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Discrimination of Species Specific DNA Markers Using RAPD and AFLP Analysis between *Atractylodes japonica* Koidz. and *Atractylodes macrocephala* Koidz.

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ABSTRACT : To identify the variation of the RAPD patterns between two *Atracylodes* species, 52 kinds of random primers were applied to each eight of *A. japonica* and *A. macrocephala* genomic DNA. Ten primers of 52 primers could be used to discriminate between the species and 18 polymorphisms among 67 scored DNA fragments (18 fragments are specific for *A. japonica* and *A. macrocephala*) were generated using these primers, 26.9% of which were polymorphic. RAPD data from the 10 primers was used for cluster analysis. The cluster analysis of RAPD markers showed that the two groups are genetically distinct. On the other hand, to identify the variation of the AFLP patterns and select the species specific AFLP markers, eight combinations of *Eco*RI/*Mse*I primers were applied to the bulked *A. japonica* and *A. macrocephala* genomic DNA. Consequently, three combinations of *Eco*RI/*Mse*I primers (*Eco*R I /*Mse* I ; AAC/CTA, AAC/CAA, AAG/CTA) used in this study revealed 176 reliable AFLP markers, 42.0% of which were polymorphic. 74 polymorphisms out of 176 scored DNA fragments were enough to clearly discriminate between two *Atractylodes* species.

Key words : *Atractylodes japonica*, *Atractylodes macrocephala*, discrimination, RAPD, AFLP

INTRODUCTION

A. japonica and *A. macrocephala*, called Packchul in Korean herb name, have been mainly cultivated and consumed in Korea. Domestically grown Packchul, *A. japonica*, has comparatively weaker competitiveness in Korea herbal market than *A. macrocephala* because of its long period of cultivation in farmer's fields and wild gathering from mountainous areas followed by low productivity. Under these circumstances, local products of *A. japonica* are currently trading at ten times higher price than the products from China.

According to the market open policy in Korea, it is expected that the importing amount of cheap Chinese products will be increased and thus, the damage of local industry will be greater and greater. In addition, it is urgently needed to establish a distribution system that can prevent Chinese origins distributed as much as Korean origin.

In order to solve the above problems and to set up new market policy, it is necessary to develop a handy discriminative method for the commercial herb medicines. In addition, new varieties which have better quality and higher yield, should be developed by using conventional breeding and genetic engineering.

The use of RAPD resulted in a potentially useful tool for species discrimination (Williams *et al.*, 1990; Demeke *et al.*, 1992; Cole & Kuchenreuther, 2001). Cultivar identification (Yang & Quiros, 1993),

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parentage determination (Elisiario *et al.*, 1999), genetic relationships evaluation (Nicese *et al.*, 1998) and estimation of population genetic variability (Harrison *et al.*, 1997) are some examples of the multiple use of the RAPD technique.

Use of AFLP technique in plant genetic studies increased with the improvement of detection methods since the first reported in 1995 (Vos *et al.*, 1995). Cultivar identification (Kim *et al.*, 1998), germplasm characterization (Cervera *et al.*, 1998), genetic diversity assessment (Zhu *et al.*, 1998) and biosystematic studies (Kardolus *et al.*, 1998) are examples of the most frequent applications of these markers.

The aim of this study is to find out the differences between two species, *A. japonica* and *A. macrocephala*, using RAPD and AFLP techniques.

MATERIALS AND METHODS

Plant materials

The medicinal plants, *A. japonica* and *A. macrocephala*, were used for RAPD and AFLP analysis. Domestic lines of *A. japonica* was collected from eight regions including Kumsan in Korea and imported lines of *A. macrocephala* was from China. The domestic lines of *A. japonica* and the imported lines of *A. macrocephala* were cultivated according to the standard cultural practices of National Crop Experiment Station, Suwon, Gyeonggi Province, Korea from 2001 to 2002 (Table 1).

Table 1. Plant samples used for RAPD and AFLP analysis.

Entry No.	Species	Collection sites
1	*Atractylodes japonica*	Geumsan, Chungnam Province
2		Uiseong, Gyeongsang Province
3		Inje, Gangwon Province
4		Hwaseong, Gyeonggi Province
5		Suwon, Gyeonggi Province
6		Gapyong, Gyeonggi Province
7		Pochon, Gyeonggi Province
8		Pyeongchang, Gangwon Province
9	*Atractylodes macrocephala*	Suwon, Gyeonggi Province
10		
11		
12		
13		
14		
15		
16		

DNA extraction

The young leaves of the plants were used for RAPD and AFLP analysis. Samples (100 ㎎ of fresh leaves and dried roots) were frozen in liquid nitrogen and ground in a mortar to become a fine powder. DNA was extracted using QIAGEN DNeasy Plant Kit (Dellaporta, 1983; Doyle & Doyle, 1987).

RAPD analysis

The RAPD analysis was carried out using the following mixture: genomic DNA (10 ng/㎕) 1 ㎕, primer (5 ㎛M) 1 ㎕, dNTPs (250 ㎛M total) 1.2 ㎕, *Taq*-polymerase (5U/㎕) 0.2 ㎕, 10× buffer 1.5 ㎕, distilled water 10.1 ㎕, for a total of 15 ㎕ reaction mixture. The *Taq*-polymerase and buffer were purchased from BIONEER (KOREA).

Fourty random primers supplied by OPERON Technologies Inc. (Alameda, CA) and 12 primers, named URP primers which are derived from a rice moderative repeated sequence, supplied by SEOULIN Technologies Inc. (KOREA), were used for the analysis. Amplification reactions were carried out on the DNA Thermal Cycler (BIOMETRA) subjected to 35 cycles of PCR as follows: 94℃, one minute; 35℃ ~ 39℃ for random primer, 55℃ ~ 57℃ for URP primers, one minute; 72℃, two minutes.

Amplification products were analysed by electrophoresis on 1 % agarose gel in 1× TBE buffer and detected by ethidium bromide staining under UV lights.

Only clear and distinct bands were scored both in agarose gels, attributing '1' to the presence and '0' to the absence of a band. The NTSYs-pc software was used for statistical analysis of data. The unweighted pair-group method with arithmetic means (UPGMA) was used to construct the genetic similarity dendrograms.

AFLP analysis

AFLP analysis was carried out with AFLP™ Analysis System I kit (Life Technologies Inc.) by Manufacturer's indications with minor modifications. Template DNA (180 ng) was double digested with 1.25 units of each *Eco*RI and *Mse*I restriction enzymes at 37℃ for three hours in a final volume of 25 ㎕. Specific adapters were ligated for two hours to the restriction fragments with one unit of T4 DNA ligase in 10 mM Tris-HCl pH 7.5, 10 mM DTT, 50 mM KCl and 50 mM K-acetate, at 20℃. Pre-amplification was done using pre-amp primer mix with dilluted template DNA, under the following conditions: 30 cycles of 94℃ for 30 seconds, 56℃ for 60 seconds and 72℃ for 60 seconds. Pre-amplified DNA was analyzed on 1% agarose gel electrophoresis.

To identify the variation of the AFLP patterns and select the species specific AFLP markers, eight combinations of *Eco*RI/*Mse*I primers, E-AAC/M-CAA, E-AAC/M-CAG, E-AAC/M-CAT, E-AAC/M-CTA, E-AAG/M-CAA, E-AAG/M-CAC, E-AAG/M-CAT and E-AAG/M-CTC, were applied to the bulked genomic DNA of *A. japonica* and *A. macrocephala*. Amplifications were carried out using a touch-down PCR program: 1 cycle of 94℃ for 30 seconds, 65℃ for 30 seconds, and 72℃ for 60 seconds, then 12 cycles with the annealing temperature lowered by 0.7℃ per cycle, followed by 23 cycles of 94℃ for 30 seconds, 56℃ for 30 seconds, and 72℃ for 60 seconds.

After the PCR reaction, 20 ㎕ of tracking dye (98% formamide, 10 mM EDTA pH 8.0, xylene cynol and bromophenol) was added. Amplified DNA were denatured at 95℃ for three minutes and 6 ㎕ of denatured samples were loaded onto the 5% sequencing gel containing 8 M urea and the gel was run in 1× TBE at 45 W constant power for about 2 hours & 30 minutes. After running, the gel was silverstained.

RESULTS AND DISCUSSION

The RAPD and AFLP analysis have been very useful implement for species discrimination (Bang *et al.*, 2002; Lee *et al.*, 2002; Gelsomina *et al.*, 2003) and cultivar identification (Yang & Quiros, 1993; Kim *et al.*, 1998; Bang *et al.*, 2001). RAPD polymorphisms, simply detected as DNA segments which amplify from one parent but not the other, are inherited in a Mendelian fashion and can be used to construct genetic maps in a variety of species (Williams *et al.*, 1990).

To identify the variation of the RAPD patterns between two *Atracylodes* species, 52 different random primers were applied to each eight of *A. japonica* and *A. macrocephala* genomic DNA. The optimal PCR conditions for most primers were the condition of 5 ng templet DNA, 4 ㎛M primer, 0.5 unit *Taq* DNA Polymerase in a 20 ㎕ total reaction volume and the PCR cycle condition of initial denaturation for 2 min at94 ℃; and 35 cycles of 30 seconds at 94℃, 30 seconds 39℃, 60 seconds 72℃; and a final 5 min. extension at 72℃.

Ten primers out of 52 primers could be used to discriminate between the species and 18 polymorphisms among 67 scored DNA fragments (18 fragments are specific for either *A. japonica* or *A. macrocephala*) were generated using these primers, 26.9% of which were polymorphic. The number of polymorphic bands ranged from 1 to 4 per primer and the amplified products varied between 0.6 kbp and 2.4 kbp. RAPD data from the 10 primers were used for cluster analysis (Fig. 1, Table 2). Cluster analysis of RAPD markers showed that the two groups are distinct genetically (Fig. 2). Consequently, the RAPD technique was useful method to discriminate between *A. japonica* and *A. macrocephala*.

The advantages of AFLP technique are the capacity to inspect an entire genome for polymorphism and its reproducibility. To identify the variation of the AFLP patterns and select the species specific AFLP markers, the eight combinations of *Eco*RI/*Mse*I primers were applied to the bulked *A. japonica* and *A. macrocephala* genomic DNA.

Consequently, the three combinations of *Eco*RI/*Mse*I primers (*Eco*R I /*Mse* I ; AAC/CTA, AAC/CAA, AAG/CTA) used in this study revealed 176 reliable AFLP markers, 42.0% of which were polymorphic ranging from 150 to 500 bp in size (Fig. 3). The variation of produced band numbers depending on the

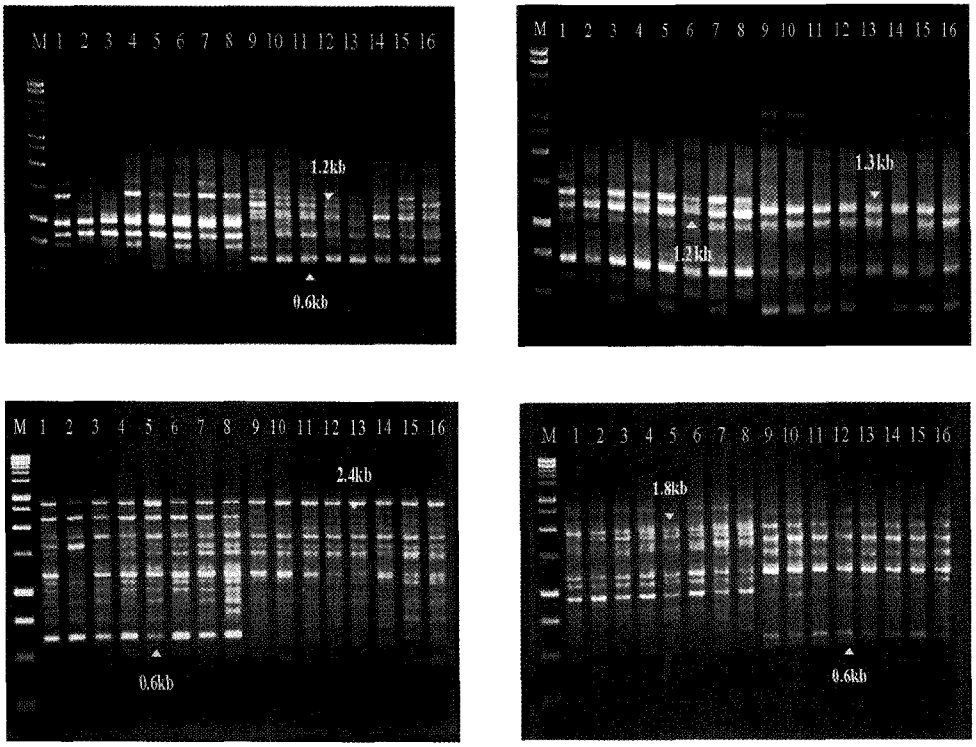

Fig. 1. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON A 12, A 17, B 05 and B 08 primer. Lane M, 1kb DNA ladder; Lane 1-8, *A. japonica*; Lane 9-16, *A. macrocephala*.

Table 2. Selected primers and number of polymorphic fragments generated by RAPD analysis.

Primers†	No. of polymorphic fragments	Polymorphic DNA fragments size (kbp)
URP02	1	1.3 (*Am*)
OPA06	1	1.9 (*Am*)
OPA08	2	1.2 (*Aj*‡), 0.6 (*Am*§)
OPA09	3	1.3 (*Am*), 1.2 (*Aj*) , 0.9 (*Aj*)
OPA11	1	1.2 (*Aj*)
OPA12	2	1.2 (*Am*), 0.6 (*Am*)
OPA15	2	1.7 (*Am*), 1.0 (*Am*)
OPA17	2	1.3 (*Am*), 1.2 (*Aj*)
OPB05	2	2.4 (*Aj*), 0.6 (*Aj*)
OPB08	2	1.8 (*Aj*), 0.6 (*Am*)
Total	18	

† Primers that reveal polymorphic fragments between *A. japonica* and *A. macrocephala*.
‡ Specific fragments in *A. japonica*.
§ Specific fragments in *A. macrocephala*.

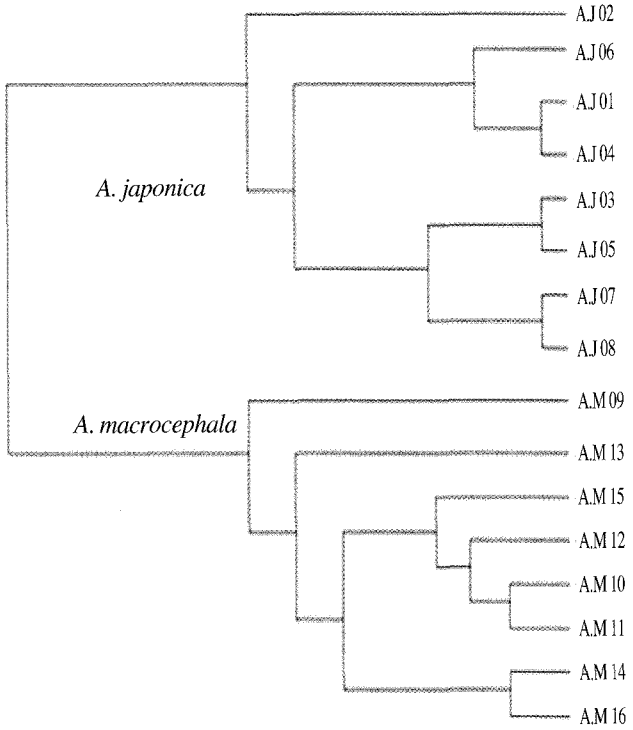

Fig. 2. Genetic relatedness dendrograms generated by UPGMA method on the basis of RAPD data.

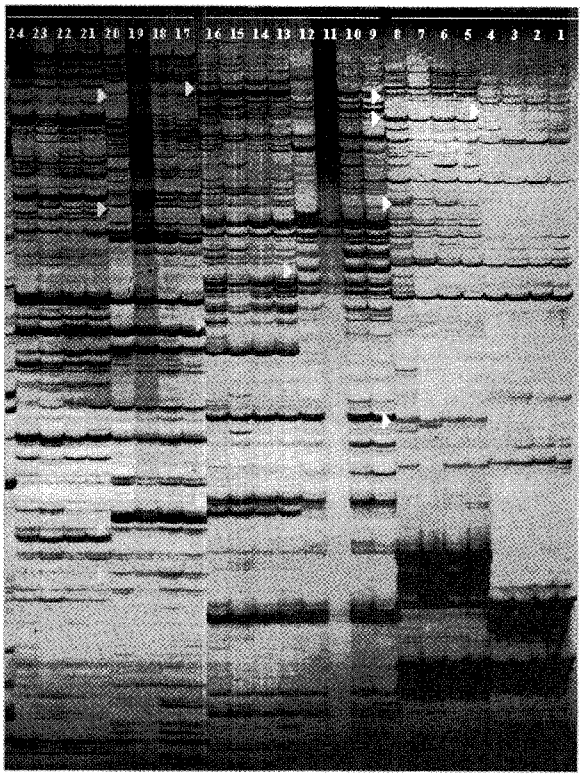

Fig. 3. AFLP profiles generated by the E-AAG/M-CTA (lane 1-8), E-AAC/M-CAA (lane 9-16), E-AAC/M-CTA (lane 17-24) primer combination. Lane 1-4, 9-12, 17-20, *A. japonica*; Lane 5-8, 13-16, 21-24, *A. macrocephala*.

Table 3. Number of scored AFLP fragments and polymorphisms.

Primers		No. of polymorphic fragments	No. of total fragments	Percentage of polymorphic fragments (%)
*Eco*R I	*Mse* I			
AAC	CTA	23	60	38.3
AAC	CAA	20	57	35.1
AAG	CTA	31	59	52.5
Total		74	176	42.0

primer sequences. The primer combination E-AAG/M-CTA revealed the highest percentage of polymorphic fragments (52.5%), while the lowest percentage (35.1%) was generated by the primer combination E-AAC/M-CAA (Table 3). 74 polymorphisms out of 176 scored DNA fragments were enough to clearly discriminate between *A. japonica* and *A. macrocephala*. The superior resolution power of the AFLP technique was proved by the achieved discrimination between *A. japonica* and *A. macrocephala*.

To select the species specific DNA fragments, we obtained 18 RAPD bands and 74 AFLP clones, respectively. The superior resolution power of the AFLP technique was proved by the achieved results discriminating between *A. japonica* and *A. macrocephala*. This is certainly due to the higher multiplex ratio of this technique, which allows much larger number of markers to be revealed per reaction (42.0 vs. 26.9 shown by the RAPD technique), and to the more stringent PCR (annealing) conditions used in this technique (Cervera *et al.*, 1998).

From now on, we are going to develop SCAR marker with species specific DNA markers through cloning and sequencing. The development of discriminative method for herbal medicines using SCAR marker will be helpful to protect the nations unique resources, set up distribution system for Korean herbal market, protect home industries, and provide reliable medicines to local consumers.

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