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Genetic Analysis of Flower Color Traits in *Calanthe discolor, C. sieboldii,* and Variants Using Molecular Linkage Map

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This study was conducted to clarify the genetic relationship between *Calanthe discolor, C. sieboldii* and variants, and the cause of flower color variations by using a molecular linkage map and a quantitative trait loci (QTL) analysis for flower and lip color in *Calanthe* species native to Korea. Twenty plants were included in three *C. discolor* and three *C. sieboldii,* and fourteen variants were obtained from their habitat, Jeju-do in Korea. The flowers of *C. discolor* were brownish red, the values of Commission Internationale de l'Eclairage (CIE) Lab were between 40 and 50. The flowers of *C. sieboldii* were yellowish, the values of CIE Lab were between 110 and 130. The variants had various mixed colors that were thought to have originated from natural hybridization between *C. discolor* and *C. sieboldii*, and the values of CIE Lab were between 50 and 70. The colors of the lips were usually divided into white and yellow. *C. discolor* had a white lip, *C. sieboldii* had a yellow one, and the variants had a white to yellow one. The CIE Lab value of each color was 90 in white and 110 to 120 in yellow lips. A molecular linkage mapping was constructed based on the segregation of 154 RAPD markers using a MAPL program. Sixteen linkage groups containing 66 markers were established. It covered a total map distance of 220.4 cM. The distance between adjacent markers ranged from 0 to 6.6 cM, with an average distance of 3.3 cM. These markers are thought to be closely associated with flower and lip color expression. Among the 16 molecular linkage groups, 3 QTLs had flower color trait loci and 1 QTL had lip color trait loci.

Key words : *Calanthe*, molecular linkage map, quantitative trait loci (QTL), flower color

Introduction

Genus *Calanthe* includes about two hundreds species which taxa were distributed in China, Korea, Japan, southeast Asia, Australia, South Africa, and middle America in template and tropical areas [8]. Five species of *Calanthe coreana* Nak., *C. discolor* Lindl., *C. replexa* Max., *C. striata* R. Br. For. *sieboldii* Ohwi., and *C. discolor* Lindl *var. bicolor* Makino were indigenous to South Korea [8]. In our study, we used *C.discolor, C. sieboldii* and variants. because these 2 species and variants flower at the same time in the same habitat. Genetic map based on DNA polymorphism is a powerful tool for the study of qualitative and quantitative traits and ultimately can be used to facilitate the cloning of gene of interest. Most crops and several famous floral plants have been researched on qualitative and quantitative traits and genetic relationship by using molecular markers, genetic map, and QTL analysis [3,4,12]. In precedent studies about flower color by using mapping and QTL, Dunemann et al. [4] studied about leaf chlorosis and selected markers related to flower color of Rhododendron by using molecular linkage map. Debener and Mattiesch [3] reported that they could find markers related to pink color of rose flower in studies of construction of molecular linkage map by using molecular markers. Abe et al. [1] reported that they found trait loci associated to anthocyanin tinting in lily asiatic hybrid 'Montreux' and 'Cunnecticut King' by using molecular linkage map and QTL analysis. There were several studies about morphological characters and genetic relationship in *Calanthe* species native to Korea [9,11,13]. But these studies were little systematic and scientific to explain the genetic relationship between species.

This study was conducted to clarify the genetic relationship between each species, and the cause of flower color variations by constructing molecular linkage map and quantita-

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tive trait loci (QTL) analysis for flower and lip color in *Calanthe* species native to Korea. These data will be useful as the basis of flower color study in *Calanthe discolor, C. sieboldii and variants.*

Materials and Methods

Plant materials

Plant materials were selected by flower color at habitat at Jeju-do in Korea (Fig. 1). Total twenty plants included three *C. discolor*, three *C. sieboldii* and 14 variants which have different flower color. These selected plants were used flower color analysis, molecular linkage map construction and QTL analysis.

Flower color analysis

We used color meter (Micro S-5, Technidyne corporation, USA) to obtain accurate and scientific color value. The data of each measured value was used for QTL analysis. The color meter used in this study presented color value in Commission Internationale de l'Eclairage (CIE) color system. Presented values, L*a*b*, of color meter was converted into CIE L*a*b* (Table 1).

Construction molecular linkage map

For RAPD analysis, genomic DNA was extracted by using modified CTAB(cetyltrimethylammonium bromide) method. And one hundred 10-based primers were purchased from Operon (Alameda, USA). Total 305 markers amplified by 100

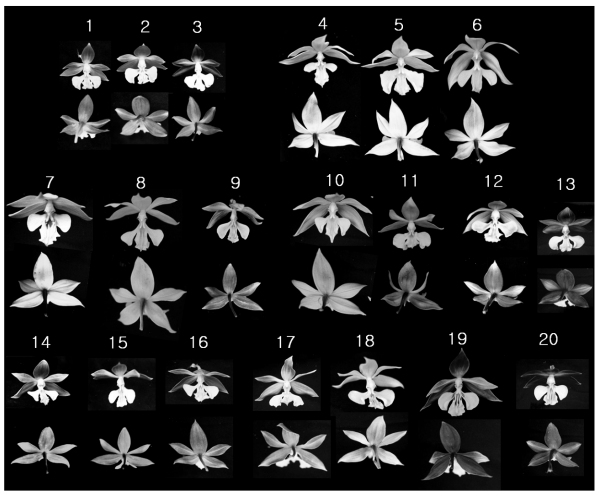

Fig. 1. Flowers of *Calanthe* species used plant materials which are included with No.1 to 3 are *C. discolor*, 4 to 6 are *C. sieboldii*, and 7 to 20 are variants.

Table 1. Equation of color expression on CIE L*a*b* system

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

primers were used to construct the molecular linkage map. The segregation ratios of all markers were compared with the expected segregation ratio of 3:1 that followed Mendelian law and used the chi-square test at 5% significant level. The primer code and the amplified fragment-size indicated the marker name. The primer code starting 'OP' designated 10-base Operon primers. Linkage map were constructed using a double pseudo-testcross strategy. A threshold of LOD value >3.0 was employed to make the linkage map. Linkages between 3:1 segregating markers were analyzed using MAPL [21] program.

QTL analysis

Flower and lip color were measured by color meter (Micro S-5, Technidyne corporation, USA). The association between markers and QTL related with this flower and lip color traits were tested using the interval mapping method with MAPL program [21]. The LOD value >3.0 was employed to declare the presence of a QTL in given region. The LOD peak was used to estimate the most likely QTL position on the molecular linkage map.

Results

Analysis of flower color

The flower color of *C. discolor* was dark purplish red or brownish red. The lightness (L*) value was 30 to 40, red-green (a*) value was 0 to 20, yellow to blue (b*) was 20 to 30, and the CIE Lab value was 40 to 50. *C. sieboldii* flowers were yellow or bright yellow. The L* value was above 80, a* value was 10, b* value was around 80, and the CIELab value was 110 to 130. Variants had diversity of mixed color perhaps originated from the cross between *C. discolor* and *C. sieboldii*. The value of CIELab in variants was between 50 to 70. The color of the lip was usually divided into white and yellow. The CIELab value of each color was 90 in white and 110 to 120 in yellow lip (Table 2). This study measured flower color using a color meter in *Calanthe* species. This flower color measuring method is

Table 2. CIE Lab value on color space of each flower in *Calanthe* species

Plant no.	L, a, b color space of flower			CIE Lab	L, a, b color space of lip			CIE Lab
	L^z	a^y	b^x		L	a	b	
1	46.70	1.22	29.32	55.2	89.70	-9.77	15.43	91.5
2	34.25	-0.89	29.22	45.0	91.34	-9.56	14.55	93.0
3	89.50	-9.77	90.12	127.4	89.73	-9.60	14.89	91.5
4	88.92	-10.12	80.45	120.3	88.23	-10.02	80.04	119.5
5	33.41	21.00	34.97	52.7	86.52	-9.92	79.23	117.7
6	88.75	-9.98	80.44	120.2	88.30	-9.34	78.90	118.8
7	83.29	1.30	90.04	122.7	90.48	-9.88	21.03	93.4
8	84.70	-10.20	91.22	124.9	88.93	-9.78	89.07	126.2
9	71.56	-15.88	67.20	99.4	87.56	-15.89	80.24	119.8
10	66.54	-15.33	66.49	95.3	87.22	-16.04	80.22	119.6
11	53.21	1.30	60.21	80.4	86.33	-10.01	64.11	108.0
12	39.79	16.44	29.89	52.4	44.35	12.89	43.09	63.2
13	29.40	7.56	30.14	42.8	74.11	-7.88	79.44	108.9
14	32.11	19.45	34.12	50.7	88.22	-10.03	79.22	119.0
15	38.22	18.20	30.22	52.0	90.35	-9.99	49.25	103.4
16	37.20	16.20	38.33	55.8	88.21	-10.02	63.88	109.4
17	55.22	17.66	66.54	88.3	88.34	-9.84	80.09	119.6
18	74.34	9.77	93.13	119.6	80.89	-9.35	100.22	129.1
19	32.12	20.35	37.33	53.3	88.09	-10.45	64.65	109.8
20	39.89	18.04	29.22	52.6	80.20	-8.75	79.05	112.9

[z]represent value of lightness; [y]represent value of red to green; [x]represent value of yellow to blue.

more scientific to take accurate value and to translate color to value for using QTL analysis.

Construction of molecular linkage map

A genetic linkage map was constructed based on the segregation of 154 RAPD markers using MAPL [21] program (Fig. 2). Sixteen linkage groups containing 66 markers were established. All of markers were generated by one- hundred of 10-based Operon primers. It covered a total map distance of 220.4 cM. The distance between adjacent markers ranged from 0 to 6.6 cM with average distance of 3.3 cM. In our study, molecular linkage map was constructed by using *C. discolor*, *C. sieboldii*, and variants, which are natural inter-specific crossed between *C. discolor*, and *C. sieboldii*.

QTL analysis

Among the 16 molecular linkage groups, 3 groups had flower color trait loci and 1 group had lip color trait loci (Fig. 2). Group 14 had 8 markers related to flower color which markers were $OPB16_{2000}$, $OPC08_{1100}$, $OPB15_{950}$, $OPE12_{850}$, $OPD03_{1900}$, $OPA13_{1700}$, $OPD19_{500}$, and $OPC17_{950}$. All the markers in group 14 scored more than LOD3.0 and

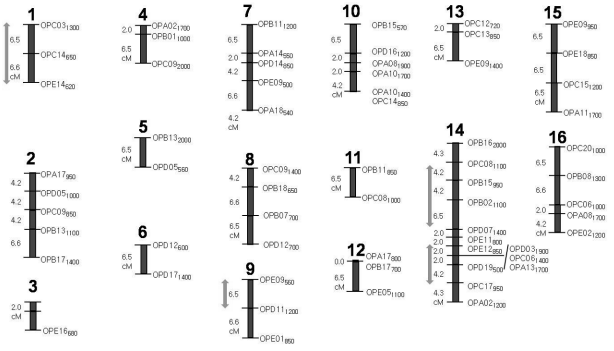

Fig. 2. Linkage map showing locations of QTL associated with flower color (arrows of group 1 and 14) and lip color (arrow of group 9). 1~16: number of linkage groups.

$OPD19_{500}$ scored 3.90 which was highest value in this group. Group 1 had 3 markers related to flower color which markers were $OPC03_{1300}$, $OPC14_{650}$, and $OPE14_{620}$. Three markers in group 1 had scores more than LOD3.0 and $OPE14_{620}$ scored 4.40 which value was highest in this trait. Group 9 had 2 markers related to trait of flower color which markers were $OPE09_{560}$ and $OPD11_{1200}$. Two markers in group N had scores more than LOD3.0 and $OPE09_{560}$ was nearest to LOD 3.89 (Table 3). In the trait of lip color, its markers were linked on group 9 which had 2 markers $OPE09_{560}$ and $OPD11_{1200}$. The $OPD11_{1200}$ was the highest LOD scored marker in this trait which was LOD 3.49 (Table 3). DNA markers linked to the trait loci for the number of petal in the carnation[19], the petal number and flower color in diploid rose[3] and leaf chlorosis and flower color in *Rhododendron* [4] have been identified and anthocyanin pigmentation in asiatic hybrid lily [1] have been identified, but no reports exists of the

Table 3. Significant QTL marker associations to flower color and lip color detected by interval mapping in *Calanthe* species

Traits	Marker	interval	Linkage group	LOD	A.E	P.V
Flower color	$OPB16_{2000}$	$OPC08_{1100}$	14	3.46	-24.652	0.543
	$OPC08_{1100}$	$OPB15_{950}$	14	3.71	-24.393	0.601
	$OPE12_{850}$	$OPD03_{1900}$	14	3.22	-22.998	0.586
	$OPA13_{1700}$	$OPD19_{500}$	14	3.90	-26.113	0.678
	$OPD19_{500}$	$OPC17_{950}$	14	3.95	-27.246	0.695
	$OPC03_{1300}$	$OPC14_{650}$	1	3.18	-24.168	0.579
	$OPC14_{650}$	$OPE14_{620}$	1	4.40	-26.425	0.703
	$OPE09_{560}$	$OPD11_{1200}$	9	3.89	-23.235	0.584
Lip color	$OPE09_{560}$	$OPD11_{1200}$	9	3.49	-7.223	0.264

LOD: logarithm of odds; A.E: additive effect;
P.V: phenotypic variation value.

mapping of useful traits loci in other ornamental crops. We believe this study is the first to construct molecular linkage map of *Calanthe* species native to Korea using PCR-based molecular markers and to map the floral trait loci associated with flower color expression. Our results are important to understand the genetic basis of the traits and to apply molecular markers linked to mapped loci for map-assisted selection (MAS).

Discussion

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 [8]. Among the five species, *C. discolor*, *C. replexa*, *C. sieboldii*, and variants were found in habitats, Jejudo, Korea. *C. discolor*, *C. sieboldii*, and variants grow naturally at the same habitat in the Mt. Halla. They flower almost at the same time from the end of March to April. *C. discolor* and *C. sieboldii* are thought to be different species according to flower color, other morphological characters, and studies about genetic relationship of *Calanthe* species [9]. Variants have intermediate flower color and other morphological characters of *C. discolor* and *C. sieboldii* [10]. *C. replexa* grows wild in different habitats from *C. discolor*, *C. sieboldii* and variants of Mt. Halla, and their flowering is summer season and their morphological characters are completely different from *C. discolor, C. sieboldii* and *C. bicolor*. Because of the difference in habitat at location, altitude and flowering season, natural hybridization between *C. replexa* and other species is impossible [10]. Molecular linkage map in this study has been constructed by using *Calanthe* taxa consisted of three *C. discolor*, three *C. sieblodii* and differently flowering twenty variants. The precedent studies of flower color were estimated by using Munsell color system [16], but this measuring method has problems to translate color to value for QTL analysis. But in this study, we used color meter (MicroS-5, Technidyne corporation, USA) to obtain accurate and scientific color value. The presented color values in Commission Internationale de I'Eclairage (CIE) color system were effective to convert each flower colors into significant values efficient for QTL analysis. To our knowledge, this study was the first attempt in the *Calanthe* species to construct molecular linkage map and analyze flower color and lip color traits by using QTL analysis in order to understand genetic background. Constructing molecular linkage maps of allogamous and vegetatively reproducing species, such as forest trees [6,17], fruit trees [5], industrial crop trees [7,14,18] and ornamental plants [1,3,4], have been constructed using a double-testcross strategy. Our linkage map had 16 linkage groups. The linkage map obtained in this study was not saturated and further marker analysis will be necessary to complete them, because the number of linkage groups is smaller than the haploid number of chromosome (n=20). In genetic analysis of trait associated with flower and lip color, among the 16 molecular linkage groups, 3 groups had flower color trait loci and 1 group had lip color trait loci. $OPD19_{500}$ of Group B scored 3.93 which was highest value in this group, and phenotypic variation was 68.6%, which is indicating a major gene, to regulate flower color, is in this chromosomal region. $OPE14_{620}$ of Group K scored 4.16 which value was highest in this trait, and phenotypic variation was 69.9%, indicating that a major gene responsible for regulating flower color is located in this chromosomal region. Identification of molecular markers closely linked to agronomically useful loci is the first step for marker-assisted selection (MAS). QTL analysis clarified that putative QTLs $OPD19_{500}$ and $OPE14_{620}$ in linkage groups of this study are closely associated with flower color regulation of *Calanthe* species. These QTLs are a target for MAS. Many genes are necessary for flower color biosynthesis. A number of structural genes, and regulatory genes have been identified and precisely characterized [15,22]. These genes were mapped on to the linkage maps in petunia [20] and in *Arabidopsis* [22]. However, when genetic analysis of trait loci for flower and fruit color was examined, a single locus that determines the presence or absence of pigments was detected as follow. A single trait locus determining pink and white flowers has been mapped on the linkage map of diploid rose [3]. The red and yellow skin color of apple fruit, which is correlated with anthocyanin accumulation, is controlled by a single dominant locus [2]. The results of precedent studies of flower and fruit color indicate that only a few genes determine the presence or absence of pigments in flowers and fruits, and a number of other genes for pigments synthesis are functional factors. In this study, therefore, it is thought that various colored flower of *Calanthe* species would be regulated by a few genes. Also, it is possibly supposed that the brownish flowered *C. discolor* has dominant genetic factors in flower color inheritance, because yellowish flowered *C. sieboldii* has lower phenotypic variation then *C. discolor* and variants (Table 3). All of these results indicated that variants

were originated from natural cross between *C. discolor* and *C. sieboldii*, and because the genes of flower color of *C. discolor* are major gene for color inheritance, it was considered that the flower color of variants have similar value of CIE Lab of *C. discolor*.

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초록 : 연관지도를 이용한 새우난초, 금새우난초, 변이종의 화색의 유전분석

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본 연구는 제주도에서 자생하는 새우난초 3개체, 금새우난초 3개체 그리고 변이종 14개체를 포함하여 총 20개체를 화색에 따라 분류하고 유전자지도를 작성하여 QTL분석을 하였다. 화색은 새우난초가 어두운 자색으로 CIE Lab값이 40~50 정도였으며, 금새우난초는 황색으로 110~130 정도였고, 변이종 개체들은 새우난초와 유사하거나 다소 높았다. PCR 결과 얻은 polymorphism이 인정되는 154개 marker에 대한 분리비 적합도 검정에서 51개 marker에서 5% 수준의 유의성이 인정되었으며, 유의성이 인정된 51개 marker 중에서 새우난초 type은 37개, 금새우난초 type은 14개 였다. Polymorphism이 인정된 154개 marker에 대하여 MAPL program을 이용하여 이들 marker 상호간의 연관관계를 분석한 결과는 16개의 연관군과 1개의 독립군으로 구분되었으며, 이들 연관군에 대한 분자연관지도는 전체 group의 크기가 220.4 cM (centi Morgan)이고, marker 간의 평균거리는 3.3 cM이었다. 양적 형질에 대한 분자연관지도상의 QTL 분석 결과, LOD 3.0 이상인 화색 과 설판색의 QTL은 각각 3개와 1개였다. 이상에서 얻어진 자료는 새우난초 속의 화색 연구에 도움이 될 것으로 사료된다.