

## Taxonomy of Korean *Calanthe* species and few of its mutants based on AFLP data

Krishnamoorthy Srikanth, Ja Choon Koo<sup>3</sup>, Jajung Ku<sup>1</sup>, Kyung Choi<sup>1</sup>, Kwang-Woo Park<sup>1</sup>,  
Soonku So<sup>2</sup>, Yong-Gook Choi and Sung Soo Whang<sup>3\*</sup>

Division of Science Education, Chonbuk National University, Jeonju 561-756, Korea

<sup>1</sup>National Arboretum, Gyenggido 487-821, Korea

<sup>2</sup>Department of Biological Sciences, Chonbuk National University, Jeonju 561-756, Korea

<sup>3</sup>The Institute of Science Education, Chonbuk National University, Jeonju 561-756, Korea

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## AFLP에 의한 한국산 새우난초속 식물과 그의 수종 돌연변이에 대한 분류학적 연구

스리칸스 · 구자춘<sup>3</sup> · 구자정<sup>1</sup> · 최 경<sup>1</sup> · 박광우<sup>1</sup> · 소순구<sup>2</sup> · 최용국 · 황성수<sup>3\*</sup>

전북대학교 과학교육학부, <sup>1</sup>국립수목원, <sup>2</sup>전북대학교 생명과학과, <sup>3</sup>전북대학교 과학교육연구소

**ABSTRACT:** Five Korean *Calanthe* species, *C. discolor*, *C. bicolor*, *C. sieboldii*, *C. reflexa*, and *C. aristulifera*, were studied using amplified fragment length polymorphism (AFLP) to assess their taxonomic and genetic relationships. Sixteen accessions belonging to five native *Calanthe* spp. and mutants with yellow tepal and white lip (YW mutants) were studied. We identified 50 putative markers using AFLP analysis. The results of AMOVA showed that genetic variance was higher between species than within species. Genetic dissimilarity when compared with the rest of the species was the lowest for individuals of the YW mutants and the highest for individuals of *C. reflexa*. The mutants clustered outside the major group. *Calanthe bicolor* clustered with *C. discolor*, suggesting that its genetic composition is closer to that of *C. discolor*. Though it is suggested to have originated as a result of natural hybridization between *C. sieboldii* and *C. discolor*, introgression is likely to have occurred in the direction of *C. discolor* based on the data of molecular marker, clustering and genetic dissimilarity. *Calanthe reflexa* and *C. aristulifera* were genetically the most diverse of the species studied. In conclusion, the results showed that there is genetic diversity in Korean *Calanthe* species, that *C. bicolor* introgressed in the direction of *C. discolor* and that the YW mutants are genetically closer to *C. sieboldii*.

**Keywords:** Korean *Calanthe*, molecular marker, genetic distance, UPGMA

**적 요:** 5개의 한국산 새우난초류 즉 새우난초, 한라새우난초, 금새우난초, 여름새우난초, 신한새우난초의 분류학적 및 유전적 유연관계가 증폭길이다형성(AFLP) 자료를 근거로 조사되었다. 위의 5개 분류군 및 노란색에 흰 설엽을 갖는 몇몇 돌연변이체 등 16 개체가 연구에 사용되었다. AFLP를 통해서 50개의 분자마커가 동정되었다. 다변량분석의 결과는 유전적 다양성이 종내에서 보다 종간에 크게 나타났다. 유전적 비유사성은 다른 분류군에 비해서 YW 돌연변이체들 간에 가장 낮았고, 여름새우난초 개체들 간에 가장 높았다. 돌연변이체들은 주된 유집군의 밖에 유집되었다. 한라새우난초는 새우난초와 먼저 유집되어 유전적 구성이 다른 종보다 새우난초에 더 가까운 것으로 판단된다. 비록 한라새우난초가 새우난초와 금새우난초의 자연잡종에 의해 기원되었다고 하여도 분자 마커, 유집분석 그리고 비유사도 자료를 통해서 유전자입이 새우난초 쪽으로 이루어진 것으로 판단된다. 결론적으로 한국산 새우난초류는 유전적 다양성이 있고, 한라새우난초는

\*Author for correspondence: whang@jbnu.ac.kr

새우난초 쪽으로 유전자이입이 있으며, YW 돌연변이체들은 유전적으로 금새우난초에 보다 가깝다.

**주요어:** 한국산 새우난초속, 분자 마커, 유전적 거리, UPGMA

The genus *Calanthe* (subtribe Blettiinae, tribe Arethuseae, Epidendroid phylad) belongs to the Epidendroideae largest subfamily within the family Orchidaceae (Dressler, 1993; Cameron et al., 1999). *Calanthe* contains about 171 species, which are widely distributed in both tropical and subtropical regions (Gale and Drinkell, 2007). Even though the highest biodiversity of *Calanthe* is in the Malay Archipelago and New Guinea, 29 *Calanthe* species are known to occur in the eastern part of China, Taiwan, Japan and Korea (Govaerts et al., 2007). In Korea, only five species, *C. discolor*, *C. bicolor*, *C. sieboldii*, *C. reflexa*, and *C. aristulifera*, have been reported, but many naturally occurring mutants within *Calanthe* are also found in the wild (Lee and Kwack, 1983; Hotsunimi et al., 1989; Kim and Kim, 1989; Hyun et al., 1999; Cho et al., 2009; Park, 2010). However, the genetic relationships between/among mutants collected from the wild and native species in Korea are still controversial because there are frequent hybridizations as well as mutation giving rise to new varieties (Kim et al., 2008).

As a consequence of industrialization, indiscriminate reclamation and massive collection, most Korean *Calanthe* species have been disappearing from the wild resulting in the government designations as endangered species (Korean Forest Service, 2006). The characterization of the genetic diversity and examination of the genetic relationships among *Calanthe* taxa are necessary for drafting protection strategies that include consideration of species distribution and effective breeding

programs, and the increased use of plant genetic resources (Wang et al., 2009; Park, 2010).

A PCRbased DNA fingerprinting technique, like amplified fragment length polymorphism (AFLP), represents an informative, costeffective, and robust approach for the assessment of genetic diversity (Vos et al., 1995; Blears et al., 1998; Schmidt et al., 2004; Mba and Tohme, 2005; Kim et al., 2008; Kim et al., 2009; Li et al., 2010). Since AFLP is performed by combining restriction enzyme digestion and PCR amplification, the data generated is reproducible and sensitive (Majer et al., 1996; Maugham et al., 1996).

This study seeks firstly to understand the taxonomic and genetic relationships among *Calanthe* species native Korea. Secondly, the study seeks to determine the genetic status of *C. bicolor* which is considered to have originated by hybridization between *C. discolor* and *C. sieboldii*. Thirdly, the study attempts to elucidate the relationships of YW (yellow tepals and white lip) mutants to naturally occurring species, and lastly to identify molecular markers for distinguishing species and the wild type from mutants. These results would be useful for drafting protection strategies to conserve endangered orchids including *Calanthe* spp.

## Materials and Methods

### Plant materials

We used 16 accessions of *Calanthe* (Table 1), of which 13

**Table 1.** List of plant materials used in this study.

Taxa	Area of collection	Accession No.	Floral features
<i>C. sieboldii</i>	Jeju Island, Korea	CS 610	yellow tepals and yellow lip
		CS 612	
		CS 613	
<i>C. discolor</i>	Jeju Island, Korea	CD 614	brown tepals and white lip
		CD 615	
		CD 616	
		CB 618	
<i>C. bicolor</i>	Jeju Island, Korea	CB 619	brown tepals and yellow lip
		CB 620	
		CR 621	
<i>C. reflexa</i>	Jeju Island, Korea	CR 623	lavender tepals and lavender lip
		CR 624	
		CA 904	
YW Mutants	Fukuoka, Japan	CV 604	white with rose tint sepals and a darker lip
		CV 607	
		CV 607	
		CV 608	
YW Mutants	Jeju Island, Anmyeon Island, other island south of the Korean peninsular	CV 604	yellow tepals and white lip
		CV 607	
		CV 608	

**Table 2.** List of primers used in this study and putative marker number for each taxon.

Primer	Fragment size range (bp)	Total number of bands	No. of Polymorphic bands	Proportion of polymorphic fragments (%)	No. of putative markers					
					<i>C. sieboldii</i>	<i>C. discolor</i>	<i>C. bicolor</i>	<i>C. reflexa</i>	<i>C. aristulifera</i>	YW mutants
E-ACG/M-CAC	262-914	38	36	94.7			1	1	1	1
E-AGC/M-CTC	288-1716	59	57	96.6	1	1	1	2		1
E-ACA/M-CAG	269-1047	54	52	96.3	2	1			1	4
E-ACA/M-CTG	258-1224	65	61	93.8	1	3		2	1	2
E-AGG/M-CAG	183-1047	63	60	95.2		1	2	1	1	1
Total		279	266		4	6	4	6	4	9
Mean		55.8	53.2	95.3						

belonged to five *Calanthe* species, *C. discolor* Lindl., *C. bicolor* Lindl., *C. sieboldii* Decne. ex Regel, *C. reflexa* Maxim., and *C. aristulifera* Hayata. The remaining three accessions were selected among 15 naturally occurring mutants in the genus, particularly mutants with yellow tepals and white lips (mentioned as YW mutants hereafter). Through a pilot study we found that there were only three different genetic lines among the 15 YW mutants that were collected, so one individual representing each genetic line was used in this study. The plants were grown at the plant growth facilities of Chonbuk National University. Fresh leaves from these plants were used for experiments. *Calanthe aristulifera* was reported to occur in Korea recently (Hong et al., 2009). Due to its limited prevalence and collection difficulties, we used *C. aristulifera* collected from Fukuoka, Japan.

### Isolation of genomic DNA

Genomic DNA was extracted from 200 mg of fresh leaves ground in liquid nitrogen according to the Cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The isolated genomic DNA was then quantified in a photometer (GeneQuant, Pharmacia Biotech, Piscataway, NJ, USA), and DNA integrity was checked on a 0.8% agarose gel against a known 1kb DNA Marker (Intron Biotechnologies, Korea), and diluted accordingly.

### AFLP analysis

AFLP was performed as described by Vos et al. (1995), and was conducted using the AFLP Analysis System I (Invitrogen Life Technologies, CA, USA) and visualized by a polyacrylamide gel electrophoresis (PAGE) system. The procedure followed was as described by the manufacturer with minor modification as described by Vuylsteke et al. (2007). First, 250 ng of DNA was digested using the restriction enzymes

*EcoRI* and *MseI*. Following this, *EcoRI* and *MseI* adapters were ligated to the restriction fragment to generate template DNA for amplification. PCR was performed in two consecutive reactions, following the Invitrogen protocol. In the preamplification step, genomic DNAs were amplified with the primers *EcoRI*+A/*MseI*+C. The PCR products resulting from the amplification reaction were diluted and used as templates for selective amplification. Selective amplifications were carried out with *EcoRI* and *MseI* (containing three selective nucleotides) primers on a thermal cycler using a Touchdown PCR cycle programmed as follows: 13 cycles at 94°C for 30 sec, 65°C (0.7°C/cycle) for 30 sec and 72°C for 1 min, followed by 23 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. The reaction products were then size fractionated on an 8% PAGE run at 15 watts for 14 hrs. The gel was made visible by silverstaining with the Power Stain silver staining kit (ELPIS Biotech Inc., Korea).

An initial screening with 40 primer combinations for five individuals' representative of the various species was performed from which five primer combinations (Table 2) that generated good polymorphic band patterns with the best resolution were chosen for further analysis.

### Statistical analysis

The gels were analyzed with the Gel image analyzing program TotalLab T1120 (Nonlinear Dynamics Ltd, UK) program. The bands generated with AFLP markers were scored as 1 (for presence) or 0 (for absence) in a binary matrix for each primer. The matrix generated by the software was also checked manually to ensure reliability. Genetic dissimilarities were calculated on the basis of the Nei and Li (1979) distance matrix using the computer program PAUP 4.02 (Swofford, 1998). The dissimilarity matrix thus obtained was used to generate an unweighted pairgroup method of arithmetic

analysis (UPGMA) (Sneath and Sokal, 1973) based on pairwise distances to understand the relationships between the species.

Nei's (1973) gene diversity and percentage of polymorphic loci were calculated to determine the genetic diversity within the species using the program Popgene 1.31 (Yeh et al., 1997). The nonparametric analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was calculated using GenAlEx (Peakall et al., 2006) for understanding the diversity within and among the species.

## Results

### Polymorphism, genetic diversity and molecular variance

After a preliminary screening of five individual specimens that were representative of all species with 40 AFLP primers, five primers that generated polymorphic, distinguishable band patterns were used for further analyses (Table 2). This resulted

in the amplification of 279 loci out of which 266 were polymorphic. Each primer pair amplified an average of 55.8 loci out of which 53.2 were polymorphic. The primer pair EAGC/MCTC was the most polymorphic at 96.6%, while the primer pair EACA/MCAG was the least polymorphic at 93.8%. The percentage of polymorphism was 95.3%.

We also identified many putative species-specific markers (Table 2). In all, 33 markers were found using AFLP PCR fingerprinting. The majority of the markers identified were specific for *C. reflexa* and *C. aristulifera*. To understand the genetic diversity within the species, Nei's (1973) gene diversity (h) index and percentage of polymorphic loci (ppl) between individuals within the species were estimated. Individuals of *C. sieboldii* were the most diverse (Table 4). The average genetic diversity among the species was 0.0862 and the percentage of polymorphic loci (ppl) was 22.15. This result shows that there is considerable diversity within the species. *C. aristulifera* was excluded from the analysis as only one accession was available.

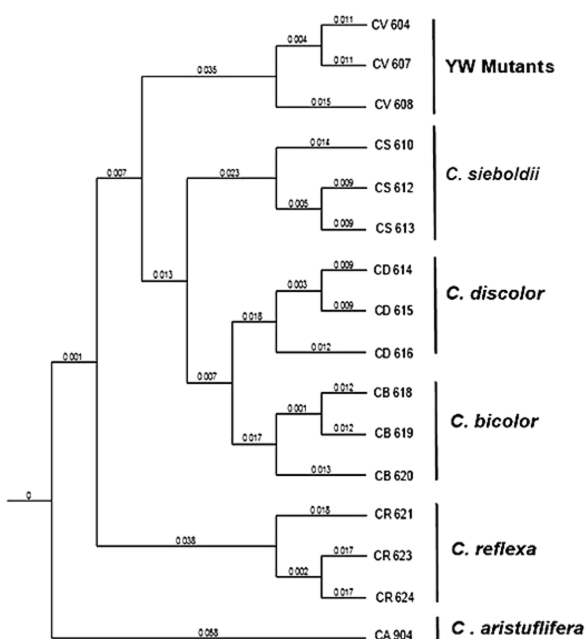
The nonparametric analysis of molecular variance (AMOVA) was performed using the data matrix generated by the markers (Table 3). The percentage of variation among the species was 66%, and within the species it was 34%. This result clearly indicates that the variation among species is higher than that within species.

### Genetic dissimilarity among species

**Table 4.** Genetic diversity of *Calanthe* species based on AFLP markers.

Species	N	ppl (%)	h
<b>Within</b>			
<i>C. sieboldii</i>	3	24.37	0.0906
<i>C. bicolor</i>	3	20.43	0.0767
<i>C. discolor</i>	3	20.43	0.0727
<i>C. reflexa</i>	3	22.58	0.0872
YW mutants	3	22.94	0.0904
MEAN	3	22.15	0.0862

N = number of individuals, ppl = percentage of polymorphic loci, h = Nei's gene diversity (1973).



**Fig. 1.** An UPGMA dendrogram of Korean *Calanthe* species and YW mutants from AFLP data. Numbers above the branches indicate the length of nodes.

**Table 3.** Analysis of molecular variance (AMOVA) on *Calanthe* species based on AFLP data.

	df	SS	Variation	% of total variation	PhiPT	pvalue
Among species	4	560.00	39.80	66		
Within species	10	206.00	20.60	34	0.590	< 0.001

0.590 < 0.001.

df = Degree of frequency (n1), SS = Sum of Squares, PhiPT =  $VAP/(VAP + VWP)$ , where VAP = variance among the population and VWP = variance within the population.

**Table 5.** Genetic dissimilarity (Nei and Li, 1979) coefficients of the *Calanthe* species based on AFLP (see the Table 1 for abbreviation).

	CV 604	CV 607	CV 608	CS 610	CS 612	CS 613	CD 614	CD 615	CD 616	CB 618	CB 619	CB 620	CR 621	CR 623	CR 624	CA 904
CV 604	-															
CV 607	0.022	-														
CV 608	0.032	0.028	-													
CS 610	0.105	0.093	0.098	-												
CS 612	0.093	0.086	0.088	0.028	-											
CS 613	0.101	0.090	0.085	0.028	0.019	-										
CD 614	0.109	0.091	0.096	0.061	0.066	0.068	-									
CD 615	0.114	0.093	0.102	0.065	0.074	0.080	0.018	-								
CD 616	0.095	0.085	0.093	0.065	0.071	0.073	0.026	0.021	-							
CB 618	0.124	0.101	0.111	0.075	0.078	0.081	0.060	0.061	0.053	-						
CB 619	0.114	0.093	0.094	0.080	0.077	0.087	0.063	0.063	0.049	0.024	-					
CB 620	0.122	0.113	0.110	0.074	0.077	0.081	0.062	0.069	0.064	0.028	0.024	-				
CR 621	0.115	0.119	0.123	0.122	0.110	0.105	0.116	0.123	0.111	0.102	0.107	0.105	-			
CR 623	0.122	0.123	0.136	0.115	0.106	0.110	0.128	0.145	0.128	0.103	0.106	0.096	0.034	-		
CR 624	0.117	0.124	0.117	0.106	0.098	0.100	0.120	0.125	0.103	0.092	0.092	0.102	0.040	0.033	-	
CA 904	0.117	0.118	0.125	0.104	0.094	0.097	0.108	0.125	0.115	0.132	0.126	0.113	0.125	0.114	0.115	-

Genetic dissimilarity (Nei and Li's coefficients) among the species were calculated based on AFLP data (Table 5). The dissimilarity values ranged from 0.0272 to 0.1152, with the pair of *C. reflexa* CR623 and *C. discolor* CD615 being the most dissimilar, and the pairs of *C. discolor* CD615 and CD614 being the least dissimilar, with an average genetic dissimilarity between all pairs of accessions ranging from 0.0272 (YW mutants) to 0.1152 (*C. aristulifera*). The genetic dissimilarity values based on AFLP data are given in Table 5. The table shows that the genetic dissimilarity between all pairs of accession was the least between the YW mutants and individuals of other species and the highest between *C. aristulifera* and individuals of other species.

### Taxonomic clustering

An UPGMA dendrogram was constructed (Fig. 1) based on pairwise distances using the AFLP data (Table 3). The

dendrogram formed two basal clusters. One group included the YW mutants, *C. sieboldii*, *C. bicolor*, *C. discolor*, *C. reflexa*, while the other group included *C. aristulifera*. The largest cluster included four subgroups, with the YW mutants, individuals of *C. sieboldii*, and individuals of *C. reflexa* making one subgroup each, while individuals of *C. discolor* were clustered with individuals of *C. bicolor* to form the fourth subgroup. *C. reflexa* formed the outermost subgroup.

### Discussion

So far, five *Calanthe* species, *C. discolor*, *C. sieboldii*, *C. reflexa*, *C. aristulifera*, and a natural hybrid, *C. bicolor*, have been reported in Korea (Hotsunimi et al., 1989; Kim and Kim, 1989; Lee and Kwack, 1983). Though there have been several studies on the genetic relationships among *Calanthe* species, they have been incomprehensive as the studies were based on

few taxa and did not include the mutants occurring naturally in the wild (Hyun et al., 1999; Cho et al., 2007; Kim et al., 2009). The present study uses AFLP PCR fingerprinting for measuring the genetic variations and determination of genetic relationships among Korean *Calanthe* taxa and YW mutants. We also attempted to determine the relationships of the YW mutants within the *Calanthe* species, as well as the genetic status of *C. bicolor*. This study was relatively comprehensive, as it involved all the Korean *Calanthe* species reported so far and also few YW mutants.

In the past, classification was primarily based on morphological characteristics. However, with the arrival of powerful PCRbased DNA fingerprinting techniques, it is now possible to investigate plant variability more precisely by direct analysis at the genetic level with a reliable data set for the reproducible estimation of genetic diversity and for investigation of the phylogenetic relationships among plant species (Schmidt et al., 2004; Mba and Tohme, 2005; Kim et al., 2008; Kim et al., 2009; Li et al., 2010). Due to the combination of restriction enzymes and PCR techniques, AFLP is reproducible and very sensitive (Majer et al., 1996; Maugham et al., 1996), thereby increasing the reliability of the data generated. AFLP was found to be very efficient for studying genetic diversity among species as it detected a high level of polymorphism of 95.7% (Table 2). In a previous study by Kim et al. (2009), 80% polymorphism was detected between *Calanthe* species using AFLP markers, compared to 95.7% polymorphic detection in our study. The higher polymorphic detection in our study could be attributed to the addition of the YW mutants and *C. reflexa* and the newly reported *C. aristulifera*. We were also able to detect 33 putative species-specific markers (Table 2), with the majority of them being specific to *C. reflexa* or *C. aristulifera*. This shows that these two species have genetically diverged from the other species, which explains the high levels of polymorphism observed in the present study.

Although an intra-species genetic diversity study was not the major objective of this study, an attempt was made to study intraspecies diversity as we felt it would provide a basis for future studies in breeding and population genetics. The levels of intraspecies diversity were much smaller compared to interspecies diversity, which was consistent with the results of Talhahas et al. (2003). The results of Nei's genetic diversity (Table 4) showed that there was considerable diversity between individuals of the same species. The ppl averaged 22.15%, and the average gene diversity was 0.0862. AMOVA showed 34% variation within the species, compared to 64% variation among species with AFLP.

Genetic dissimilarity between all pairs of accessions was the

lowest between the YW mutants and individuals of other species, while the highest dissimilarity occurred between *C. aristulifera* and individuals of other species; this clearly shows that *C. aristulifera* has genetically diverged from the rest of the species. In general, pairs of accessions belonging to the same species were the least dissimilar.

The UPGMA dendrogram constructed using AFLP marker data revealed the genetic relationship among the species. The clustering, although consistent with what had been reported previously (Hyun et al., 1999), improved primarily due to the fact that earlier studies did not involve all the reported Korean *Calanthe*, and did not include the YW mutants. One of the major objectives of this study was to determine the genetic status of *C. bicolor* and the relationship between the YW mutants and the rest of the species. The dendrogram showed the clustering of *C. bicolor* with *C. discolor* and the YW mutants forming the outermost subgroup. This led us to conclude that *C. bicolor* is genetically more similar to *C. discolor* than to *C. sieboldii*. Though *C. bicolor* is suggested to have originated by natural hybridization between *C. sieboldii* and *C. discolor*, from the band pattern, dendrogram and dissimilarity value it was considered that introgression must have occurred in the direction of *C. discolor*. In addition, from the dissimilarity values, the YW mutants used in this study were found to be genetically closer to the individuals of *C. sieboldii* than to the rest of the species suggesting that they must be mutants of *C. sieboldii* though further studies are needed to prove this. *Calanthe reflexa* and *C. aristulifera* had diverged the most genetically among the species studied.

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