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Comparative Studies of the *Hanabusaya asiatica* and its Allied Groups by RAPD Analysis

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Abstract **The phylogenetic relationships between *Hanabusaya asiatica* and its allied groups were assessed by Randomly Amplified Polymorphic DNA (RAPD) analysis of genomic DNA, and their taxonomic status was reevaluated at the molecular level. The analyzed plants consisted of 36 populations of eight genera and 27 taxa. Forty-two out of 101 primers(10-mer) screened were able to amplify DNA. Only 19 primers, however, actually succeeded in DNA amplification, resulting in 523 randomly amplified DNA fragments. The analyzed taxa showed very high polymorphism, allowing each individual taxon to be identified based on RAPD analysis. All eight genuses were differentiated from each other at the 0.86 level of similarity index value. In particular, *Hanabusaya asiatica* was distinguished from its closely related genus. Intraspecific and interspecific relationships were quite close, at levels ranging from 0.77 to 0.99. Our RAPD analysis supports the previous data based on morphological and palynological studies.**

Key words: **RAPD *analysis*, PCR, *Hanabusaya asiatica*, phylogeny.**

Hanabusaya asiatica was reported as a new genus in 1911 by Nakai. Its origin and the phylogenetic relationship between *Hanabusaya asiatica* and closely related taxa have been discussed intensively because *Hanabusaya asiatica* was known to be similar to *Adenophora remotiflora* and *Campanula punctata* (Lee, 1969), based on flower morphology (Lee et al., 1986). Lee et al. (1986, 1988) investigated the phylogenetic relationship among these genera based on palynological studies. The characters of pollen grains were reported also to be variable in other species (Hara, 1988). It can thus be suggested that the use of modern molecular approaches will be necessary for the resolution of systematic problems.

Recently, genomic polymorphism has been reported in many species using RAPD technique (Welsh and McClelland, 1990; Hu and Quiros, 1991; Cho et al., 1995; Shin et al., 1995). The RAPD technique provides a faster and easier approach for exploring genetic polymorphism in molecular taxonomic studies (Williams et al., 1990, 1993; Welsh and McClelland, 1990). In the course of a comprehensive phylogenetic study of Korean Campanulaceae based on morphological, anatomical, ultrastructural, and molecular analysis (Yoo, 1995), we have obtained some evidence by RAPD analysis that might support the palynological studies done by Lee et al. (1986, 1988).

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Materials and Methods

1. *Plant Materials*

Leaf samples of Korean Campanulaceae, 8 genera and 27 taxa (including 9 variation types), totaling 36 taxa, were obtained from plants grown in the field and a greenhouse and stored at $-80\geq$ °C until use (Table 1). Specimens have been maintained in the Herbarium of the Department of Biology, Kangwon National University, Korea.

2. *DNA Extraction and Polymerase Chain Reaction*

For each population, bulk leaf samples were ground in liquid nitrogen. Grinding was continued after addition of extraction buffer (0.2 M Tris-Cl pH 8.0, 0.05 M EDTA pH 8.0, 0.5 M NaCl, 0.5% SDS). An equal volume of chloroform : isoamyl alcohol (24:1) was immediately added, and the solution was mixed and incubated on ice for 5 min. After centrifugation at 13,000 g for 7 min, the supernatant was collected; to it 2 volumes of ethanol were added, mixed, and left in a freezer (-20°C) for one hour. After centrifugation at 13,000 g for 7 min, the supernatant was removed. The DNA pellet was washed twice in 70% (v/v) ethanol, dried in a vacuum dryer, and resuspended in TE buffer containing 10 μg/ml RNAse. The presence of total genomic DNA was confirmed by electrophoresis on a 0.7% (w/v) agarose gel and quantified by measuring absorbance at 260 nm by a Beckman spectrophotometer.

Nineteen arbitrary primers (10-mer, UBC, Canada) (Table 2) were used for polymerase chain reaction

Table 1. Materials and collection data of *Hanabusaya asiatica* and its allied groups.

Scientific name	Collection site and date
Adenophora Sect. Remotiflorae	
A. grandiflora Nakai	KW* : Mt. Sorak (Aug. 12, 1992)
	Mt. Odae (Aug. 12, 1993)
A. remotiflora (S. et Z.) Miquel	KW : Mt. Daryong (Sep. 19, 1992)
	Mt. Sorak (Aug. 25, 1992)
var. hirticalycis J. Lee et S. Lee	CN : Mt. Jiri, Nogodan (Aug. 24, 1993)
Sect. Thyrsanthae	
A. coronopifolia Fischer	CJ : Mt. Halla (Sep. 30, 1992)
A. polyantha Nakai	KG : Dukjukdo (Aug. 30, 1991)
A. stricta Miquel	KG : Dukjukdo (Sep. 1, 1992)
var. lancifolia Honda	KG : Dukjukdo (Sep. 30, 1991)
A. taquetii Léveillé	CJ : Mt. Halla (Sep. 7, 1993)
Sect. Platyphyllae	
A. divaricata Fr. and Sav.	KW : Mt. Daryong (Sep. 19, 1992)
	Kongkeun (Aug. 5, 1994)
A. racemosa J. Lee et S. Lee	KW : Mt. Odae (Jul. 23, 1993)
	Mt. Jumbong (Aug. 20, 1993)
A. triphylla var. *japonica* Hara	KW : Hyangnobong (Aug. 15, 1991)
A. verticillata Fischer	KW : Mt. Daryong (Oct. 4, 1992)
	Kongkeun (Aug. 5, 1994)
	CJ : Cheju-do, 1100 Goji (Sep. 27, 1992)
var. abbreviata Léveillé	CJ : Mt. Halla (Sep. 30, 1992)
var. angustifolia Regel	KW : Hongcheon Yulcheonri (Sep. 7, 1993)
var. hirsuta Fr. Schmidt	KN : Mt. Kaya (Sep. 30, 1991)
Asyneuma japonicum Miquel	KW : Chunseong Jiamri (Sep. 21, 1993)
Campanula glomerata var. *dahurica* Fischer	KW : Kongkeun (Sep. 12, 1992)
C. punctata Lamarck	KW : Kongkeun (Jun. 22, 1992)
	Mt. Daryong (Jun. 18, 1993)
C. takesimana Nakai	KB : Ulleungdo (Aug. 18, 1992)
Codonopsis lanceolata (S. et Z.) Trautv.	KW : Mt. Daryong (Sep. 19, 1992)
	Kongkeun (Sep. 22, 1992)
C. minima Nakai	CJ : Mt. Halla (Jul. 16, 1991)
C. pilosula (Fr.) Nannf.	KW : Mt. Jumbong (Jun. 22, 1992)
C. ussuriensis (Rupr. et Maxim.) Hemsley	KW : Heongsung Sambaeri (Jul. 29, 1991)
Hanabusaya asiatica Nakai	KW : Mt. Sorak(Aug. 15, 1992)
	Mt. Jumbong (Aug. 20, 1993)
Peracarpa carnosa var. *circaeoides* (Fr. Schmidt) Makino	CJ : Cheju-do Kyore (Aug. 5, 1993)
Platycodon grandiflorus (Jacq.) A.DC.	KW : Ganseong Myongwolri (Aug. 17, 1991)
Wahlenbergia marginata (Thunb.) A. DC.	CJ : Seokwipo Seohori (Aug. 3, 1993)

(Notes) All samples are colleted from Korea by the authors.

* KG: Kyeonggido, CJ: Chejudo, CN: Chollanamdo, KW: Kangweondo, KN: Kyongsangnamdo: KB: Kyongsangbukdo.

(PCR) based on the protocol of Williams et al. (1990), with minor modifications. PCR was performed in a volume of 25 μl reaction solution containing 1 × KCl buffer, 4 mM dNTPs, 50 ng of DNA, 0.8 unit of *Taq* polymerase (Promega), and 0.2 μM of primer. Twenty μl of mineral oil was added over the reaction solution. PCR was performed in a DNA Thermal Cycler (Perkin Elmer Cetus 9600). DNA was amplified using the following program: 94°C for 1 min, 35°C for 1 min, 72°C for 2 min, 45 cycles; 72°C for 10 min, 1 cycle. Amplified

DNA products were analyzed by electrophoresis in 1.5% agarose gels and detected by staining the gels with ethidium bromide. The gel was photographed under UV light with Polaroid 667 film. A PCR tube containing all components except genomic DNA was run as a control with each primer in order to check for contamination. To check the molecular weight of each DNA fragment separated on the gel, 3 Kb ladder DNA was used for comparison.

3. *Phylogenetic Relationship*

Amplified and separated DNA fragments on agarose gels were scored for presence (1) or absence (0) of bands for each of the 36 populations using 19 primers. Only reproducible bands were considered, and faint bands which appeared unstable in multiple runs were ignored (Hashizume et al., 1993). In some cases no band was detected, possibly due to insufficient homologies between primers and the DNA template. These were counted as missing values due to the possibility that they arose by the failure of the PCR caused by some other variation in the reaction. Similarity coefficients were calculated employing the following equation (Nei and Li, 1979):

$$S=\frac{2n_{xy}}{n_x+n_y}$$

The NTSYS program (Exeter Software) was used to produce a phenogram for which the UPGMA (unweighted pair-group method with arithmetic average) was employed (the NTSYS tree program).

Results and Discussion

1. *RAPD Analysis*

The phylogenetic relationships between *Hanabusaya asiatica* and its allied groups including 8 genera and 27 species, totaling 36 populations, were investigated at the DNA level using RAPD method. Only 19 out of 101 primers screened gave rise to a total of 523 randomly amplified DNA fragments. Generally, base composition of primers influences the ability of DNA amplification (Williams et al., 1990), and higher ratio of G+C content has been shown to be positively correlated with ability and strength of DNA amplification (Fritsch et al., 1993). Nineteen of the primers screened for this study successfully amplified the genomic DNAs, and G+C contents of all the primers were higher than 60% (Table 2).

Each primer amplified 6 (primer B) to 40 (primer J) DNA fragments with a size range between 298 and 2036 bp. No monomorphic band appeared in all analyzed plants, resulting in very high polymorphisms among them. This was expected because of the large morphological variation and the wide range of taxa studied.

In the comparative studies, interspecific polymorphisms found in each genera showed higher inter-variety consistency (63.3–82.1%) in the banding patterns of individual plants: *Adenophora*, 69.9%, 64 bands; *Campanula*, 72.1%, 75 bands; and *Codonopsis*, 82.1%, 92 bands. In each section of *Adenophora*, 23, 24, and 50 bands were detected in Remotiflorae, Thyr-

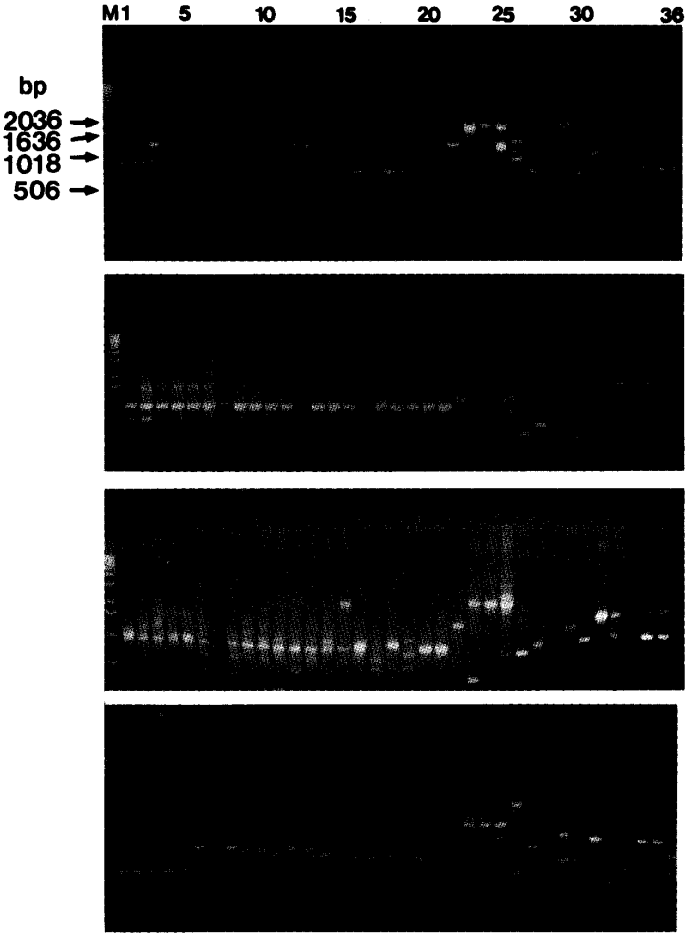

Fig. 1. RAPD profiles of the analyzed plants. The sequence of each primer in D, E. J, K is shown in Table 2.
M. DNA Marker, Lane 1. *Adenophora remotiflora* var. *hirticalycis*, 2. *A. remotiflora* (Mt. Sorak), 3. *A. remotiflora* (Mt. Daryong), 4. *A. grandiflora* (Mt. Odae), 5. *A. grandiflora* (Mt. Sorak), 6. *A. coronopifolia*, 7. *A. taquetii*, 8. *A. stricta* var. *lancifolia*, 9. *A. stricta*, 10. *A. polyantha*, 11. *A. racemosa* (Mt. Jumbong), 12. *A. racemosa* (Mt. Odae). 13. *A. divaricata* (Kongkeun), 14. *A. divaricata* (Mt. Daryong), 15. *A. triphylla* var. *japonica*, 16. *A. verticillata* (Kongkeun), 17. *A. verticillata* (Chejudo), 18. *A. verticillata* (Mt. Daryong), 19. *A. verticillata* var. *angustifolia*, 20. *A. verticillata* var. *hirsuta*, 21. *A. verticillata* var. *abbreviata*, 22. *Campanula glomerata* var. *dahurica*, 23. *C. punctata* (Kongkeun), 24. *C. punctata* (Mt. Daryong), 25. *C. takesimana*, 26. *Asyneuma japonicum*, 27. *Platycodon grandiflorus*, 28. *Hanabusaya asiatica* (Mt. Sorak), 29. *H. asiatica* (Mt. Jumbong), 30. *Wahlenbergia marginata*, 31. *Peracarpa carnosa* var. *circaeoides*, 32. *Codonopsis lanceolata* (Mt. Daryong), 33. *C. lanceolata* (Kongkeun), 34. *C. minima*, 35. *C. ussuriensis*, 36. *C. pilosula*.

Table 2. List of primers used for the RAPDs analysis by PCR.

Primer	Sequence (5′ → 3′)	Primer	Sequence (5 → 3′)
A	CCT GGG CTG G	K	GAG GGC GAG C
B	CCT GGG CCT A	L	GAG GGC AAG A
C	ACA GGG CTC A	N	GGG CAC GCG A
D	CCG GCC TTA G	O	GAG CAC CTG A
E	CCG GCC TTC C	P	GAG CAC CAG G
F	CCG GCC CCA A	Q	GAG CAC GGG G
G	AAA ACC GGG C	R	GGG CCC GAG G
H	TTG GCC GAG C	S	GGG CGC GAG T
I	AGG GGC GGG A	T	GGG AGG AGG G
J	GAG GGC GTG A		

santhae, and Platyphyllae, respectively (Table 3).

2. *Genetic Similarity and Phylogenetic Relationship*

The similarity indices among Korean Campanulaceae are presented as a phenogram in Fig. 2. The 36 populations showed high levels of similarity indices ranging from 0.77 to 0.99, and each of the genera was well distinguished by a similarity index value of 0.86 (Fig. 2).

Each species of *Adenophora* including numerous taxa was distinguished at the similarity coefficient of 0.95. Classifications of sections and series in the genus *Adenophora* were dependent upon external morphologies such as inflorescence, leaf arrangement, and shapes of disc, corolla, and calyx lobe (Hong, 1983; Fedorov, 1957), but there was no distinct except for section Remotiflorae and series Verticillatae (*A. verticillata, A. verticillata* var. *abbreviata, A. verticillata* var. *angustifolia* and *A. verticillata* var. *hirsuta*). This results indicated that a great deal of diversities in morphology and phylogenetic relationship in *Adenophora* was due to the latest differentiation in the Campanulaceae (Arano and Saito, 1975).

In the *Campanula* species, *C. glomerata* var. *dahurica* is distinguished from *C. punctata* and *C. takesimana* by the types of inflorescence and shapes of corolla. RAPD results also showed the distinct differences in phylogenetic relationship among these species. Lee (1971) reported that *C. takesimana* and *C. punctata* growing in Japan and Korea were the same species based on morphological characters and chromosome numbers. In our RAPD analysis, however, they were well distinguished from each other.

Codonopsis with climbing habit of stem was distinguished by other 7 genera with similarity index of 0.77. Among them *C. pilosula* having differences in leaf morphology and corolla appeared to have lower similarity index of 0.86 when compared with the other 3 species possessing the higher similarity indices above 0.94.

Hanabusaya asiatica displayed high polymorphisms in the intraspecific populations. Also, comparing with *Campanula* and *Adenophora* previously known as a similar taxon by palynological and morphological characters (Lee et al., 1986, 1988), *Hanabusaya asiatica* was quite different from other taxa.

Table 3. Number of bands by RAPD product.

Taxa	No. of species	No. of populations	Total bands	Polymorphic bands	% polymorphic bands
Adenophora	15	21	92	64	69.6
Sect. Remotiflorae	3	5	63	23	36.5
Sect. Thyrsanthae	5	5	62	24	38.7
Sect. Platyphyllae	7	11	79	50	63.3
Campanula	3	4	104	75	72.1
Codonopsis	4	5	112	92	82.1
Asyneuma	1	1	54		
Hanabusaya	1	2	68		
Peracarpa	1	1	39		
Platycodon	1	1	56		
Wahlenbergia	1	1	41		

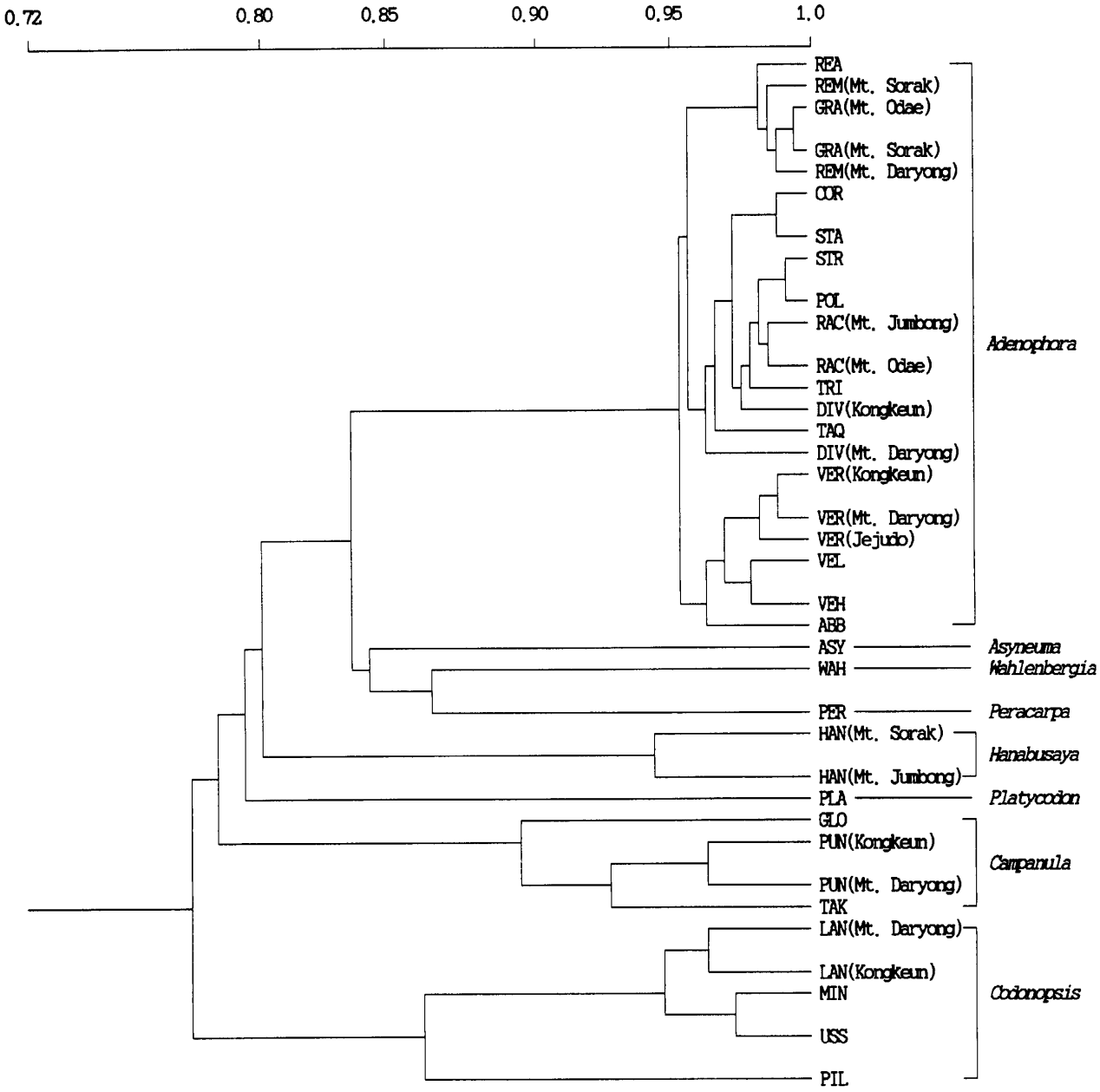

Fig. 2. Phenogram of *Hanabusaya asiatica* and its allied groups based on analysis of PCR amplified fragments produced by 19 arbitrary 10-mer RAPD primers.
(Notes) POL. *Adenophora polyantha,* REM. *A. remotiflora,* REA. *A. remotiflora* var. *hirticalycis,* ABB. *A. verticillata* var. *abbreviata,* DIV. *A. divaricata,* GRA. *A. grandiflora,* RAC. *A. racemosa,* STR. *A. stricta,* STA. *A. stricta* var. *lancifolia,* COR. *A. coronopifolia,* TAQ. *A. taquetii,* TRI. *A. triphylla* var. *japonica,* VER. *A. verticillata,* VEL. *A. verticillata* var. *angustifolia,* VEH. *A. verticillata* var. *hirsuta,* PUN. *Campanula punctata,* GLO. *C. glomerata* var. *dahurica,* TAK. *C. takesimana,* ASY. *Asyneuma japonicum,* LAN. *Codonopsis lanceolata,* MIN. *C. minima,* PIL. *C. pilosula,* USS. *C. ussuriensis,* HAN. *Hanabusaya asiatica,* PER. *Peracarpa carnosa* var. *circaeoides,* WAH. *Wahlenbergia marginata,* PLA. *Platycodon grandiflorus.*

Above the results *Hanabusaya asiatica* were identified allied groups in Korean Campanulaceae. Also RAPD data were matched very well with previous data based on morphological and palynological studies.

But, *Symphyandra*, known to be the most similar taxon in terms of external morphology to *Hanabusaya asiatica* being distributed in mainly Russia could not be found in Korea. Thus, complete comparative analysis of phylogenetic relationship was somewhat limited in our study. Other approaches for molecular taxonomy such as RFLP and gene sequencing are undergoing for the resolution of systematic problems.

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