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Evaluation of Genetic Diversity among the Genus *Viola* by RAPD Markers

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Abstract - The genetic diversity among the genus *Viola* was evaluated using the random amplified polymorphic DNA (RAPD) method. A total of 142 distinct amplification fragments by 18 random primers were scored to perform the cluster analysis with UPGMA. *Viola* species from the subsection Patellares were clustered into group I to IV. The groups from I to IV were consistent with its morphological taxonomy, series Pinnatae, Chinensis, Variegatae, and Patellares in the subsection Patellares, respectively. Even though *V. albida* and *V. albida* var. *takahasii* were classified in Chinensis, they were assigned into group I. The cluster analysis separated other subsections from Patellares in the section Nomimium. Interestingly, *V. verecunda* and *V. grypoceras* in subsections Biobatae and Trigonocarpae, respectively, were clustered into group C with a high similarity coefficient. Therefore, RAPD analysis can be used for providing an alternative classification system to identify genotypes and morphological characters of *Viola* species.

Key words - Genetic diversity, *Viola*, RAPD, Cluster analysis, Morphological taxonomy

Introduction

Viola species in the family Violaceae are widely cultivated as ornamental plants (Woodland, 1991). The family is of little commercial value except for the *Viola* species, which contains the garden pansies, a hybrid group (*V. tricolor*). Wild *Viola* species native to Korea have promising features as important genetic resources of pansy breeding for heat tolerance (Lee, 1993). In spite of horticultural importance, at present, no information on molecular markers of *Viola* species is available for genetic study and classification.

The genus *Viola* in Korea consists of three sections, Dischidium, Chamaemelanium, and Nomimium, based on morphological characters (Lee, 1993). Most of *Viola* species belong to the section Nomimium which consists of 6 subsections. The classification of *Viola* has been reported by the screening of phenolics from the leaf (Stebbins *et al.*, 1963), the morphological study of leaf epidermis and seeds (Lee and Lee, 1968), and the screening of flavonoids and alkaloids from flowers (Lee and Yook, 1975). However, since the above evaluations are subject to genotype environment interactions, the genetic variation of these plants could not be fully analyzed. Therefore, random amplified polymorphic DNA (RAPD) analysis which does not have genotype environment interactions is a valuable alternative method for the classification of the genus *Viola*.

The application of RAPD markers generated by the polymerase chain reaction (PCR) has resulted in the detection of nuclear DNA polymorphisms (Welsh and McClelland, 1990; Williams *et al.*, 1990). Since the RAPD method is technically simple, it has been widely used to evaluate genetic relationships of *Allium* (Wilkie *et al.*, 1993), buckwheat (Kump and Javornik, 1996), *Lens* (Abo-elwafa *et al.*, 1995), lentils (Sharma *et al.*, 1995) and watermelon (Lee *et al.*, 1996), etc.

In this study, RAPD analysis has been used not only to analyze the genetic diversity among the genus *Viola*, but also to facilitate the classification of *Viola* species.

Materials and Methods

Plant material and DNA extraction

Twenty-five *Viola* species selected from sections Dischidium and Nomimium were used in this study (Table 1). *Viola* species were chosen from as broad a genetic base as possible. Total genomic DNA of *Viola* species was isolated from soil-grown seedlings as described by Junghans and Metzlaff (1990) with minor modifications. Each seedling was ground in an 1.5mL tube with lysis buffer [0.05 M Tris (pH 7.6), 0.1 M NaCl, 0.05 M EDTA, 0.5% SDS, 0.01 M 2-mercaptoethanol]. The tubes were incubated for 15 min with occasional mixing. This mixture was extracted with phenol/chloroform and centrifuged. The resulting supernatant was precipitated with isopropanol, dissolved in TNE buffer [0.01 M Tris (pH 8.0), 0.1 M NaCl, 1mM EDTA], and treated with RNase (10mg/mL). The final pellet was rinsed with 70% ethanol and dissolved in TE buffer.

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Selection of random primers and RAPD amplification

In order to select primers that can amplify informative PCR products, preliminary RAPD screening was carried out with random primers from OPA to OPE (Operon Technologies Inc., USA). Through the tests, 18 random primers were selected for evaluating genetic diversity among the genus *Viola* (Table 2). Amplification reactions were carried out in volumes of 10μL containing 5 ng genomic DNA, 50mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 2.5mM $MgCl_2$, 0.2mM dNTPs, 400nm random primer, and 0.5 unit *Taq* DNA polymerase (Promega, USA). Amplification was performed in a thermocycler (Perkin Elmer/Cetus 9600, USA) programmed as follows: initial denaturation at 94℃ (10 min), 45 cycles of 94℃ (1 min)/36℃

Table 1. Taxon and sample numbers of *Viola* secies used in this RAPD study

Taxa			Sample number	Scientific name
Section	Subsection	Series		
Nomimium	Patellares	Pinnatae	1	*V. chaerophylloides*
		Chinensis	2	*V. albida* var. *takahashii*
			3	*V. albida*
			4	*V. ibukiana*
			5	*V. mandsurica*
			6	*V. mandshurica* for. *albescence*
			7	*V. patrinii*
			8	*V. yedoensis*
			9	*V. japonica*
			10	*V. seoulensis*
			11	*V. phalacrocarpa*
			12	*V. scarbrida*
			13	*V. ishidyana*
			14	*V. hirtipes*
		Variegatae	15	*V. variegata* var. *ircutiana*
			16	*V. variegata*
			17	*V. variegata* var. *chinensis*
		Patellares	18	*V. selkirkii* var. *albiflora*
			19	*V. boissieuana*
	Plagiostigma	-	20	*V. blandaeformis*
	Hypocarpae	-	21	*V. collina*
	Bilobatae	Verecundae	22	*V. verecunda*
	Trigonocarpe	Rostratae	23	*V. grypoceras*
		Campylostylae	24	*V. acuminata*
Dischidium	-	-	25	*V. biflora*
-	-	-	26	*V. tricolor* 'Helen Mount'

Table 2. Nucleotide sequences of primers and number of informative RAPD markers amplified with them in the genus *Viola* used in this study

Primer	Sequence (5'-3')	Number of informative RAPD mark	Primer	Sequence (5'-3')	Number of informative RAPD mark
OPA14	TCTGTGCTGG	10	OPC07	GTCCCGACGA	10
OPA17	GACCGCTTGT	10	OPC08	TGGACCGGTG	11
OPB04	GGACTGGAGT	7	OPD02	GGACCCAACC	7
OPB05	TGCGCCCTTC	14	OPD15	CATCCGTGCT	9
OPB08	GTCCACACGG	10	OPD20	ACCCGGTCAC	10
OPB10	CTGCTGGGAC	8	OPE11	GAGTCTCAGG	6
OPB14	TCCGCTCTGG	5	OPE12	TTATCGCCCC	4
OPB17	AGGGAACGAG	5	OPE14	TGCGGCTGAG	7
OPC02	GTGAGGCGTC	5	OPE18	GGACTGCAGA	4
Total		142			

(1 min)/72℃ (2 min), final extension at 72℃ (10 min). Amplification products were electrophoresed on 1.5% agarose gels and detected by staining with ethidium bromide. The 100 bp ladder (Promega, USA) was used as the size marker.

Data analysis

Reproducible bands were scored for their presence (1) or absence (0) for all *Viola* species studied. Data were entered into the Numerical Taxonomy and Multivariate Analysis System program package for PC (NTSYS-pc version 1.70) (Rohlf, 1992). The cluster analysis was performed with the unweighted pair-group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1993), and dendrograms were constructed.

Results and Discussion

A total of 100 random primers were preliminarily screened on each genomic DNA of *Viola* species (Table 1) to examine RAPD patterns.

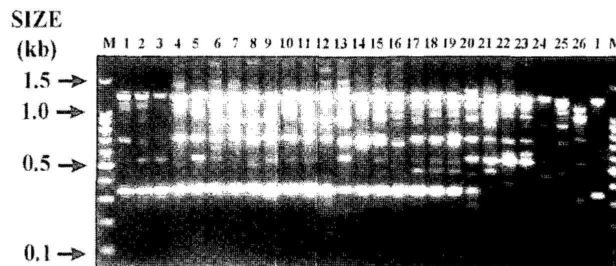

Fig. 1. RAPD profiles generated from 26 genomic DNA of *Viola* species using primer OPB04.
Lanes numbers correspond to the sample number in Table 1.
M indicates molecular markers of 100 bp ladders.

Although some primers generated polymorphic products, the primers that amplified faint and/or unreproducible ones were not chosen. Eighteen primers were selected for analysis, because they amplified easily-scorable and reproducible PCR products. A total of 142 distinct amplification fragments by the selected primers were scored and showed polymorphisms between species (Table 2). Each primer amplified a different number of informative bands, varying from 4～14

Table 3. Morphological characteristics of *Viola* species used in this study

Scientific name	Sample number	Leaf			Stigma shape[z]
		Form	Vein[y]	Margin	
V. chaerophylloides	1	Palmately compound	CMC	Palmately lobed	S
V. albida var. *takahashii*	2	Palmatifid	MC	Palmately parted	W
V. albida	3	Triangular elliptical	E	Erosely serrate	W
V. ibukiana	4	Ovate	E	Erosely lobed	W
V. mandsurica	5	Lanceolate	E	Wide crenate	W
V. mandshurica for. *albescence*	6	Lanceolate	E	Wide crenate	W
V. patrinii	7	Lanceolate	E	Wide crenate	W
V. yedoensis	8	Lanceolate	E	Wide crenate	W
V. japonica	9	Ovate	E	Serrate	W
V. phalacrocarpa	10	Narrow ovate	E	Serrate	W
V. seoulensis	11	Ovate	E	Serrate	W
V. scarbrida	12	Narrow ovate	E	Serrate	W
V. ishidyana	13	Ovate	E	Serrate	W
V. hirtipes	14	Ovate	E	Serrate	W
V. variegata var. *ircutiana*	15	Roundish cordate	AB	Serrate	WP
V. variegata	16	Roundish cordate	AB	Serrate	WP
V. variegata var. *chinensis*	17	Roundish cordate	AB	Serrate	WP
V. selkirkii var. *albiflora*	18	Cordate	AB	Serrate	WP
V. boissieuana	19	Ovate	AB	Wide crenate	WP
V. blandaeformis	20	Cordate	AB	Serrate	WS
V. collina	21	Cordate	AB	Crenate	H
V. verecunda	22	Reniform	AB	Crenate	U
V. grypoceras	23	Cordate	AB	Serrate	SC
V. acuminata	24	Cordate	AB	Serrate	SH
V. biflora	25	Reniform	R	Crenate	Y

[z](H) Hooked, (S) Spherical head, (SC) Straight or slightly curved cylindrical, (SH) Shortly hooked, with rear marginal hair, (U) Upright marginal collared, (W) Worm head, (WP) Worm head with projected apical margin, (WS) Worm head with slanted apical margin, (Y) Y-shaped.
[y](AB) Actino-brochidodromous, (CMC) Compound & mixed crospedodromous, (MC) Mixed crospedodromous, (E) Eucamptodromous, (R) Reticulate actinodromous.

per primer. One of typical RAPD patterns is shown in Fig. 1, and the range of PCR products was 0.3 ~ 1.3 kb.

Twenty-six *Viola* species were analyzed with informative bands by UPGMA. Nineteen *Viola* species in the subsection Patellares were assigned into group I to IV (Table 1, Fig. 2). On the other hand, *V. verecunda* (22) and *V. grypoceras* (23) were assigned into group V. *V. blandaeformis* (20), *V. collina* (21), *V. acuminata* (24), *V. biflora* (25) and *V. tricolor* (26) were not assigned into any groups. The groups from I to IV assigned by cluster analysis were consistent with its morphological taxonomy, series Pinnatae, Chinensis, Variegatae, and Patellare in the subsection Patellares, respectively, except *V. albida* (3) and *V. albida* var. *takahasii* (2) in Chinensis.

Viola species in the series Chinensis [except *V. albida* (3) and *V. albida* var. *takahasii* (2)] were assigned into group II (Fig. 2). *Viola* species in group II were divided into two small groups according to the cluster analysis. Also, two small groups could be differentiate from each other by leaf forms. *V. ishidoyana* (13), *V. scabrida* (12), *V. phalacrocarpa* (11), and *V. seoulensis* (10) in group II are morphologically characterized by ovate- or narrow ovate-shaped leaves, eucamptodromous leaf veins, serrate leaf margins, worm head-shaped stigmas and colored flowers. Other *Viola* species [*V. ibukiana* (4), *V. mandsurica* (5), *V. mandshurica* for. *albescence* (6), *V. patrinii* (7), *V. yedoensis* (8), *V. japonica* (9)] in group II have lanceolate-shaped leaves, eucamptodromous leaf veins, and wide creanate leaf margins.

V. chaerophylloides (1), and *V. albida* (3), *V. albida* var. *takahasii* (2) were classified into the series Pinnatae and Chinensis, respectively, with different leaf shapes. However, they were clustered into the group I with a high similarity coefficient of 0.88 (Fig. 2).

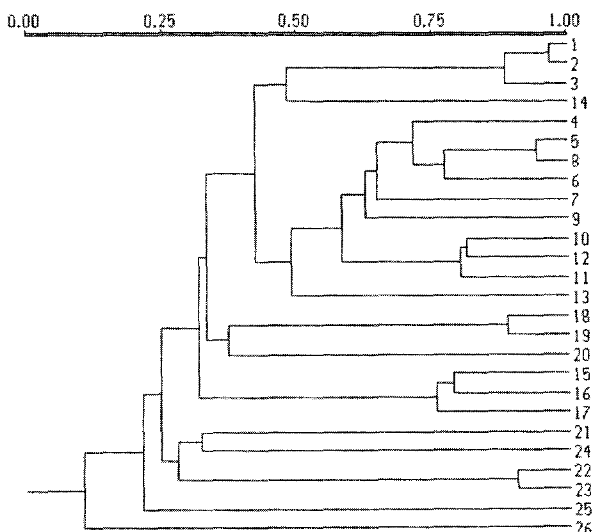

Fig. 2. UPGMA dendrogram of genetic relationships of 25 species in the genus *Viola* based on 142 RAPD products.
Numbers correspond to the sample number in Table 1.

Viola species in the series Variegatae and Patellares were clustered into group III and IV, respectively. *Viola* species in group III are characterized by morphological patterns, such as roundish cordate leaves, actino-brochidodromous veins, and worm head-shaped stigmas with protected apical margins. *Viola* species in group IV are morphologically characterized by actino-brochidodromous veins and worm head-shaped stigmas with protected apical margins, but have different leaf forms and veins.

V. verecunda (22) and *V. grypoceras* (23) in group V showed the high similarity coefficient of 0.90, even though they were classified into subsections Biobatae and Trigonocarpae, respectively, by morphological characteristics.

The present studies demonstrate that the genetic relationships of the genus *Viola* derived from RAPD analysis are in agreement with the conventional taxonomy based on morphological characteristics, except *V. albida* (3) and *V. albida* var. *takahasii* (2) in Chinensis. Therefore, RAPD analysis can be used for providing an alternative classification system to identify genotypes and morphological characters of *Viola* species.

In conclusion, it is suggested that RAPD analysis could be successfully applied for studying genetic relationships and identifying genotypes of *Viola*. These studies on DNA polymorphisms provide an essential basis on which to classify *Viola* species and to assist hybrid breeding programs.

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