Journal of Life Science 2007 Vol. 17. No. 3. 312~317 ©JLS / ISSN 1225-9918

Morphological characteristics and genetic diversity of *Calanthe* species native to Korea

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Received December 6, 2006 / Accepted February 13, 2007

This study was conducted to research the morphological characteristics and analyze the genetic diversity by using RAPD in *Calanthe* species native to Korea. Nine samples were selected by flower color and 19 morphological characteristics. In the length and width of leaf, dorsal sepal, the lateral sepal, the petal, the central lip, and the lateral lip, *C. discolor* was the shortest and narrowest, respectively, but *C. sieboldii* was the longest and the widest, respectively. The flower stalk length was the shortest in *C. discolor*, and the longest in *C. sieboldii*. Three variants were the intermediate between *C. discolor* and *C. sieboldii* in the above morphological characteristics, but spur length was the longest in *C. discolor*, the shortest in *C. sieboldii*, and intermediate in the variants. The ovary length in *C. discolor* was shortest and *C. sieboldii* and variants were similar with each other. The flower color of *C. discolor* were brownish red, the value of CIE Lab was between 40 and 50. The flower color of *C. sieboldii* was yellowish, the value of CIE Lab was between 110 and 130. And variants had various colors between 50 to 70 in the value of CIE Lab. By analyzing multiple band patterns of PCR products, 154 bands were selected as polymorphic RAPD markers. The analysis of genetic similarity of *Calanthe* species using RAPD showed that *C. discolor* and *C. sieboldii* are more distant from each other than variants, and these results demonstrated that genetic position of variants located between *C. discolor* and *C. sieboldii*.

Key words – *Calanthe*, morphological characteristics, CIE Lab, genetic diversity, RAPD

Introduction

One or two hundred *Calanthe* species are distributed in south China, south Korea, Japan, south east Asia, Australia, South Africa, and middle America in template and tropical areas [7,16,31]. Five species of *Calanthe coreana* Nak., *C. discolor* Lindl., *C. replexa* Max., *C. striata* R. Br. for. *sieboldii* Ohwi., *C. discolor* Lindl var. *bicolor* Makino are indigenous to South Korea [7,31]. However, there has been little systematic research on *Calanthe* species. Recently, interest in *Calanthe* species has increased in light of recognition of horticultural values of gorgeous flower color, fragrance of flower, long flowering period, and etc.

Random amplified polymorphic DNA (RAPD) analysis is a technique for amplification of specific segments of genomic DNA using random arbitrary primers [21,29]. The RAPD technique provides a faster and easier approach for exploring genetic polymorphism, requires only small amounts of DNA, and involves no radioactivity [8,13,27,30]. The current analysis assessed the genetic diversity within and among populations [1,4,5,26], and elucidated the phylogenetic relationship among cultivated varieties [3,6].

Our study was conducted to investigate morphological characteristics, to analyze the genetic diversity and phylogenetic relationship by using RAPD in *Calanthe* species native to Korea.

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Materials and Methods

Morphological characteristics investigation

Nine plant materials were selected randomly at habitats in Jeju, Korea. Plant materials were consisted of 3 plants of *C. discolor* and 3 plants of *C. sieboldii*, and the 3 plants of variants which thought to be crossed between *C. discolor* and *C. sieboldii* (Fig. 1). Morphological characteristics sur-

vey was conducted from middle April after flowering and survey methods were as follows.

The number of leaves counted after eliminating old leaves completely. Leaf length and width was measured the longest leaf in the new leaves. The number of flowers was counted after flowering of all the flowers. The length and width of floral parts, such as dorsal sepal, lateral sepal, petal, central lip, and lateral lip, were measured and used average values of data. The length of spur, ovary, and flower stalk was measured. Flower color was measured using a color meter (Micro S-5, Technidyne corporation, USA), which presents color value in Commission Internationale de l'Eclairage (CIE) color system. CIE L*a*b* is the most complete color model used conventionally to describe all the colors visible to the human eye. It was developed for this specific purpose by the International Commission on Illumination (Commission Internationale d'Eclairage, hence its CIE initialism). The * after L, a and b are part of the full name, since they represent L*, a* and b*, derived from L, a and b(Table 1).

Genomic DNA isolation and RAPD analysis

To understand the genetic diversity of *Calanthe* species native to Korea, 3 plants of *C. discolor*, 3 of *C. sieboldii*, and 3 of variants were selected a same habitat in Jeju, Korea.

Fig. 1. Flowers of *Calanthe* species used for plant materials.

Table 1. Equation of color expression on CIE Lab[z]

Equation	Meaning of value
$\triangle L^* = L2^* - L1^*$	Difference of lightness
$\triangle a^* = a2^* - a1^*$	Red - Green
$\triangle b^* = b2^* - b1^*$	Yellow - Blue
$\triangle E^* = (\triangle L^{*2} + \triangle a^{*2} + \triangle b^{*2})^{1/2}$	Difference of color

[z] The three parameters in the model represent the lightness of the color (L*, L*=0 yields black and L*=100 indicates white), its position between magenta and green (a*, negative values indicate green while positive values indicate magenta) and its position between yellow and blue (b*, negative values indicate blue and positive values indicate yellow).

The DNA of each plant was extracted from young leaves using cetyltrimethylammonium bromide (CTAB) method.

To analyze the DNA of individuals, we selected one hundred 10-mer random arbitrary primers of OPA, OPB, OPC, OPD, and OPE-set (Operon Technologies, California, USA). DNA amplification reactions were performed in 0.6 ml tubes containing 25 $\mu\ell$ of the reaction buffer, 10 mM Tris-HCL, pH 8.8, 50 mM $MgCl_2$, 100 μM each of dATP, dTTP, dGTP, dCTP, 0.2 mM primer, 2.1 units Taq DNA polymerase, and 25 ng of genomic DNA. The amplification products were separated by electrophoresis on 1.0 % agarose gels, stained with ethidium bromide, and photographed under UV light using Polaroid 667 film.

Genetic analysis

Each polymorphic fragment detected by RAPD analysis was treated as a unit character which was quantified by 1 for presence of fragment and 0 for absence of fragment from 0 to 2,000 base pair. Phylogenetic similarity coefficients of each strain were quantified using the Ntsys ver. 2.11 computer program [25]. A cluster analysis was done using the unweighted pair group method with arithmetic (UPGMA).

Results

Morphological characteristics

Nine samples were selected by flower color and 19 horticultural traits were investigated to study morphological characteristics of *Calanthe* species native to Korea. (Table 2). The number of leaves was 3 to 4. There was no difference between 2 species and variants. Leaf length was the longest in *C. sieboldii*, the shortest in *C. discolor*, and variants

Table 2. Morphological characteristics of *Calanthe* species

Plant no.	LN (ea)	LL (㎝)	LW (㎝)	FN (ea)	FSL (㎝)	PL (㎜)	PW (㎜)	DSL (㎜)	DSW (㎜)
1	3.6±0.5*	21.5±3.9	7.2±1.0	11.0±3.7	26.4±6.3	13.7±2.1	5.0±0.6	14.9±1.9	7.5±1.0
2	3.6±0.5	20.5±2.7	7.3±1.2	12.0±2.3	26.6±6.5	15.6±2.0	5.4±0.5	15.2±2.3	7.8±0.8
3	3.6±1.2	23.4±4.0	6.9±1.2	10.5±3.0	23.5±5.3	13.6±1.8	5.2±0.4	15.0±2.1	6.9±0.9
4	3.3±1.0	34.5±3.8	8.5±1.2	12.9±3.4	34.5±7.8	25.7±2.2	8.8±0.8	23.2±3.2	13.2±0.8
5	3.5±0.6	30.2±4.0	8.4±2.7	13.0±3.3	32.5±8.0	22.9±2.4	8.8±0.7	28.6±2.9	12.6±1.0
6	3.2±0.4	32.3±3.9	8.6±2.0	13.5±3.6	33.5±6.9	25.5±1.9	8.8±0.9	24.3±3.5	12.6±0.7
7	3.7±0.5	27.4±0.9	7.3±1.1	13.7±3.3	31.7±5.5	17.7±2.0	6.7±0.7	19.2±2.5	9.7±1.0
8	3.2±0.4	27.4±3.1	8.3±1.0	14.8±4.6	34.1±6.5	17.1±2.3	6.8±0.4	18.6±3.2	10.1±1.4
9	3.5±0.4	31.4±3.4	9.8±1.3	12.1±1.5	43.4±5.3	23.5±2.4	9.7±1.2	27.1±0.6	11.5±0.6

Plant no.	LSL (㎜)	LSW (㎜)	CLL (㎜)	CLW (㎜)	LLL (㎜)	LLW (㎜)	SL (㎜)	OL (㎜)
1	15.5±2.2	5.7±0.8	9.2±0.7	5.0±1.0	8.1±0.9	7.9±0.3	13.8±1.4	15.0±2.2
2	16.9±2.5	5.7±0.6	10.0±0.9	5.6±0.6	8.4±0.4	7.0±0.9	13.3±1.2	15.2±2.0
3	16.2±3.0	5.2±0.4	9.9±0.6	4.8±0.8	8.0±0.8	7.2±0.5	14.2±1.0	16.3±1.8
4	30.8±3.3	10.2±1.0	17.3±1.6	9.4±0.6	15.0±1.4	10.3±1.0	11.5±1.0	15.3±1.5
5	25.2±2.0	11.1±0.9	17.3±1.5	8.9±1.5	13.4±1.5	10.3±0.9	10.7±1.1	14.8±1.7
6	30.4±2.8	10.6±1.2	17.5±1.8	9.3±2.0	14.8±4.2	11.3±1.0	10.9±0.8	16.2±1.8
7	19.1±2.2	7.4±0.7	12.4±1.5	8.8±1.0	11.7±0.8	8.0±0.6	13.7±1.3	15.3±1.4
8	20.4±2.0	7.7±1.2	13.8±1.2	7.9±0.4	13.1±1.2	10.8±0.8	13.1±0.8	14.6±0.4
9	29.8±1.6	9.7±0.9	18.8±1.8	9.5±2.0	14.6±0.8	13.0±1.0	13.7±0.9	17.5±0.8

* Figures were represented as mean value ± standard deviation
LN: no. of leaves; LL: leaf length;LW: leaf width; FN: no of flowers; FSL: flower stalk length; PL: petal length; PW: petal width; DSL: dorsal sepal length; DSW: dorsal sepal width; LSL: lateral sepal length; LSW: lateral sepal width; CLL: central lip length; CLW: central lip width; LLL: lateral lip length; LLW: lateral lip width; SL: spur length; OL: ovary length

were intermediate between *C. discolor* and *C. sieboldii*. Leaf width was widest in *C. sieboldii*, the narrowest was *C. discolor*, variants were intermediate between *C. discolor* and *C. sieboldii*. The number of flowers was about 15 in *C. discolor* and *C. sieboldii*, but some samples had 20 to 24 flowers. Flower stalk length showed that *C. discolor* was around 25 cm, and *C. sieboldii*was more than 30 cm. The petal length of *C. discolor*was around 15 mm, *C. sieboldii* was around 25 mm, and variants were between *C. discolor* and *C. sieboldii*. Petal width showed that *C. discolor* was around 5 mm, *C. sieboldii* was 8 to 9 mm, and variants were 6 to 7 mm. The dorsal sepal length of *C. discolor* was around 15 mm, *C. sieboldii* around 25 mm, and variants around 20 mm. The dorsal sepal width had the same tendency as dorsal sepal length. The lateral sepal length of *C. discolor* was 15 to 16 mm, *C. sieboldii* around 30 mm, and variants around 20 mm. The lateral sepal width was around 5 mm in *C. discolor*, around 10 mm in *C. sieboldii*, and 7 to 8 mm in the variants. The central lip length was around 10 mm in *C. discolor*, 15 to 18 mm in *C. sieboldii*, and 12 to 14 mm in variants. The central lip width was 5 mm in *C. discolor*, 9 mm in *C. sieboldii*, and 6 to 8 mm in variants. Lateral lip length was 8mm in *C. discolor*, 14 to 15mm in *C. sieboldii*, and 10 to 13 mm in variants. The lateral lip width was 7 to 8 mm in *C. discolor*, and 10 to 11 mm in *C. sieboldii* and variants. The spur length was 13 to 14 mm in *C. discolor*, 10 to 11 mm in *C. sieboldii*, and similar or shorter than *C. discolor* in variants. The ovary length showed that *C. discolor* was 15 to 16 mm, *C. sieboldii* was 14 to 16 mm, and variants were similar with two species. The flower color of *C. discolor* was dark purplish red or brownish red. The L* (lightness) value was 30 to 40, a* (Red-Green) value was 0 to 20, b* (Yellow to Blue) was 20 to 30, and the CIE Lab value was 40 to 50. *C. sieboldii* flowers was yellow or bright yellow. The L* value was above 80, a* value was -10, b* value was around 80, and the CIE Lab value was 110 to 130. The value of CIE Lab of variants was between 50 to 70. The CIE Lab value of lip color was 90 in white lip and 110 to 120 in yellow lip (Table 3, Fig. 1).

Genetic relationship

The genetic relationship of 3 of *Calanthe discolor*, 3 of *C. sieboldii*, and 3 of variants was investigated using RAPD. The number of amplified bands for each ten-mer primer varied from 2 to 8, with an average of around 4 bands per primer accounting for a total of 305 bands from 100

Table 3. CIE Lab value on color space of each flower in *Calanthe* species

Sample no.	L, a, b color space of flower			CIE Lab[w]	L, a, b color space of lip			CIE Lab
	L[z]	a[y]	b[x]		L	a	b	
1	48.61	1.22	30.83	57.6	90.40	-9.81	15.56	92.3
2	32.80	0.77	28.29	43.3	90.44	-9.67	15.44	92.3
3	31.41	20.55	35.82	51.9	90.38	-9.57	15.39	92.2
4	87.94	-10.11	79.34	118.9	87.33	-9.88	78.68	118.0
5	87.50	-9.51	89.26	125.4	87.31	-9.89	78.61	117.9
6	87.85	-10.04	79.30	118.8	87.28	-9.78	78.60	117.9
7	31.44	20.38	35.25	51.4	87.33	-9.76	78.57	117.9
8	35.40	15.71	37.55	53.9	87.24	-10.40	62.85	108.0
9	31.39	20.42	35.74	51.8	87.16	-10.34	62.75	107.9

[z] represent value of lightness; [y] represent value of red to green; [x] represent value of yellow to blue.
[w] See foot note of Table 1.

primers. After analyzing the multiple band patterns of the PCR products, 154 bands of 305 bands were selected as polymorphic RAPD markers. The size of the amplified products ranged from 0.5 kb to 2.0 kb. 154 polymorphic bands were analyzed with the Ntsys ver. 2.11 [25] program for phylogenetic tree (Fig. 2). The phylogenetic tree is separated into two major groups. One group contained the majority of *C. sieboldii*, which are closely related with about 70% similarity. This group consists of yellowish and large flowered plants. The other group contained two small groups which were divided into *C. discolor* and variants. The group of *C. discolor* is closely related with about 90%. This group consists of plants with dark brownish and small flowered. The genetic similarity between *C. discolor* and *C. sieboldii* was about 52%. This result was thought

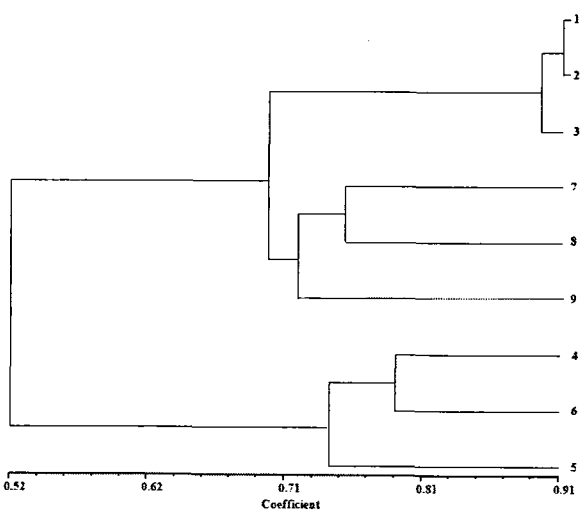

Fig. 2. Dendrogram of genotype of *Calanthe* species based on UPGMA analysis system. Coefficient value is the similarity of each other plants.

that these 2 species are almost genetically different species, because they have higher genetic diversity between 2 species than variants, and they have different morphological characteristics, such as flower color and flower size. The genetic position of variants is between *C. discolor* and *C. sieboldii*, and variants is highly related with *C. discolor*.

Discussion

Most studies used Munsell color system (1923) to measure flower color. However, this study was the first to measure flower color using a color meter in *Calanthe* species. This data will be useful as the basis of flower color study in *Calanthe* species. Hyun et al. (1999) investigated leaf length and width, sepal length and width, petal length and width, lip length and width, spur length, and ovary length in *C. discolor*, *C. siboldii*, and *C. bicolor*. *C. bicolor* is a putative hybrid between *C. discolor* and *C. sieboldii*. Leaf length was longest in *C. sieboldii*, the shortest in *C. discolor*, and *C. bicolor* was intermediate between the two species. The length and width of dorsal and lateral sepal, petal, and central and lateral lip were longest and widest in *C. sieboldii*, the shortest in *C. discolor*, and intermediate in *C. bicolor*. The spur length was the longest was *C. discolor*, the shortest in *C. sieboldii* *C. bicolor* was intermediate between the two species. The ovary length of *Calanthe* species was the longest in *C. sieboldii*, the shortest in *C. discolor*, and *C. bicolor* was intermediate between the two species. These results are similar with our studies. However, Kim and Kim (1989) reported that length and width of leaf were the longest in putative hybrid between *C. discolor* and *C. sieboldii*, the shortest in *C. discolor*. In addition, in spur length, *C.*

discolor was the shortest. The result of spur length disagreed with our study and Hyun et al. (1999) which was thought that plant materials had some problems in step of selection or errors in measuring.

The use of RAPD markers to genetically fingerprint plants which are morphologically similar or indistinguishable has been established as a reliable, efficient, and very informative tool. We analyzed 9 plants of *Calanthe* species based on genetic variation. The current study confirms previous studies which showed that molecular markers can more readily dissect genetic differences between closely related genotypes as compared to isozymes. Evidence from recent analysis of marker data from other crops has shown that isozyme agreement with pedigree data is poor (Dudley, 1994). This is due to the low number of isozyme markers available to obtain adequate representation of the genome [11]. The *Calanthe* species fingerprinted in this study were similar enough in appearance to be grouped together taxonomically; however, their DNA fingerprints indicate significant genetic diversity.

RAPD markers appear to be a good choice for assessing genetic relationships in *Calanthe* species with polymorphism levels sufficiently high to establish informative fingerprints with relatively few markers. RAPDs are currently used routinely by plant breeders to identify genetic variation [10,14,22], to locate regions of the genome linked to agronomically important genes [17,18,23,24], and to facilitate introgression of desirable genes into commercial crops [15,28]. We plan to use these markers in genetic populations being developed to tag gene(s) associated with tool of flower color selection in *Calanthe* species.

Acknowledgment

This work was supported for two years by Pusan National University Research Grant.

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초록 : 한국 자생 새우난초의 형태적 특성 및 유전적 다양성

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본 연구는 자생 새우난초의 형태적 특성을 조사하고, RAPD법을 이용하여 유전적 다양성을 분석하고자 수행되었다. 자생지에서 화색을 포함한 19가지의 형태적 특성 분류 기준에 따라 새우난초, 금새우난초, 변이종을 각각 3종류씩 9종류를 선발하였다. 잎의 길이와 넓이, 주판(dorsal sepal), 부판(lateral sepal), 꽃잎(petal), 중심 설(central lip), 측면 설(lateral lip)은 길이와 넓이에 있어서 새우난초가 가장 짧고 좁았으며, 금새우난초가 가장 길고 넓었다. 화경(flower stalk)의 길이는 새우난초가 가장 짧았고, 금새우난초가 가장 길었으며, 변이종은 위의 각 기관의 길이와 넓이에 있어서 새우난초와 금새우난초의 중간정도였다. 그러나 거(spur)의 길이는 새우난초가 가장 길었으며, 변이종, 금새우난초의 순이었다. 자방(ovary)의 길이는 새우난초가 가장 짧았고, 금새우난초와 변이종은 비슷하였다. 새우난초의 화색은 CIE Lab 값이 40에서 50 사이의 갈색계통이었으며, 금새우난초는 CIE Lab 값이 110에서 130 사이의 밝은 황색계통이었다. 변이종은 CIE Lab 값이 50에서 70사이의 다양한 색을 나타내었다. 유전적 유연관계를 조사하기 위하여 multiple band의 양상을 분석한 결과, 총 305개의 band 중 154개의 polymorphic band를 선발하였다. 이들의 유연관계는 새우난초와 금새우난초가 가장 멀어 새우난초와 금새우난초는 다른 종에 속해있음을 알 수 있었고, 변이종은 유전적으로 새우난초와 금새우난초의 중간에 위치하고 있음을 알 수 있었다.