Journal of Plant Biology, June 1999, 42(2) : 168-173

Molecular Evidence for the Phylogenetic Position of *Hanabusaya asiatica* Nakai (Campanulaceae), an Endemic Species in Korea

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The phylogenetic relationship of the Korean endemic genus, *Hanabusaya*, to other campanulaceous genera has been controversial since it was described by Nakai in 1911. Three genera of Campanuloideae, *Symphyandra*, *Adenophora*, and *Campanula*, have been considered closely related by various taxonomists on the basis of anther shape, gross morphology, and pollen characters, respectively. We have tested these competing taxonomic hypotheses using the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA from 12 taxa representing 7 genera of Campanulaceae. The molecular phylogeny indicates strongly that *Hanabusaya* is more closely allied to *Adenophora* than to *Campanula* or *Symphyandra*. The phylogenetic affinity of *Hanabusaya* and *Adenophora* is supported by a 100% bootstrap value and a high decay index (13). The average sequence divergence value (Kimura's 2-parameter method) between *Hanabusaya* and the *Adenophora* species is 2.58. The value is significantly (about ten times) lower than the ones observed between *Hanabusaya* and the species of *Campanula* (average of 23.52) and between *Hanabusaya* and *Symphyandra* (24.95). The ITS sequence phylogeny suggests that some morphological characters, such as fused anthers and corolla shape, are homoplastic in the Campanulaceae genera.

keywords: Campanulaceae, *Hanabusaya*, ITS, Korean edemic genus, phylogeny

Hanabusaya asiatica Nakai (Campanulaceae) is a well known endemic in Korea. The species was first described as *Symphyandra asiatica* by Nakai (1909) because of its synantherous stamens. However, Nakai (1911) segregated the species into a new genus, *Hanabusaya*, based on its distinctive morphological characters, such as the absence of basal leaves and appendages between the calyx lobes. Nakai (1921) added another species to the genus, *Hanabusaya latisepala*, but this species is considered a variety of *H. asiatica* (Lee, 1996).

Since Lee's (1969) brief statement that *H. asiatica* is very similar to *Adenophora remotiflora*, there have been several systematic studies employing various approaches including morphology (Yoo and Lee, 1995, 1996), palynology (Lee et al., 1986, 1988; Lee, 1988), and randomly amplified polymorphic DNA (RAPD) (Yoo et al., 1997). Unfortunately, most of the studies have centered on emphasizing the unique nature of *H. asiatica* and on confirming the segregation of the species as a distinct endemic genus. A notable exception was the study by Lee et al. (1986) which proposed that the species might be congeneric with *Campanula*, although this idea was abandoned in a later study (Lee et al., 1988). Thus, the phylogenetic affinity of *H. asiatica* to other campanulaceous genera, especially to *Symphyandra*, *Campanula*, and *Adenophora* remains unresolved.

In this paper, DNA sequences from the internal transcribed spacer (ITS) regions of the nuclear ribosomal repeat are analyzed to infer the phylogenetic relationship of *H. asiatica* with putatively related genera. We selected the ITS regions because they have been useful phylogenetically in a wide range of taxa at generic and specific levels (Baum et al., 1994; Baldwin et al., 1995; Downie and Katz-Downie, 1996; Kim and Jansen, 1996; Suh et al., 1996; Yuan et al., 1996; Francisco-Ortega et al., 1997; Alice and Campbell, 1998). Taxonomic and evolutionary implications of the molecular phylogeny are also discussed.

MATERIALS AND METHODS

Plant Samples, PCR and Sequencing

Twelve taxa representing seven genera were selected to reconstruct the ITS phylogeny of *H. asiatica* and putatively related genera, including *Campan-*

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Table 1. Taxa in the Campanulaceae examined for ITS of nuclear rDNA. Voucher specimens are deposited at the HAU (Hallym University Herbarium), SKK (Sung Kyun Kwan University Herbarium), and BH (Bailey Hortorium).

Taxa	Voucher
Hanabusaya asiatica Nakai	YDK 98-1 (HAU), Mt. Hwaak, Korea
Adenophora remotiflora (S. et Z.) Miq.	Lee 41 (SKK), Mt. Kebang, Korea
Adenophora erecta S. Lee, J. Lee et S. Kim	Lee 44 (SKK), Is. Ulleung, Korea
Adenophora wawreana Zahlbr.	Lee 20 (SKK), Lushan, China
Adenophora coronopifolia Fisch.	Lee 21198 (SKK), Mt. Halla, Korea
Campanula punctata Lam.	YDK 98-2 (HAU), Chunchon, Korea
Campanula glomerata L.	*AF090722, AF090723
Asyneuma japonicum Miq.	YDK 98-5 (HAU), Gapyong, Korea
Edraianthus graminifolius (L.) A. DC.	T. Ayers 88-195 (BH), cultivated
Symphyandra hofmannii Pant.	T. Ayers 88-225 (BH), cultivated
Codonopsis pilosa (Fr.) Nannf.	YDK 96-1 (HAU), Mt. Sorak, Korea
Codonopsis lanceolata (S. et Z.) Trautv.	YDK 96-7 (HAU), Chulwon, Korea

*The sequence of *Campanula glomerata* was obtained from the Genbank.

ula, *Symphyandra*, and *Adenophora* (Table 1). Two species from *Codonopsis* were chosen as outgroups because the genus has been shown to be one of the basal members of Campanulaceae (Cosner, 1993). Total genomic DNA was isolated from fresh leaf tissue using a modified CTAB procedure (Doyle and Doyle, 1987), followed by purification using ultracentrifugation in a cesium chloride/ethidium bromide gradient. The nucleotide sequence of the ITS regions of nuclear rDNA was determined directly from the PCR products using the snap-chill method (Winship, 1989). PCR was carried out in a 100 uL reaction containing about 50-100 ng of template DNA, 10 uL of 10X reaction buffer, 2.5 units of ExTaq (Takara Co.), 8 uL of 200 uM dNTPs in an equimolar ratio, and 50 pmol of each primer. The PCR and sequencing primers were identical to the ones designed by White et al. (1990) except ITS1, which differed by the two underlined bases (5'-GGAAGGAGAAGTCGTAACAAGG-3'). Five percent DMSO was added for some difficult DNA samples which produced fuzzy or double bands under initial PCR amplifying conditions. The thermal cycler was programmed to perform an initial 3 min denaturation at 95°C, 1 min annealing at 50°C, and 1 min extension at 72°C. This was followed by 30 cycles with 1 min denaturation at 95°C, 1 min annealing at 50°C, and 1 min extension at 72°C. The product was terminated with a final extension for 10 min at 72°C and soaking at 15°C. To remove unused amplifying primers and dNTPs, the PCR product was purified by a Geneclean kit (Bio 101) according to the manufacturer's protocol. The purified double stranded DNA was sequenced using the Sequenase (ver. 2.0, USB), ^{35}S and the four sequencing primers (White et al., 1990). For some DNA samples, dITP was used in place of dGTP to prevent base compression.

Sequence and Phylogenetic Analyses

The boundaries of ITS 1, the 5.8S coding region, and ITS 2 were determined by comparison with known sequences (Suh et al., 1993; Kim and Jansen, 1994). ITS sequences were aligned using ClustalW (ver. 1.6, Thompson et al., 1995). Gaps introduced from the alignment were treated as missing characters in subsequent analyses. Sequence distance values, neighbor-joining (Saitou and Nei, 1987), and maximum parsimony (branch and bound option) trees were obtained using PAUP (ver. 4.0b1, Swofford, 1998). To evaluate the degree of support for given clades, the bootstrap (Felsenstein, 1985) and decay analyses (Bremer, 1988; Donoghue et al., 1992) were performed using PAUP (ver. 4.0b1, Swofford, 1998) and AutoDecay (ver. 4.0.1, Eriksson, 1998), respectively.

RESULTS

Complete and aligned DNA sequences of the ITS regions for *H. asiatica* and related taxa are provided in Figure 1, and their characteristics are summarized in Table 2. Alignment of ITS sequences introduced 66 gaps (mainly due to aligning sequences of *Codonopsis* species) and resulted in a matrix of 554 nucleotide sites. Of the 554 sites, 241 sites were variable and 182 sites were phylogenetically informative.

Fitch parsimony analysis resulted in a fully resolved single most parsimonious tree (Fig. 2). The tree had a

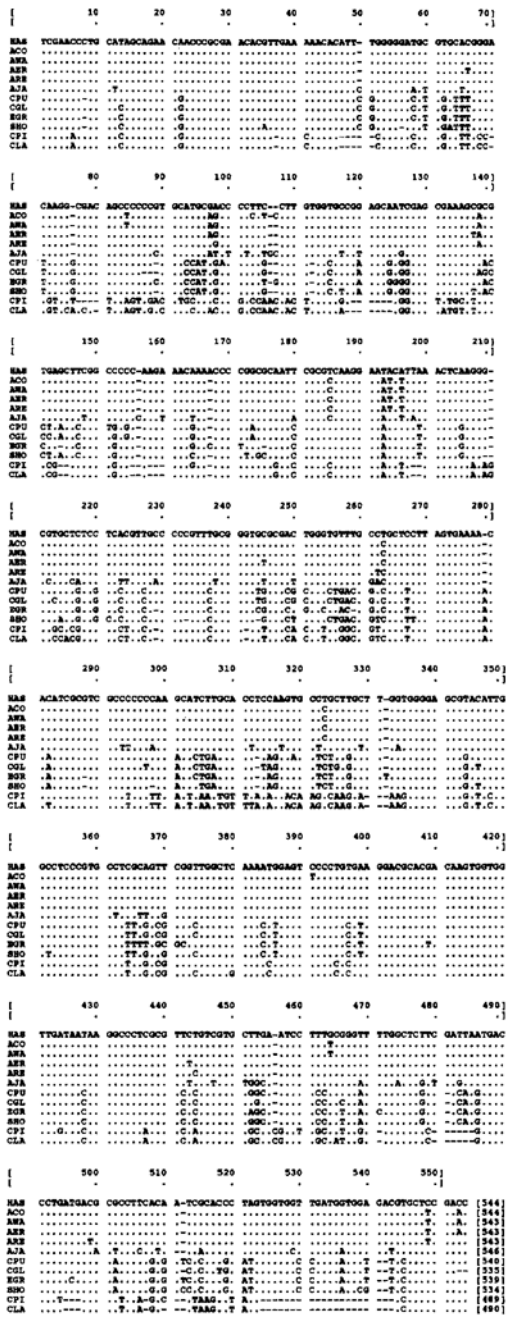

Figure 1. Aligned DNA sequences of the ITS regions in 18S-26S nuclear ribosomal DNA from the 12 taxa examined. ITS 1 ranges from position 1 to 282; ITS 2 extends from position 283 to 554. Sequences of the 5.8S subunit are excluded. Hyphens are gaps required for alignment. Dots in the sequences denote same nucleotide state as the first taxon, *H. asiatica*. Taxon abbreviations are: HAS (*H. asiatica*), ACO (*Adenophora coronopifolia*), AWA (*Adenophora wawreana*), AER (*Adenophora erecta*), ARE (*Adenophora remotiflora*), AJA (*Asyneuma japonica*), CPU (*Campanula punctata*), CGL (*Campanula glomerata*), EGR (*Edraianthus graminifolius*), SHO (*Symphyandra hofmannii*), CPI (*Codonopsis pilosa*), CLA (*Codonopsis lanceolata*).

Table 2. Size and G + C% of ITS 1 and 2 of *H. asiatica* and related taxa.

TAXA	ITS1		ITS2	
	length	G+C%	length	G+C%
Hanabusaya asiatica	275	58.2	269	57.2
Adenophora coronopifolia	275	58.5	269	56.2
Adenophora wareana	274	58.4	269	56.6
Adenophora erecta	274	57.7	269	56.5
Adenophora remotiflora	274	58.4	268	57.1
Campanula punctata	274	60.9	266	63.6
Campanula glomerata	272	62.4	263	61.6
Asyneuma japonicum	278	55.1	268	52.2
Edraianthus graminifolius	273	63.0	266	61.7
Symphyandra hofmannii	270	58.5	264	62.1
Codonopsis pilosa	253	64.0	236	59.8
Codonopsis lanceolata	255	62.3	235	59.6
*after alignment	282	–	272	–

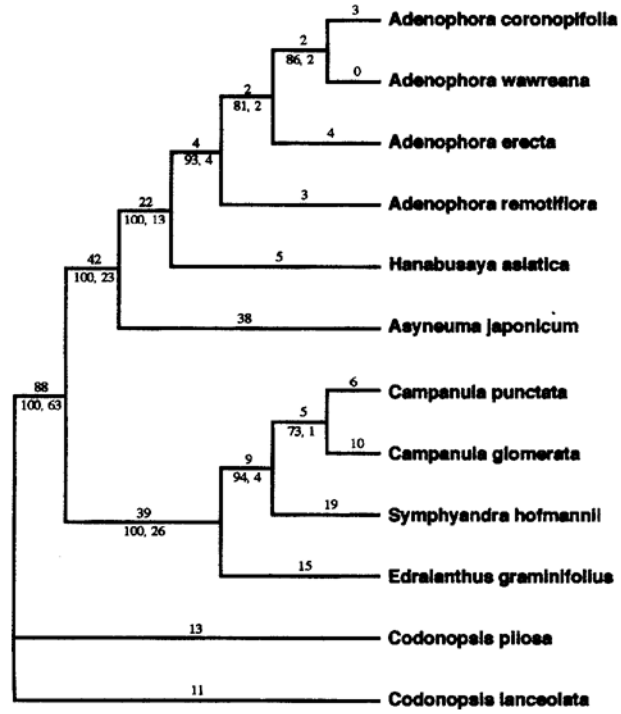

Figure 2. The single most parsimonious tree of *H. asiatica* and related taxa based on ITS sequence data. Numbers above branches are nucleotide changes. Numbers below branches represent bootstrap support (percentage) and decay indices, respectively.

length of 340 steps with a consistency index (CI) of 0.84 excluding uninformative characters and retention index (RI) of 0.91. The ingroup taxa were divided into two major clades, which consisted of *Adenophora/Hanabusaya/Asyneuma* and *Campanula/Symphyandra/Edraianthus*. *H. asiatica* and the species

Table 3. Pairwise sequence distance between taxa examined. Absolute distances are shown above the diagonal, and sequence divergence values (X100) by Kimura's two parameter method are below the diagonal.

		1	2	3	4	5	6	7	8	9	10	11	12
1	HAS	–	16	13	15	11	64	106	108	106	112	132	134
2	ACO	3.01	–	3	9	13	67	113	115	112	119	137	135
3	AWA	2.44	0.56	–	6	10	66	111	113	110	117	137	135
4	AER	2.82	1.69	1.12	–	12	64	110	112	109	114	134	132
5	ARE	2.25	1.87	1.30	1.68	–	65	108	110	107	112	134	134
6	AJA	12.82	13.48	13.27	12.84	13.28	–	125	127	122	127	142	140
7	CPU	22.90	24.72	24.19	23.94	23.42	27.90	–	4	30	29	129	130
8	CGL	24.14	25.19	24.63	24.39	24.12	30.35	3.07	–	31	31	130	131
9	EGR	23.30	24.88	24.41	24.15	23.63	27.53	5.85	6.95	–	40	133	136
10	SHO	24.95	26.84	26.28	25.46	24.93	29.01	5.67	6.58	8.01	–	128	128
11	CPI	34.58	36.24	36.32	35.27	35.29	38.03	33.75	33.16	34.96	33.83	–	22
12	CLA	35.10	35.34	35.41	34.40	35.12	37.12	33.95	33.01	35.91	33.69	4.69	–

Refer Figure 1 for taxon abbreviations.

of *Adenophora* formed a strong monophyletic group as indicated by a 100% bootstrap value and a decay index of 13. The clade consisting of *Campanula* and *Symphyandra* was remotely related to *H. asiatica*.

The nucleotide sequence distance calculated by Kimura's 2-parameter method (Kimura, 1980) varied from 0.56 to 38.03 (Table 3). The sequence distance values between *H. asiatica* and other species ranged from 2.25 (*A. remotiflora*) to 35.10 (*C. lanceolata*). The topology of the neighbor-joining tree (not shown) was identical to that of the parsimony tree.

DISCUSSION

The ITS sequence data indicate a very close phylogenetic relationship between *Hanabusaya* and *Adenophora*. This is a rather surprising because very few characters have been proposed to support a close relationship between these two genera. The paucity of morphological characters supporting a sister group relationship between *Hanabusaya* and *Adenophora* is due, at least in part, to the fact that most previous studies on *Hanabusaya* were designed to justify its endemic status. These previous studies provided limited discussion on the phylogenetic relationship of the genus (Lee et al., 1988; Yoo and Lee, 1995, 1996; Yoo et al., 1997). We found that some of the characters examined previously, such as micromorphology of pollen (Lee et al., 1988) and seeds (Yoo and Lee, 1995), may indeed support sister group relationship between *Hanabusaya* and *Adenophora*.

It is noteworthy that the average sequence distance between *Hanabusaya* and *Adenophora* is 2.63, which is substantially lower than the distance between the two species of *Codonopsis* (4.69). In spite of the strong support for a close relationship between the two genera in the ITS tree, *Hanabusaya* is markedly different from *Adenophora* in floral (synantherous) and vegetative (absence of basal leaves) structures (Yoo and Lee, 1996). Furthermore, the four species of *Adenophora*, examined from three sections of the genus, form a strongly supported monophyletic group. Thus, the generic circumscription of *Hanabusaya* and *Adenophora* should be maintained until more definitive evidence (i.e., paraphyly of *Adenophora*) is provided from expanded sampling.

The remote relationship of *Hanabusaya* and *Campanula/Symphyandra* in the ITS tree appears to contradict the previous studies. Fedorov (1957) indicated that the general appearance of *H. asiatica* is extremely similar to *C. punctata*. In a morphological cladistic analysis for the Korean Campanulaceae, Yoo (1995) also proposed a sister group relationship between *Hanabusaya* and *Campanula*. Moreover, Lee et al. (1986) and Lee (1988) indicated that the two genera are so similar in pollen characters and they may be even congeneric. High sequence divergence (ca. 23.99) between *Hanabusaya* and the *Campanula/Symphyandra* clade suggest that these two lineages of Campanulaceae have been separated for a relatively long time. It is evident that the campanulate corolla shared by *Hanabusaya* and *Campanula* is a homoplastic character because the former is more closely related to *Asyneuma*, which has a deeply lobed corolla.

The placement of *Hanabusaya* and *Symphyandra* in different clades suggests that synantherous stamens have evolved independently in these genera. Neoteny, extension of the earlier phases of development

into maturity (Takhtajan, 1991), is a possible evolutionary mechanism for the origin of the synantherous condition in those genera. In early stages of floral development, the anthers of the campanulaceous plants typically form a tube in which pollen is shed and through which the style with collecting hairs grow. As the flower opens, the closely positioned anthers eventually become free and wither in most genera of the family (Kovanda, 1985; Mabberley, 1990). We postulate that the fused anthers in *Hanabusaya* and *Symphyandra* originated independently by the retention of the juvenile stamen condition. The connate anthers are also found in the related family Lobeliaceae (Mabberley, 1990). Diverse comparative approaches including developmental and molecular genetic studies with more vigorous taxon sampling are required to address the evolution of this unusual character in the Campanulaceae.

Our study indicates that ITS sequences are very useful for examining phylogenetic relationships of *Hanabusaya*. Recently, Cosner et al. (1994) demonstrated that *rbc*L sequences have utility in determining phylogenetic positions of problematic genera (i.e., *Nemacladus*, *Pentaphragma*, *Cyphia*), which have often been included in the Campanulaceae. Integration of DNA sequence data from expanded sampling will provide valuable information on various systematic issues such as familial and generic circumscription, intrafamilial classification, and morphological evolution in the Campanulaceae. Additional gene sequence data will also help to assess the mode of chloroplast genome evolution in the campanulaceous genera, which exhibits highly complicated structural rearrangements (Cosner, 1993; Cosner et al., 1997).

ACKNOWLEDGMENTS

We thank to Dr. K.O. Yoo for valuable comments and Tina Ayers for providing leaf material. This study was supported by the internal grant from the Hallym University.

Received April 29, 1999; accepted June 22, 1999.

LITERATURE CITED

Alice LA, Campbell CS (1998) Phylogeny of *Rubus* (Rosaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. Amer J Bot 86: 81-97

Baldwin BG, Sanderson MJ, Porter JM, Wokciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. Ann Missouri Bot Gard 82: 247-277

Baum DA, Sytsma KJ, Hoch PC (1994) A phylogenetic analysis of *Epilobium* (Onagraceae) based on nuclear ribosomal DNA sequences. Syst Bot 19: 363-388

Bremer K (1988) The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution 42: 795-803

Cosner ME (1993) Phylogenetic and molecular evolutionary studies of chloroplast DNA variation in the Campanulaceae. Ph.D. thesis. The Ohio State University, Columbus

Cosner ME, Jansen RK, Lammers TG (1994) Phylogenetic relationships in the Campanulales based on *rbc*L sequences. Pl Syst Evol 190: 79-95

Cosner ME, Jansen RK, Palmer JD, Downie SR (1997) The highly rearranged chloroplast genome of *Trachelium caeruleum* (Campanulaceae): miltiple inversions, inverted repeat expansion and contraction, transposition, insertion/deletions, and several repeat families. Curr Genet 31: 419-429

Donoghue MJ, Olmstead RG, Smith JF, Palmer JD (1992) Phylogenetic relationships of Dipsacales based on *rbc*L sequences. Ann Missouri Bot Gard 79: 333-345

Downie SR, Katz-Downie DS (1996) A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. Amer J Bot 83: 234-251

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11-15

Eriksson T (1998) AutoDecay ver. 4.0.1 (Hypercard program distributed by the author). Botaniska Institutionen, Stockholm University, Stockholm

Fedorov AA (1957) Flora of the U.S.S.R. Vol 24.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791

Francisco-Ortega J, Santos-Guerra A, Hines A, Jansen RK (1997) Molecular evidence for a Mediterranean origin of the Macronesian endemic genus *Argyranthemum* (Asteraceae). Amer J Bot 84: 1595-1613

Kim K-J, Jansen RK (1994) Comparisons of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*, Asteraceae): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. Pl Syst Evol 190: 157-185

Kim Y-D, Jansen RK (1996) Phylogenetic implications of *rbc*L and ITS sequence variation in the Berberidaceae. Syst Bot 21: 381-396

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120

Kovanda M (1985) Campanulaceae. *In* VH Heywood, ed, Flowering plants of the world, Croom Helm, London and Sydney, pp 254-256

Lee S, Ahn YM, Park KR (1986) Palynological relationship of *Hanabusaya asiatica* Nakai within the Campanulaceae. Kor J Plant Tax 16: 25-37

Lee S (1988) Palynological evaluation of some endemic genera in Korea. Kor J Plant Tax 18: 19-31

Lee S, Chung YJ, Lee J (1988) A palynotaxonomic study of the Korean Campanulaceae. Kor J Plant Tax 18: 115-131

Lee WC (1969) A discussion on Korean endemic plants. Kor J Plant Tax 1: 15-21

Lee WC (1996) Coloured standard illustrations of Korean plants. Academy Press, Seoul

Mabberley DJ (1990) The plant book: A portable dictionary of the higher plants, Cambridge University Press, Cambridge

Nakai T (1909) Plantae Novae Asiaticae. Bot Mag Tokyo 23: 185-192

Nakai T (1911) A preliminary note on new genus of Campanulaceae found in Korea. Bot Mag Tokyo 25: 160-162

Nakai T (1921) Notulae ad plantas Japoniae et Koreae XXV. Bot Mag Tokyo 35: 147

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425

Suh Y, Cho H-J, Kim S, Park C-W (1996) Comparative analysis of ITS sequences from *Acer* species (Aceraceae) in Korea. J Plant Biol 39: 1-8

Suh Y, Thien LB, Reeve HE, Zimmer EA (1993) Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. Amer J Bot 80: 1042-1055

Swofford DL (1998) PAUP: phylogenetic analysis using parsimony, ver. 4.01b, Smithsonian Institution, Washington, DC

Takhtajan A (1991) Evolutionary trends in flowering plants. Columbia University Press, New York

Thompson JD, Higgins DG, Gibson TJ (1995) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* M Innis, D Gelfand, J Sninsky, T White, eds, PCR protocols: A guide to methods and applications, Academic Press, San Diego, pp 315-322

Winship PR (1989) An improved method for direct sequencing of PCR amplified material using dimethyl sulphoxide. Nucleic Acids Res 17: 1266

Yoo KO (1995) Taxonomic studies on the Korean Campanulaceae. Ph.D. thesis, Kangwon University, Chunchon

Yoo KO, Lee WT (1995) Seed morphology of Korean Campanulaceae. Kor J Plant Tax 25: 61-70

Yoo KO, Lee WT (1996) External morphology of Korean Campanulaceae. Kor J Plant Tax 26: 77-104

Yoo KO, Lee WT, Kim NS, Lim HT (1997) Comparative studies on the *Hanabusaya asiatica* and its allied groups by RAPD analysis. Plant Species Biol 12: 49-54

Yuan Y-M, Kupfer P, Doyle JJ (1996) Infragenetic phylogeny of the genus *Gentiana* (Gentianaceae) inferred from nucleotide sequences of the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA. Amer J Bot 83: 641-652