C for 10 min. An equal volume of Dilution Solution was added to the extract.

Each 20 µl polymerase chain reaction (PCR) solution contained 0.5 U *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA), 2 µl PCR buffer (Invitrogen), 1 mM $MgSO_4$, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, and 1 µl DNA extract. The following primers were used for amplifying and sequencing: primers c (forward; 5′-CGAAATCGGT AGACGCTACG-3′) and d (reverse; 5′-GGGGATAGAGG GACTTGAAC-3′) of Taberlet *et al*. (1991, 2007) for the *trnL*(UAA) intron; and primers A (forward; 5′-AAAGATCT AGATTTCGTAAACAACATAGAGGAAGAA-3′) and B (reverse; 5′-ATCTGCAGCATTTAAAAGGGTCTGAGGT TGAATCAT-3′) of Nakamura *et al*. (1997) for amplification of the 3′ region of the *rpl16* gene and the *rpl16-rpl14* IGS. The PCR protocol for the *trnL*(UAA) intron comprised pre-denaturation for 2 min at 95°C, followed by 35 cycles (denaturation at 95°C for 30 sec, annealing at 55°C for 40 sec and extension at 68°C for 40 sec) and final extension at 68°C for 10 min. The PCR protocol for the 3′ region of the *rpl16* gene and the *rpl16-rpl14* IGS differed in that in each cycle annealing was at 52°C for 30 sec and extension at 68°C for 1 min. Excess primers and free nucleotides were removed from the PCR products with the GenElute PCR clean-up kit (Sigma-Aldrich) according to the manufacturer's instructions. The purified PCR products were subjected to cycle sequencing using the Big-Dye® Terminator version 1.1 cycle sequencing kit and resolved on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo).

Chloroplast DNA sequence analysis

Nucleotide sequences were aligned manually to minimize the number of gaps inserted. The *trnL*(UAA) intron, the *rpl16* coding region and the *rpl16-rpl14* IGS sequences were combined into a single data set and analyzed using the neighbor-joining (NJ) approach (Saitou and Nei 1987) or the maximum parsimony (MP) analysis with PAUP* version 4.0 (Swofford 2002). Insertion/deletion events (indels) were removed from all data sets prior to analysis. The number of nucleotide substitutions per site was estimated with Kimura's (1980) two-parameter method and used to estimate the genetic distance prior to construction of the NJ tree. MP analysis was run with all sites weighted equally and unordered character states. The heuristic search option was used with a stepwise addition sequence set at random; branch-swapping method tree bisection-reconnection (TBR)

Table 1. List of *Gentiana* accessions investigated in the study, with vouchers, DNA data bank of Japan (DDBJ) accessios numbers and 2C DNA contents

Species	ssp/var/cv	Sections[a]	Vouchers[b]	DDBJ accession No.		Haplo-types[d]	2C[e,f] (pg)	Chromosome numbers[g] (2n)
				trnL intron	*rpl16-rpl14*[c]			
G. verna L.	subsp. *verna*	*Calathianae*	U 047	AB453054	AB453105		5.44	28
G. verna L.	subsp. *oschtenica* (Kusnezow) Halda	*Calathianae*	U 049	AB453055	AB453106		7.14	–
G. zollingeri Fawcett		*Chondrophyllae s.s.* (Ser. *Fastigiatae*)	A 027	AB453056	AB453107		9.10	20
G. jamesii Hemsley		*Chondrophyllae s.s.* (Ser. *Fimbriatae*)	U 017	AB453057	AB453108		5.05	36
G. rubicunda Franchet	var. *rubicunda*	*Chondrophyllae s.s.* (Ser. *Rubicundae*)	B 017	AB453058	AB453109		4.98	72
G. acaulis L.		*Ciminalis*	B 001	AB453059	AB453110		9.61	36
G. acaulis L.	cv. Sedanensis	*Ciminalis*	A 004	AB453060	AB453111		–	36
G. angustifolia Villars		*Ciminalis*	A 007	AB453061	AB453112		9.85	36
G. clusii Perrier & Songeon		*Ciminalis*	U 009	AB453062	AB453113		11.83	36
G. clusii Perrier & Songeon	cv. Dark Blue JP	*Ciminalis*	A 011	AB453063	AB453114		9.34	36
G. occidentalis Jakowatz		*Ciminalis*	A 020	AB453064	AB453115		9.77	36
G. cruciata L.	subsp. *cruciata*	*Cruciata*	B 005	AB453065	AB453116	A	9.59	26, 52
G. straminea Maximowicz		*Cruciata*	B 022	AB453066	AB453117	A	7.65	26, 52
G. tianschanica Ruprecht		*Cruciata*	B 023	AB453067	AB453118	A	6.47	–
G. tibetica King ex J. D. Hooker		*Cruciata*	B 026	AB453068	AB453119	A	7.98	52
G. dahurica Fischer	var. *dahurica*	*Cruciata*	B 006	AB453069	AB453120	C	3.65	26
G. decumbens L.		*Cruciata*	B 007	AB453070	AB453121	C	3.63	26
G. gracilipes (=*G. dahurica* var. *dahurica*) Turill		*Cruciata*	B 010	AB453071	AB453122	C	3.68	26
G. macrophylla (Regel & Winkler) Ma & K. C. Hsia	var. *fetissowii*	*Cruciata*	B 014	AB453072	AB453123	C	3.63	26 (+2B)
G. siphonantha Maximowicz ex Kusnezow		*Cruciata*	A 024	AB453073	AB453124	C	3.71	26
G. wutaiensis (=*G. macrophylla* var. *fetissowii*) Marquand		*Cruciata*	A 026	AB453074	AB453125	C	3.57	26 (+2B)
G. lutea L.	subsp. *lutea*	*Gentiana*	U 020	AB453075	AB453126		8.07	40
G. punctata L.		*Gentiana*	U 029	AB453076	AB453127		7.27	40
G. cachemirica Decaisne in Jacquemont		*Kudoa* (Ser. *Monanthae*)	B 004	AB453077	AB453128	B	3.63	16
G. cachemirica Decaisne in Jacquemont		*Kudoa* (Ser. *Monanthae*)	U 008	AB453078	AB453129	B	3.51	16
G. hexaphylla Maximowicz ex Kusnezow × *G. lawrencei* Burkill	cv. Elisabeth Eschmann	*Kudoa*	A 016	AB453079	AB453130		9.50	–
G. × 'Inverleith' (*G. lawrencei* × *G. veitchiorum*)	cv. Blue Flame	*Kudoa* (Ser. *Ornatae*)	A 018	AB453080	AB453131		7.30	–
G. ornata (Wallich ex G. Don) Grisebach		*Kudoa* (Ser. *Ornatae*)	B 015	AB453081	AB453132		–	–
G. sino-ornata I. B. Balfour × *G. veitchiorum* Hemsley	cv. Stevenagensis	*Kudoa* (Ser. *Ornatae*)	A 023	AB453082	AB453133		7.02	–
G. yakushimensis Makino		*Kudoa* (Ser. *Verticillatae*)	B 035	AB453083	AB453134		5.62	26
G. rigescens Franchet in Forbes & Hemsley		*Monopodiae*	B 016	AB453084	AB453135		4.99	24
G. asclepiadea L.	cv. Rosea	*Pneumonanthe*	U 003	AB453085	AB453136		7.58	36, 44
G. freyniana Bornmüller ex Freyn		*Pneumonanthe*	B 008	AB453086	AB453137	B	3.49	26
G. gelida Bieberstein		*Pneumonanthe*	B 009	AB453087	AB453138	B	–	26
G. kolakovskyi (=*G. septemfida* subsp. *kolakovskyi*) Doluch.		*Pneumonanthe*	U 037	AB453088	AB453139	B	3.49	26
G. lagodechiana (=*G. septemfida* subsp. *septemfida*) (Kusn.) Grossh.		*Pneumonanthe*	A 022	AB453089	AB453140	B	3.57	26
G. paradoxa Albov		*Pneumonanthe*	U 023	AB453090	AB453141	B	3.78	–
G. scabra (Miquel) Maximowicz	var. *buergeri*	*Pneumonanthe*	A 021	AB453091	AB453142	A	–	26
G. scabra Bunge	cv. Alta	*Pneumonanthe*	B 020	AB453092	AB453143	A	11.75	26
G. scabra Bunge	cv. Ao-corin	*Pneumonanthe*	B 018	AB453093	AB453144	A	11.35	26
G. scabra Bunge	cv. Momo-corin	*Pneumonanthe*	B 019	AB453094	AB453145	A	11.40	26
G. × hybrid (*G. scabra* × *G. triflora* var. *japonica*)	cv. Albireo	*Pneumonanthe*	B 036	AB453095	AB453146	A	10.27	–
G. × hybrid (*G. scabra* × *G. triflora* var. *japonica*)	cv. Polarno Blue	*Pneumonanthe*	B 038	AB453096	AB453147	A	10.57	–
G. × hybrid (*G. scabra* × *G. triflora* var. *japonica*)	cv. Polarno White	*Pneumonanthe*	B 037	AB453097	AB453148	A	10.57	–
G. triflora Pallas	var. *japonica* line Bandai	*Pneumonanthe*	B 028	AB453098	AB453149	A	9.11	26
G. triflora Pallas	var. *japonica* line Iho-EW	*Pneumonanthe*	B 030	AB453099	AB453150	A	–	26
G. triflora Pallas	var. *japonica* line Ezo	*Pneumonanthe*	B 027	AB453100	AB453151	A	9.29	26
G. triflora Pallas	var. *japonica* line Yahaba	*Pneumonanthe*	B 031	AB453101	AB453152	A	9.30	26
G. triflora Pallas	var. *japonica* cv. Giovanni	*Pneumonanthe*	B 034	AB453102	AB453153	A	–	26
G. triflora Pallas	var. *japonica* cv. Iiha-tobo	*Pneumonanthe*	B 033	AB453103	AB453154	A	–	26
Gentianella saxosa (G. Forster) Holub			U 051	AB453104	AB453155		7.03	36[h]

[a] Designated by Ho and Liu (2001).
[b] A, Iwate Agricultural Research Center (J. Abe); U, Iwate University (Y. Takahata); B, Iwate Biotechnology Research Center (M. Nishihara).
[c] 3′ coding region of the *rpl16* gene with the *rpl16-rpl14* IGR sequence.
[d] Haplotypes based on sequences of three regions (see Fig. 1 and Fig. 2).
[e] Nuclear DNA contents estimated by flow cytometry.
[f] "–": not determined
[g] Summarized in Ho and Liu (2001).
[h] Favarger (1952).

and MAXTREES were set to auto-increase. A strict consensus tree was constructed from the multiple equally shortest trees. Relative branch support was assessed using nonparametric bootstrap analysis (Felsenstein 1985) with 1000 replicates in PAUP*. Nucleotide diversity (π) (Nei and Li 1979) was calculated by pairwise comparison in three coding and non-coding regions using DnaSP version 3.0 (Roza and Roza 1999).

Flow cytometric analysis

Nuclear DNA extracts from the *Gentiana* accessions were prepared from fresh young leaf tissue (approx. 25 mm^2). Extracts from leaves of *Hordeum vulgare* cv. Sultan (2C = 11.12 pg; Johnston *et al.* 1999) or *Petunia hybrida* cv. Mitchell Diploid (2C = 3.13 pg; this study) were used as an internal standard. Sample tissues were chopped (Galbraith *et al*. 1983) with a razor blade in a plastic Petri dish in 0.4 ml Extraction buffer (Cystain PI absolute P; Partec, Münster, Germany). The crude nuclear extract was filtered through a 30 µm nylon mesh filter into a test tube, following incubation for 1 min at room temperature, 1.6 mL Staining solution (Cystain PI absolute P; Partec) containing propidium iodide (PI) and RNase was added to the test tube and the solution was mixed. After incubation in the dark for 30 min at room temperature, the fluorescence intensity of the nuclei was measured with an Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA, USA). Fluorescence was excited at 488 nm with an argon ion laser and detected through a 610 nm band pass filter. Most measurements were repeated at least twice and an average was calculated.

Results

Analysis of chloroplast DNA sequence data

Successful PCR amplifications and complete sequences were obtained for all of the taxa studied, and the accession numbers of the sequences for each taxon were assigned and shown in Table 1. The sequence characteristics for the three cpDNA regions are summarized in Table 2. The length of the *trnL*(UAA) intron and the *rpl16-rpl14* IGS sequences ranged from 330 bp (*G. rubicunda*) to 387 bp (*G. asclepiadea*, *G. lutea*, *G. punctata* and *G. verna*), and from 118 bp (*G. lutea*) to 134 bp (*G. angustifolia* and *G. occidentalis*), respectively. Proportionally, the most variable region was the *rpl16-rpl14* IGS in which 21.2% nucleotide substitution was recorded. Nine indels were inserted in the *trnL*(UAA) intron after alignment of sequences with the outgroup taxon (*G. saxosa*). Many indels were inserted in the *rpl16-rpl14* IGS, making it difficult to align this region; consequently, ambiguous sites were removed from the data set before the phylogenetic analysis. In contrast, no length variation was found in the *rpl16* coding region.

The NJ and strict consensus trees based on the combined cpDNA sequence data set are shown in Fig. 1 and Fig. 2, respectively. The topologies of the NJ tree were not completely congruent with the strict consensus tree, e.g., the position of the node of the section *Calathianae*, or the nodes of the section *Gentiana* and *Ciminalis*; however, clades of each section were retained in both trees. The three species (*G. rubicunda*, *G. jamesii* and *G. zollingeri*) belonging to section *Chondrophyllae* Bunge *s.s.* formed a monophyletic clade with 100% bootstrap support. Four taxa of section *Kudoa* (Ser. *Ornatae*) were grouped with *G. rigescens* in a clade with high bootstrap support.

Three major cpDNA haplotypes (designated A, B and C) were assigned (see Table 1, Fig. 1 and Fig. 2). These three haplotypes were characterized by one synapomorphic single nucleotide polymorphism (SNP) in each of the *rpl16* coding region and the *rpl16-rpl14* IGS. Of relevance for *Gentiana* breeding in Japan, the two most important Japanese-endemic species, *G. triflora* and *G. scabra*, possess haplotype A. Species placed in section *Pneumonanthe* possess either haplotype A or B. Species classified in section *Cruciata* have haplotypes A and C.

A genetic distance matrix is shown in Table 3. Among the 50 *Gentiana* accessions sampled, genetic distances for the *trnL*(UAA) intron ranged from 0 to 0.073. Genetic distances between haplotypes A, B and C, and other taxa ranged 0.003 (*G. yakushimensis*) to 0.037 (*G. jamesii*). Among sections, the greatest difference was shown between sections *Calathianae* and *Chondrophyllae* (0.063–0.073). The nucleotide diversity (π) in each section (*Gentiana*, *Pneumonanthe*, *Ciminalis*, *Cruciata*, *Kudoa*, *Chondrophyllae*, and *Calathianae*) of the genus *Gentiana* was 0.00421, 0.00562, 0.00721, 0.00140, 0.00964, 0.02575, and 0.00140 in the combined chloroplast regions excluding indels, respectively. The highest π value of 0.02575 was observed in the section *Chondrophyllae*.

Table 2. Characteristics of DNA sequences in the *trnL*(UAA) intron, the *rpl16* coding and the *rpl16-rpl14* IGS among *Gentiana* accessions

Region	Length (bp)	Length after alignment with outgroup taxa	Nucleotide subsitution sites including outgroup taxa (%)	Genetic Distances[a] (Average)	Parsimony informative sites including outgroup taxa
trnL(UAA) intron	330–387	302	50 (16.6)	0.000–0.073 (0.030)	25
rpl16 coding[b]	358	358	37 (10.3)	0.000–0.046 (0.018)	14
rpl16-rpl14 IGS	118–134	52	11 (21.2)	0.000–0.103 (0.042)	6

[a] Kimura's two-parameter method (Kimura 1980). Average number was caluculated for all combinations excluding outgroup taxa.

[b] Partial sequences of the *rpl16* coding region.

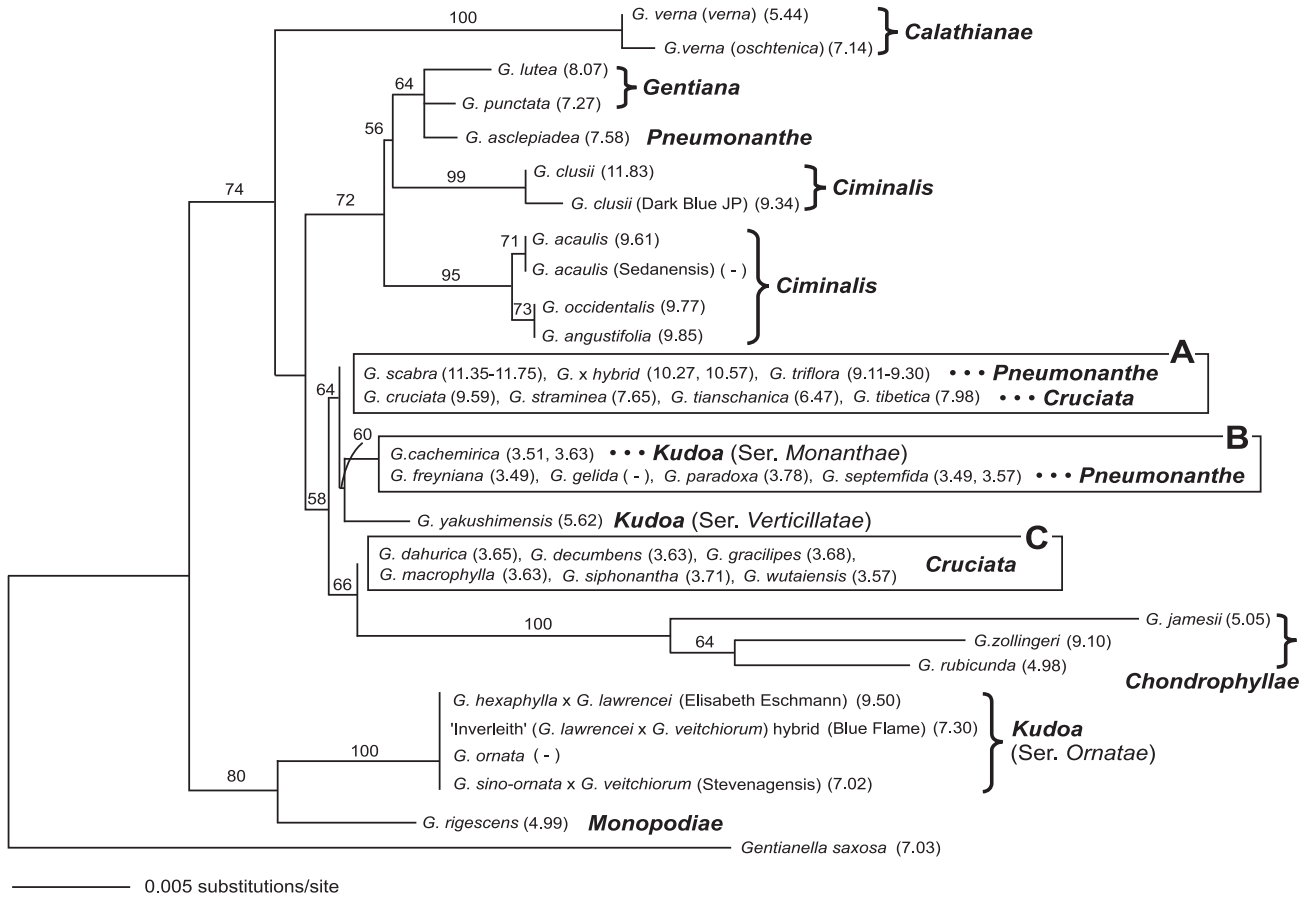

Fig. 1. Neighbor-joining tree for 50 *Gentiana* samples constructed from the combined cpDNA data set comprising sequence data for the *trnL*(UAA) intron, the *rpl16* coding region, and the *rpl16-rpl14* intergenic spacer. Values above the branches are bootstrap percentages >50% from 1,000 bootstrap replications. The three major haplotypes are labeled as A, B and C. The number in parentheses after the accession name indicates the nuclear DNA content (pg). The section within *Gentiana* to which the accessions belong is indicated in bold to the right of the brackets.

Flow cytometric analysis

Nuclear DNA contents measured for 43 *Gentiana* accessions ranged from (2C =) 3.49 pg for *G. freyniana* and *G. kolakovskyi* to 11.83 pg for *G. clusii*, indicating that highly differentiated nuclear DNA contents exist within *Gentiana* (Table 1, Fig. 1 and Fig. 2). The chromosome number of species in section *Pneumonanthe* is reported to be $2n = 26$, with the exception of *G. asclepiadea* which is $2n = 36$ or 44 (Ho and Liu 2001). In contrast, three distinct classes of nuclear DNA content coincident with the cpDNA haplotypes were found among the sampled accessions within section *Pneumonanthe*: higher nuclear DNA contents (9.11 to 11.75 pg) were recorded in *G. triflora* and *G. scabra* (both haplotype A); lower nuclear DNA contents (3.49 to 3.78 pg) occurred in four taxa possessing haplotype B; and *G. asclepiadea* had an intermediate value of nuclear DNA (7.58 pg).

Discussion

Analysis of chloroplast DNA sequence data

The NJ tree showed that the European-endemic section *Ciminalis* was paraphyletic with two distinct clades resolved: one clade comprised the two accessions of *G. clusii*, and the other clade comprised the sampled accessions of *G. acaulis*, *G. occidentalis* and *G. angustifolia* (Fig. 1). The strict consensus tree also did not show the monophyletic clade of the section *Ciminalis* (Fig. 2). These results are consistent with the findings of Gielly and Taberlet (1996), who analyzed *trnL*(UAA) intron sequences. The three species classified in section *Chondrophyllae* Bunge *s.s.* (*G. rubicunda*, *G. jamesii* and *G. zollingeri*) were monophyletic with 100% bootstrap support. Yuan and Küpfer (1997) and Gielly and Taberlet (1996) incorporated other species of section *Chondrophyllae* Bunge *s.l.* (including the sections *Chondrophyllae s.s.* and *Dolichocarpa* T.N. Ho) in analyses of nuclear internal transcribed spacer (ITS) and *trnL*(UAA) intron sequences, which supported the monophyly of the section *Chondrophyllae s.l.* complex. In a neighbor-joining analysis of *trnL*(UAA) intron data from these previous studies combined with those sequences obtained in the present study, a monophyletic section *Chondrophyllae s.l.* was retrieved (data not shown). In agreement with the present study, sections *Cruciata* and *Pneumonanthe* were shown to

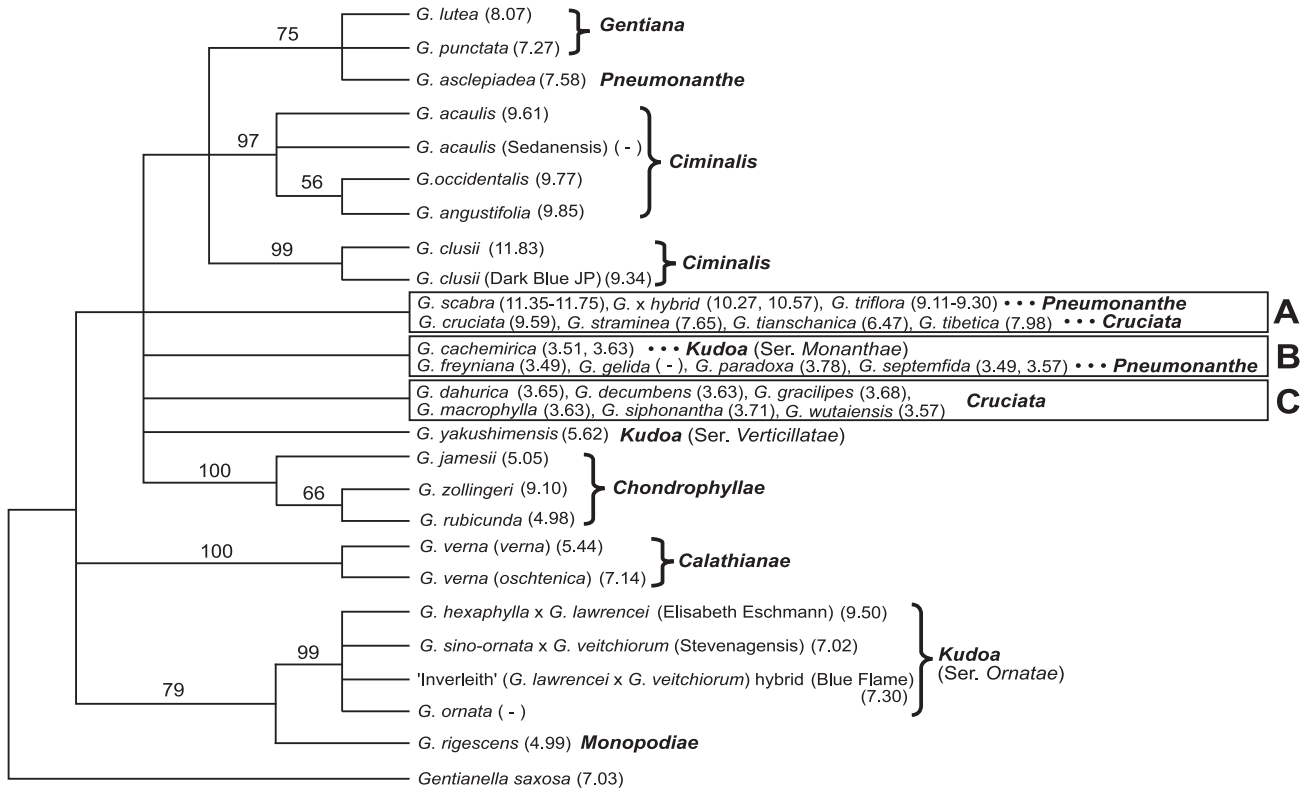

Fig. 2. The strict consensus of five trees with a length of 115 steps for 50 *Gentiana* samples constructed from the combined cpDNA data set comprising sequence data for the *trnL*(UAA) intron, the *rpl16* coding region, and the *rpl16-rpl14* intergenic spacer. The consistency index (CI) excluding uninformative characters of these trees was 0.8246 and the retention index (RI) was 0.9259. Values above branches are bootstrap percentages >50% from 1,000 bootstrap replications. The three major haplotypes are labeled A, B and C. The number in parentheses after the accession name indicates the nuclear DNA content (pg). The section within *Gentiana* to which the accessions belong is indicated in bold to the right of the brackets.

be the sister group of section *Chondrophyllae s.l.* complex in the ITS phylogeny, but further investigation is needed to elucidate phylogenetic relationships within section *Chondrophyllae s.l.*. A phylogenetic analysis of ITS sequence data for 20 *Gentiana* species by Yuan *et al*. (1996) yielded a topology essentially congruent with previous morphological classifications, except for the phylogenetic position of *G. asclepiadea*, which is classified in section *Pneumonanthe* (Ho and Liu 2001). In the present study, *G. asclepiadea* clustered with two species from section *Gentiana*, which agrees with the results of Yuan *et al*. (1996). This indicates that *G. asclepiadea* is phylogenetically much closer to section *Gentiana* than to section *Pneumonanthe*. Although the trees based on cpDNA data in the present study was not completely supported by high bootstrap values for all nodes, the relationships indicated are generally consistent with the previous results described above. Our results, therefore, can be concluded to be a plausible representation of the genetic relationships among the taxa studied.

In the present study, three major cp DNA haplotypes were characterized and all the species classified in sections *Pneumonanthe* (except *G. asclepiadea*) and *Cruciata* have any of the three haplotypes. Exceptionally, *G. cachemirica*, which was placed in section *Kudoa* by Ho and Liu (2001), possesses haplotype B, whereas the other 4 accessions of the section *Kudoa* formed a distantly related clade. Consistent with this result, a recent morphological phylogenetic analysis by Davitashvili and Karrer (2007) suggested that *G. cachemirica* belongs to *Gentiana* sect. *Pneumonanthe* rather than sections *Isomeria* or *Kudoa*. Especially, their result showed that *G. septemfida* had the closest affinity to *G. cachemirica*, which fits the present haplotype characterization.

The lowest genetic distance from haplotype A was *G. yakushimensis* (0.003), which indicates that *G. yakushimensis* is the closest relative of haplotype A among the species sampled except haplotypes B and C. In the NJ tree, haplotype A clustered with haplotype B and *G. yakushimensis*. Although this species was classified in section *Kudoa* by Ho and Liu (2001), different researchers have placed *G. yakushimensis* in different sections (*Frigidae*, *Monopodiae* or *Pneumonanthe*) (Ho and Liu 2001). Species of *Gentiana* sect. *Kudoa* are distributed in East Asia except for *G. yakushimensis*, which is an endemic species of Yakushima Island in Japan (Toyokuni 1960, Ho and Liu 2001). In addition, the reported chromosome number (2n = 26) for *G. yakushimensis* is the same as those of the species of *Gentiana* sect. *Pneumonanthe* (except

Table 3. Genetic distances of the *trnL*(UAA) intron sequence among 23 *Gentiana* samples as well as *Gentianella saxosa* according to the Kimura's two-parameter method

	G. lut	*G. pun*	*G. asc*	*G. aca*	cv. Sed	*G. occ*	*G. ang*	*G. clu*	cv. Dar	*G. ver*	sb. *osc*	*G. rig*	cv. Eli	cv. Ste	cv. Blu	*G. orn*	*G. yak*	Hap. B	Hap. A	Hap. C	*G. jam*	*G. zol*	*G. rub*	*G. sax*
G. lutea	0																							
G. punctata	0.003																							
G. asclepiadea cv. Rosea	0.007	0.003																						
G. acaulis	0.013	0.010	0.013																					
G. acaulis cv. Sedanensis	0.013	0.010	0.013	0																				
G. occidentalis	0.017	0.013	0.017	0.003	0.003																			
G. angustifolia	0.017	0.013	0.017	0.003	0.003	0																		
G. clusii	0.013	0.010	0.013	0.013	0.013	0.017	0.017																	
G. clusii cv. Dark Blue JP	0.017	0.013	0.017	0.017	0.017	0.020	0.020	0.003																
G. verna subsp. *verna*	0.034	0.030	0.034	0.034	0.034	0.030	0.030	0.034	0.037															
G. verna subsp. *oschtenica*	0.037	0.034	0.037	0.037	0.037	0.034	0.034	0.037	0.041	0.003														
G. rigescens	0.027	0.024	0.027	0.027	0.027	0.024	0.024	0.027	0.030	0.034	0.037													
cv. Elisabeth Eschmann	0.031	0.027	0.030	0.031	0.031	0.027	0.027	0.031	0.034	0.038	0.041	0.010												
cv. Stevenagensis	0.031	0.027	0.030	0.031	0.031	0.027	0.027	0.031	0.034	0.038	0.041	0.010	0											
cv. Blue Flame	0.031	0.027	0.030	0.031	0.031	0.027	0.027	0.031	0.034	0.038	0.041	0.010	0	0										
G. ornata	0.031	0.027	0.030	0.031	0.031	0.027	0.027	0.031	0.034	0.038	0.041	0.010	0	0	0									
G. yakushimensis	0.013	0.010	0.013	0.013	0.013	0.017	0.017	0.013	0.017	0.034	0.038	0.020	0.024	0.024	0.024	0.024								
Haplotype B	0.010	0.007	0.010	0.010	0.010	0.013	0.013	0.010	0.013	0.031	0.034	0.017	0.020	0.020	0.020	0.020	0.003							
Haplotype A	0.010	0.007	0.010	0.010	0.010	0.013	0.013	0.010	0.013	0.031	0.034	0.017	0.020	0.020	0.020	0.020	0.003	0						
Haplotype C	0.010	0.007	0.010	0.010	0.010	0.013	0.013	0.010	0.013	0.031	0.034	0.017	0.020	0.020	0.020	0.020	0.003	0	0					
G. jamesii	0.044	0.041	0.044	0.048	0.048	0.052	0.052	0.048	0.051	0.070	0.073	0.051	0.055	0.055	0.055	0.055	0.041	0.037	0.037	0.037				
G. zollingeri	0.045	0.041	0.044	0.045	0.045	0.048	0.048	0.045	0.048	0.066	0.070	0.048	0.052	0.052	0.052	0.052	0.038	0.034	0.034	0.034	0.027			
G. rubicunda	0.041	0.037	0.041	0.041	0.041	0.045	0.045	0.041	0.044	0.063	0.066	0.048	0.052	0.052	0.052	0.052	0.034	0.030	0.030	0.030	0.034	0.024		
Gentianella saxosa	0.059	0.055	0.059	0.059	0.059	0.055	0.055	0.059	0.062	0.066	0.070	0.045	0.055	0.055	0.055	0.055	0.052	0.048	0.048	0.048	0.088	0.085	0.081	0

G. asclepiadea), whereas species of *Gentiana* sect. *Kudoa* have various chromosome numbers (2n = 16, 24, 26, 48) (Ho and Liu 2001). Taking into consideration the facts with our present results, *G. yakushimensis* might be phylogenetically much closer to section *Pneumonanthe* than to section *Kudoa*, although the bootstrap support for the relevant clades are rather low (Fig. 1). On the basis of the present investigation of cpDNA sequence variation, the sampled taxa possessing the cpDNA haplotypes A, B, and C as well as *G. yakushimensis* are likely to be important initial targets for incorporation in Japanese gentian breeding programs.

Flow cytometric analysis

The cpDNA haplotype groupings for the *Gentiana* accessions are supported by nuclear DNA contents obtained in the present study, but these species groupings are not consistent with the prevailing morphology-based taxonomic classification. Species in section *Kudoa* possessed divergent nuclear DNA contents, which was in agreement with phylogenetic relationships suggested by the cpDNA sequence data (Fig. 1). Concomitantly, the section *Kudoa* species also had high nucleotide diversity (0.00964) among the sections analyzed. On the other hand, it is clear that nuclear DNA content is homoplasious in the *Gentiana* phylogeny. Interestingly, there was a certain degree of variation in nuclear DNA contents (from 6.47 to 11.75 pg) among the taxa possessing haplotype A. This implies that relatively recent and frequent genome rearrangements have occurred, which may account for the homoplasious variation of nuclear DNA content in *Gentiana*. In future studies, further analyses of nuclear DNA contents and chromosome counting in additional specimens would be necessary to clarify the genetic relationship among three haplotypes and within each haplotype.

The present data also showed the variation of nuclear DNA contents between an inter-specific hybrid and its parent species. Nuclear DNA contents of the three *G. scabra* × *G. triflora* cultivars were intermediate (10.27–10.57 pg) between *G. scabra* (11.35–11.75 pg) and *G. triflora* (9.11–9.30 pg). This finding indicates that nuclear DNA content would be useful to identify progeny of interspecific crosses between *Gentiana* species. Nuclear DNA content and the reported chromosome number of *G. asclepiadea* (7.58 pg; 2n = 36, 44) was closer to those of *G. lutea* (8.07 pg; 2n = 40) and *G. punctata* (7.27 pg; 2n = 40) in section *Gentiana* than those of the species in section *Pneumonanthe* (3.49–3.78 pg and 9.11–11.75 pg; 2n = 26). This finding is congruent with the result of cpDNA analysis and suggests that *G. asclepiadea* may have a genetic composition similar to that of the species in section *Gentiana*. Consequently, nuclear DNA content may reflect the genetic background in the *Gentiana* species, and could confer information useful for future gentian breeding in combination with cpDNA sequence analyses.

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