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The Use of AFLP Markers for Cultivar Identification in *Hydrangea macrophylla*

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Abstract : The principal morphological characters used for identification of *hydrangea* cultivars are often dependent on agroclimatic conditions. Furthermore, information on the selection or the genetic background of the hydrangea breeding is so rare that a molecular marker system for cultivar identification is needed. Amplified fragment length polymorphism (AFLP) markers were employed for fingerprinting *Hydrangea macrophylla* cultivars and candidate cultivars of *H. macrophylla* selected in Korea. One AFLP primer combination was sufficient to distinguish 17 *H. macrophylla* cultivars and 4 candidate cultivars. The profile of 19 loci that can minimize the error of amplification peak detection was constructed. AFLP markers were efficient for identification, estimation of genetic distances between cultivars, and cultivar discrimination. Based on the observed AFLP markers, genetic relationship was reconstructed by the UPGMA method. Seventeen *H. macrophylla* cultivars and *H. macrophylla* for. *normalis* formed a major cluster, and candidate cultivars selected in Korea formed another cluster.

Key words : Hydrangea macrophylla, *AFLP, fingerprinting, cultivar identification*

Introduction

Saxifragaceae family is a large and diverse family of evergreen or deciduous herbs and includes ornamental plants such as hydrangea. The genus *Hydrangea* native to southern and eastern Asia and North and South Americais is best known for its widely cultivated garden species. Despite its high ornamental value, the genus *Hydrangea* has been poorly studied genetically. Genetic knowledge is essential for the management of genetic resources and plant breeding (Mortreau *et al.*, 2003). Cultivar identification is useful for describing a new cultivar, testing genotype purity and speeding up DUS (distinctness-uniformity-stability) test for candidate cultivar (Chowdhury *et al.*, 2002).

Morphological characters are the most important markers for cultivar identification of crops and garden plants. However, morphological characters are often dependent on agroclimatic conditions (Bourgoin-Greneche *et al.*, 1995).

The principal characters used for identification of hydrangea cultivars are macroscopic ones: inflorescence type, sepal shape and color, and leaf shape (Bertrand, 1992). Flower color of hydrangea is a vital characteristic for cultivar identification and ornamental value. The flower color is predominantly determined by two types of pigment: flavonoids and carotenoids (Forkmann, 1991), and the range of color is mostly controled genetically. However, because of available aluminum in soil, hydrangea flower shows higher color variation than other garden plants. Aluminum is more available in lower pH soils and less available in higher pH soils.

Therefore, not only morphological characters but other marker systems are also needed for cultivar identification and breeding of hydrangea. Soller and Beckmann (1983) proposed to use molecular markers as an additional tool for varietal description. DNA markers have the advantage of being independent of environmental effects and providing direct information on the genome of each individual.

AFLP markers have the potential to resolve genetic differences at the level of 'DNA fingerprints' for individual identification and parentage analysis. In the ideal case, a few primer combinations will suffice to generate an adequate number of polymorphic markers. In the worst case, many AFLP markers have to be generated with a series of primer combinations to reveal differences between closely related inbred individuals or to confirm a lack of differences for clonality (Mueller and

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Wolfenbarger, 1999).

The aim of the study presented here was to test the usefulness of AFLP markers for cultivar identification in hydrangea, and to determine genetic relationship between hydrangea cultivar and candidate cultivar selected in Korea.

Material and Methods

1. Plant materials

Four hydrangea cultivar candidates were selected by ornamental value in Korea and propagated in Korea National Arboretum. The morphological characters of these cultivar candidates were identified for cultivar registration (Table 1). The principal characters for identification were based on Bertrand (1992).

For AFLP analysis, 22 plant samples were used : 17 hydrangea cultivars from Chollipo Arboretum, Korea, 4 cultivar candidates selected for this study and a wild species, *H. macrophylla* for. *Normalis* (Table 2).

2. DNA extraction

Leaves were frozen in liquid nitrogen and ground by mortar and pestle. Total genomic DNA was extracted by NucleoSpin Plant DNA extraction kit (MACHEREY-NAGEL, Germany).

3. AFLP procedure

AFLP reactions were performed using the Perkin-Elmer/Applied Biosystem (PE/ABI, USA). AFLP™ plant mapping kit for small genomes was used following the manufacturer's instructions. Modifications to the original protocol were as follows: a modified core mix was employed for preselective amplification and selective amplification reactions which consisted of 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1×PCR Buffer II (Perkin-Elmer), and 0.2 U *AmpliTaq* (Perkin-Elmer, USA). The number of cycles in the last step of the selective reactions was 23 for NED and FAM, 25 for JOE, and 27 for TAMRA fluorescent dyes.

Table 2. *Hydrangea macrophylla* cultivars used in the identification and cluster analysis.

Hydrangea macrophylla 'Ayesha'
Hydrangea macrophylla 'Hamburg'
Hydrangea macrophylla 'Everblooming'
Hydrangea macrophylla 'Glowing Embers'
Hydrangea macrophylla for. *Normalis*'[a]
Hydrangea macrophylla 'Goliath'
Hydrangea macrophylla 'Tovelit'
Hydrangea macrophylla 'Mariesii'
Hydrangea macrophylla 'Merritt's Supreme'
Hydrangea macrophylla 'Blue Prince'
Hydrangea macrophylla 'Tosca'
Hydrangea macrophylla 'Juliana'
Hydrangea macrophylla 'General Patton'
Hydrangea macrophylla 'Maculata variegated leaves'
Hydrangea macrophylla 'Holstein'
Hydrangea macrophylla 'Red Cap'
Hydrangea macrophylla 'Red Emperor' Eori
Hydrangea macrophylla 'Nigra'
Hydrangea macrophylla 'Pink fish'[b]
Hydrangea macrophylla 'Skyblue'[b]
Hydrangea macrophylla 'Autumn moon'[b]
Hydrangea macrophylla 'Eori'[b]

a: a wild species
b: candidate cultivars selected in Korea

4. Primer selection and reproducibility experiments

Primers were selected taking account of the total number of fragments amplified and clearness of amplification. Primer combinations that were suited for cultivar identification of hydrangea were selected and used for this study. Each reaction was conducted three times to assess the repeatability.

5. Electrophoresis and Data collection

0.4 μL of selective amplification product was mixed with 1.2 μL of loading buffer (0.75 μL of deionized formamide, 0.15 μL of blue dextran/25 mM EDTA loading

Table 1. Morphological characters of candidate cultivars selected in Korea.

		Pink fish	Skyblue	Autumn moon	Eori
Foliage	leaf color	green	green	green	green
	leaf shape	ovate	elliptic	ovate	ovate
Inflorescences	flower heads	globose	flat	globose	flat
Sterile flowers	number of sepal	4-5	4-5	4-5	4-5
	sepal edge	dentate	entire	entire	entire
	sepal overlapping	partial	total	partial	partial
	sepal shape	pointed	pointed	pointed	pointed
	sepal colour	pink	blue, pink	blue	pink

solution and 0.3 μL of GeneScan 500 ROX size standard), heated at 95°C for 3 minutes, placed on ice, and loaded on 5% polyacrylamide gels. Electrophoresis was performed at 2500 volts for 4 hours using an automated DNA sequencer (ABI P_{RISM} 377, PE/ABI, USA) equipped with GeneScan analysis software (version 3.1, Applied Biosystems, USA)

The presence/absence of each fragment was recorded directly by Genotyper DNA fragment analysis software (version 2.1).

6. Data analysis

For AFLP analyses, only clear and unambiguous bands were visually scored as either present (1) or absent (0) for all cultivars, resulting in binary data matrix. Each band was interpreted as one allele. Bands with the same mobility were assumed to be homologous (van der Voort *et al.*, 1997).

Genetic distance between cultivars and candidate cultivars were estimated by using the procedure described by Nei and Li (1979). A dendrogram was drew based on genetic distance using UPGMA (unweighted pair group method using arithmetic average) method (Sneath and Sokal, 1973). Computer software, TREECON (Van de Peer and De Wachter, 1994), was used to calculate genetic distances and to reconstruct the genetic relationships among samples.

Results

1. Primer Selection and Polymorphism

For cultivar identification and estimation of genetic distance, amplification results of 64 primer combinations (8 EcoRI primers×8 MseI primers) were tested. In most primer combinations, 20-100 amplification fragments were observed. Taking account of the total number and clearness of fragments amplified, two primer combinations of EcoRI-ACA/MseI-CTT and EcoRI-ACC/MseI-CAC were selected and used in the analysis (Table 3).

The only differences observed among different reaction in the reproducibility experiments were in the intensity of bands. One hundred twenty DNA fragments were scored, ranging from 75 to 450bp, with an average of 101.4 fragments per individual and 60 bands per primer combination. In the 22 hydrangea individuals, there were 53 polymorphic markers (44.2%).

Table 3. Primers used, fluorescent dye label and number of total and polymorphic bands obtained in the analysis of *Hydrangea macrophylla* cultivars with AFLPs.

AFLP primers	Colour dye	Total no. of bands	Polymorphic bands
EcoRI-ACA/MseI-CTT	FAM	68	31
EcoRI-ACC/MseI-CAC	NED	52	22

Table 4. DNA fingerprints of hydrangea cultivars by AFLP analysis (*Eco*RI-ACA Primer/*Mse*I-CTT Primer)

Loci[a]	127	139	150	182	184	214	227	255	263	269	272	283	286	310	351	358	371	376	383
Ayesha	+	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	+	–	+
Hamburg	+	–	–	–	+	+	–	–	–	+	–	+	–	–	+	–	–	–	+
Everblooming	+	–	–	+	+	–	+	–	+	+	–	–	–	+	+	+	–	–	–
Glowing Embers	+	+	–	–	+	+	+	–	+	+	–	–	+	–	+	+	+	–	+
Goliath	+	–	–	–	+	–	–	–	+	+	–	+	–	–	+	+	–	–	+
Tovelit	+	+	–	–	+	–	+	–	+	–	–	+	+	+	+	–	–	–	+
Mariesii	+	–	–	–	+	–	+	–	+	+	–	–	–	+	–	+	–	–	-
Merritt's Supreme	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	-
Blue Prince	+	+	–	+	+	+	+	–	–	+	–	–	–	+	+	+	–	–	+
Tosca	+	–	–	+	+	–	+	–	–	+	–	–	+	+	+	–	+	–	+
Juliana	+	+	–	+	+	+	+	–	+	+	–	–	+	+	+	+	+	–	+
General Patton	+	+	–	+	+	–	+	–	–	+	–	–	–	–	+	+	+	+	+
Maculata	+	–	–	–	+	–	–	+	+	+	–	–	–	+	+	+	+	–	+
Holstein	+	+	–	–	+	–	–	+	+	+	–	–	–	+	+	–	–	–	+
Red Cap	+	+	–	+	+	+	+	–	+	+	–	–	+	+	+	+	–	+	+
Red Emperor	+	–	–	–	+	+	–	–	+	+	–	+	+	+	+	+	–	–	+
Nigra	+	+	–	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	+
Pink fish*	–	–	+	–	–	–	–	–	+	–	+	–	–	+	–	+	–	–	–
Skyblue*	–	–	+	–	+	–	–	–	+	–	–	–	–	+	–	+	–	–	–
Autumn moon*	–	–	–	+	–	–	–	–	+	–	+	–	–	+	–	+	–	–	–
Eori*	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–

a: the number means the size of amplified fragments

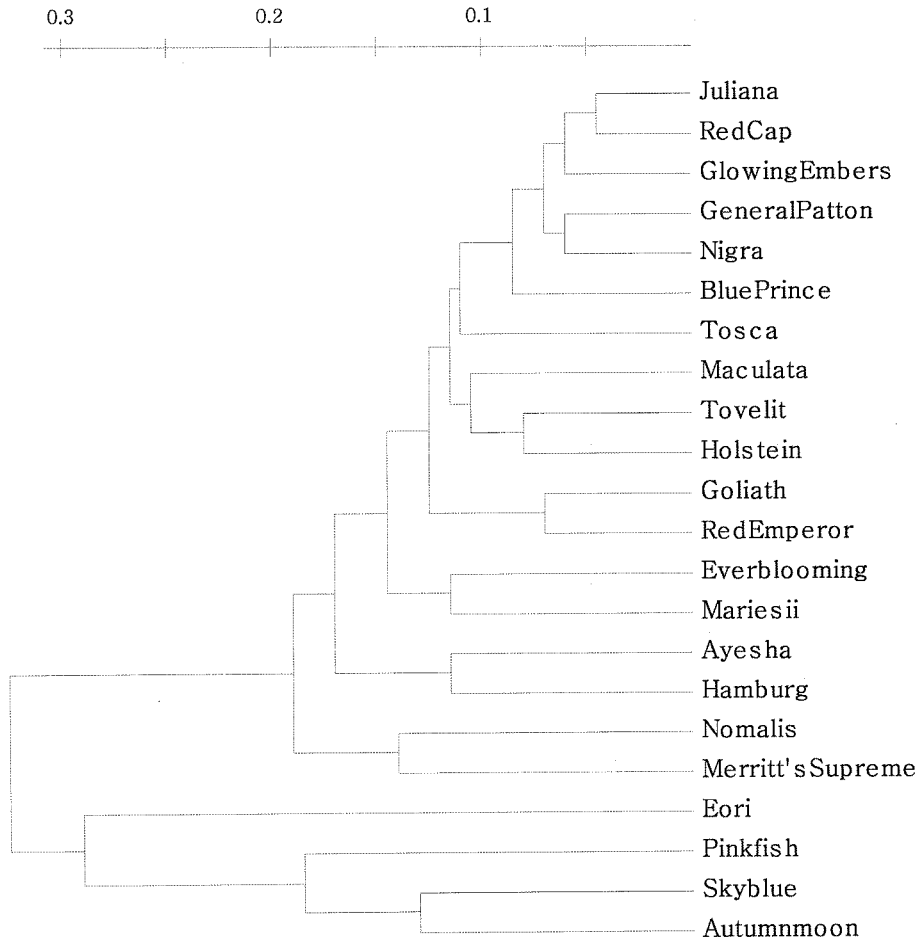

Figure 1. Dendrogram of *Hydrangea macrophylla* cultivars and candidate cultivars based on data from AFLP (The scale represents Nei's genetic distance).

2. Cultivar Identification

The polymorphism observed among cultivars is so high that all culivars and candidate cultivars could be distinguished by a single combination. There was no cultivar specific markers among 120 fragments which were amplified by EcoRI-ACA/MseI-CTT and EcoRI-ACC/MseI-CAC primer combinations. To minimize error of amplification peak detection by Genotyper program for cultivar identification, 19 loci which showed constant amplification intensity in the reproducibility experiment and no comigrated DNA fragment within the range of 2bp were selected (Table 4). With this profile of 19 loci, 22 *H. macrophylla* individuals analyzed in this study could be distinguished.

3. Genetic Relationship of Cultivars

Genetic distance was estimated using 120 AFLP markers. Two major cluster were revealed by UPGMA dendrogram (Figure 1). Seventeen *H. macrophylla* cultivars and a wild species of *H. macrophylla* for. *normalis* formed a major cluster, and candidate cultivars selected in Korea (Eori, Pink fish, Autumn moon and Sky blue) formed another cluster.

Discussion

The capacity of AFLP markers to resolve extremely small genetic differences has been demonstrated in several studies. For example, AFLPs have been used to distinguish near-isogenic lines of soybean that differ at only a single, small region in the entire genome (Maughan *et al.*, 1996).

The high reliability of AFLP markers could also be used in the identification of economically valuable cultivars (de Proft *et al.*, 2003; Kashkush *et al.*, 2001). However, because of their largely dominant nature, AFLP markers are unlikely to outcompete co-dominant markers, such as microsatellites, which allow more powerful pedigree or relatedness analysis needed for plant breeding (Mueller and Wolfenbarger, 1999). Therefore, AFLPs are appropriate for quick identification of cultivars of which *a priori* knowledge of genome structure are not known.

Information on genetic relationships among breeding

lines is critical in crop improvement. A comparison of genetic and phenotypic variation among cultivars released in different decades provides a means to assess how genetic improvement on yield potential or quality may affect crop diversity (Ortiz *et al.*, 1998).

Most of *H. macrophylla* cultivars have been bred in Europe and Japan. However, the information of the selection or the genetic background of the breeding is so rare that it is difficult to discuss the pedigree or genetic relationship of cultivars. In this study, *H. macrophylla* for. *normalis* and other cultivars formed the same cluster. *H. macrophylla* for. *normalis* is the shrub considered as original wild species of the cultivated *H. macrophylla* (Kurata, 1976). On the other hand, candidate cultivars selected in Korea formed the other cluster and show relatively high genetic distance with *H. macrophylla* cultivars. Better knowledge of the genetic similarity of parents could help to maintain genetic diversity and sustain long-term selection gain (Chowdhury *et al.*, 2002). This encourages the possibility of utilizing Korean hydrangea population as genetic source to make a new cultivar.

Conclusion

The AFLP markers are effective tools for cultivar identification in *Hydrangea macrophylla*. In the present study, one AFLP primer combinations was sufficient to distinguish 17 *H. macrophylla* cultivars and 4 candidate cultivars.

In the UPGMA dendrogram based on data from AFLPs, candidate cultivars selected in Korea formed a cluster and show relatively high genetic distance with *H. macrophylla* cultivars. This encourages the possibility of hydrangea breeding and hybridization using Korean hydrangea as a new genetic source.

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