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Identification of *Atractylodes japonica* and *A. macrocephala* by RAPD analysis and SCAR Markers

By M. K. HUH[1),*)] and K. H. BANG[2)]

Abstract

The Chinese plant, "Packchul", (*Atractylodes japonica* or *A. macrocephala*), is a very important Chinese medicinal herb plant and is called Sabju in Korea. The levels of the active components are different between these two species, but these medicines are sold in Korean herbal markets without discrimination. This study was carried out to develop a method that could be used to discriminate between *A. japonica* and *A. macrocephala* based on molecular markers. To discriminate between *A. japonica* and *A. macrocephala*, RAPD analyses were used to develop SCAR markers. Eighteen species-specific RAPD bands were obtained from 52 OPERON and URP primer sets. Two SCAR markers were developed from these RAPD clones. Both SCAR markers were cloned into pGEM-T-Easy vectors and then subjected to nucleotide sequence analysis and designated *AjR1* (1,117 bp) and *AmR1* (1,325 bp). These two markers were sufficient to discriminate between samples of *A. japonica* and *A. macrocephala*.

Key words: Atractylodes japonica, *A. macrocephala*, RAPD, SCAR.

Introduction

"Packchul", which is commonly called atractylodis rhizoma, white atractylodes rhizome, or simply atractylodes, is used as a Chinese herbal medicine. Packchul consists of two species with the botanical names of *Atractylodes japonica* Koidz and *A. macrocephala* Koidz. It has been reported that Packchul has various health benefits, such as regulating the function of the stomach, replenishing vigor, strengthening the spleen, stopping sweating, and preventing miscarriage (SAKAMOTO et al., 1996).

The Korean botanical name "Sabju" represents the genus *Atractylodes* of the family Compositae and is divided into two types, "Packchul" and "Changchul". In Korean pharmacopoeia. Packchul is defined as the rhizome and peeled rhizome of *A. japonica* and Changchul is defined as those of *A. lancea* (Thunb.) DC. or *A. chinensis* Kitamura. In the Chinese pharmacopoeia, however, Packchul includes not only *A. macrocephala*, but also includes *A. ovata* DC. and Changchuls, which includes *A. japonica*, *A. lancea*, and *A. chinensis* (KIM et al., 1998). This discrepancy has caused a confusion and difficulty in the use of these herbal medicines.

During the last decade several novel DNA markers have been developed for genome analysis. Among the types of molecular markers, "random amplified polymorphic DNA" (RAPD) techniques have greatly improved detection methods since their first reported use in 1990 (WILLIAMS et al., 1990; NICESE et al., 1998).

The RAPD technique is sensitive to reaction conditions, which results in poor reproducibility. To overcome the problems associated with RAPD and to improve their utility in marker-assisted selection, longer primers have been developed from RAPD fragments (PARAN and MICHELMORE, 1993). These longer primers generate a "sequence-characterized amplified region" (SCAR), which can be particularly useful to follow the inheritance of the marked region of the genome. SCAR markers are preferred over RAPD because they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into allele-specific markers. SCAR markers have been developed for crops (DENG et al., 1997; HERNANDEZ et al., 1999; ARDIEL, 2002). The conversion of a linked marker to SCAR has been applied successfully in a number of cases involving RAPD (NAQVI and CHATTOO, 1996; BARRET et al., 1998; LAHOGUE et al., 1998).

In *Atractylodes* species, several studies using RAPD and RFLP analyses have been reported but the use of RAPD analysis and SCAR markers to discriminate between the species has not been reported.

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The rapid expansion of industries producing foods for health improvement and materials for oriental medicine has resulted in a decrease in the use of Packchul as a herbal medicine. On the other hand its industrial use is increasing. As a result of this change, Chinese Packchul (*A. macrocephala*) was ranked second in herbal medicine imports following licorice. It has been reported that imported Chinese Packchul was less valuable than Korean *A. japonica* with respect to their components and effects. Korean Packchul is currently trading at a price 10 times higher than the Chinese source. These two species can be identified by leaf morphology, flower color and size, and rhizome shape (BANG et al., 2003). However, it is impossible to distinguish between the two species when the rhizomes are sliced. Therefore, in herbal markets the Chinese Packchul is illegally sold either without the correct label or by mixing it with Korean Packchul. The aim of this study is to develop methods to distinguish the differences between the two species, *A. japonica* and *A. macrocephala*, at the molecular level.

Materials and Methods

Materials and DNA extraction

A. japonica and *A. macrocephala* were used for RAPD analyses. Plants of *A. japonica* were collected from eight regions in Korea and imported plants of *A. macrocephala* were obtained from China. The plants of *A. japonica* and *A. macrocephala* were cultivated according to the standard cultural practices of National Crop Experiment Station, Suwon, Kyonggi Province, Korea from 2001 to 2002 (*Table 1*).

The 30 young plants per source were used for RAPD analyses. Samples were frozen in liquid nitrogen and ground in a mortar to a fine powder. DNA was extracted using QIAGEN DNeasy Plant Kit (DOYLE and DOYLE, 1987).

RAPD analysis

The RAPD analysis was carried out using the following mixture: 50 ng genomic DNA, 0.5 µM primer 250 µM dNTPs, 5 U Taq-polymerase, 10x buffer, and distilled water.

Forty random primers supplied by OPERON Technologies Inc. (Alameda, CA) and 12 primers (Seolin Technologies Inc., Korea) were used for RAPD the analysis. The amplification reaction was carried out using a DNA Thermal Cycler (Applied Biosystematics). Amplification products were analyzed by electrophoresis on 1.5% agarose gel in TBE buffer and detected by ethidium bromide staining under UV lights.

All the reactions were repeated twice and only reproducible bands were scored for analyses. 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 following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by YEH et al. (1999): the percentage of polymorphic loci (Pp), mean numbers of alleles per locus (A), and gene diversity (H) (NEI, 1973). The NTSYs-pc software was used for statistical analysis of data. The unweighted pair-group method with arithmetic means (UPGMA) was used to construct dendrograms.

SCAR analysis

To convert a selected RAPD band to a SCAR marker, each RAPD band was isolated from agarose gel using a Geneclean II kit, and the isolated DNA was separated by electrophoresis in 1.0% agarose gel to confirm DNA

Table 1. – Samples used for RAPD analyses. The percentage of polymorphism (Pp), mean number of alleles per locus (A), and gene diversity (H).

Codes	Collection sites	Pp	A	H
Atractylodes japonica				
AJ-1	Kumsan, Chungnam Province	26.6	1.28	0.12
AJ-2	Uiseong, Kyongsang Province	25.4	1.41	0.14
AJ-3	Inje, Gangwon Province	23.5	1.27	0.12
AJ-4	Hwaseong, Kyonggi Province	27.3	1.20	0.14
AJ-5	Suwon, Kyonggi Province	28.1	1.36	0.12
AJ-6	Gapyong, Kyonggi Province	30.3	1.28	0.11
AJ-7	Pochon, Kyonggi Province	22.7	1.15	0.10
AJ-8	Pyongchang, Gangwon Province	31.1	1.61	0.13
Mean		26.9	1.32	0.12
A. macrocephala				
Am-1	China	25.5	1.27	0.11

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Table 2. – RAPD analysis and SCAR markers used for discrimination of *A. japonica* and *A. macrocephala*.

RAPD	Sequences of SCAR marker primer	Size of RAPD markers (bp)
AjR1	Forward primer: 5' GGAAGGAATCGAGAAGGCTAACGC 3'	1117
	Reverse primer: 5' AATGGCCGCCATGGTTGAAG 3'	
AmR1	Forward primer: 5' CCGTCAATAAACCAAACATCACTG 3'	1325
	Reverse primer: 5' TCCTTGATGCCTACCTCCTGTTAG 3'	

AjR1 stands for *Atractylodes japonica*.
AmR1 stands for *A. macrocephala*.

size. The DNA were then ligated into the pGEM-T-Easy vector (Promega) and transformed into *E. coli* DH5α. Nucleotide sequences were determined using the automatic DNA sequencer (ABI377). The similarities of DNA sequence data were analyzed using BLAST (ALTSCHUL et al., 1997).

The SCAR marker sequences were designed by identifying the original 10 bp sequences of the RAPD primer and adding the next approximately 10 bp in the DNA sequence. These species-specific SCAR primer were then synthesized by Seolin Technologies Inc. Each of the designed SCAR primer pairs (one forward and one reverse SCAR primer) was tested by means of sub samples of both species. To establish the optimum annealing temperature, five annealing temperatures from 35 to 39 °C, with one degree increase were tested with the PCR condition of 5 ng template DNA, 4 μM primer, 0.5 unit *Taq* DNA Polymerase in a 20 μl total reaction volume. The optimum PCR condition was found to be an initial denaturation for two minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds 39 °C, and 60 seconds 72 °C; and a final five minutes of extension at 72 °C.

Results

RAPD analysis

To identify the variation of the RAPD patterns between two Atracylodes species, 52 different random primers were applied to the genomic DNAs of *A. japonica* and *A. macrocephala*.

Ten primers out of the 52 primers could be used to discriminate between the subspecies. Eighteen polymorphic bands out of 67 scored DNA fragments were generated using these primers. The number of polymorphic bands ranged from one to three per primer and the amplified products varied between 0.6 and 2.4 kbp. Five fragments are specific for *A. japonica* and do not show at *A. macrocephala*. On the other hand, three fragments are specific for *A. macrocephala*. These species-specific DNA fragments seemed to be useful to discriminate between *A. japonica* and *A. macrocephala* and were used to develop the SCAR markers.

Although the typical populations of *A. japonica* were small, isolated, and patchily distributed for accessions, they maintained a high level of genetic diversity (*H*) (*Table 1*). In a simple measure of intraspecies variability by the percentage of polymorphic bands, the AJ-7 exhibited the lowest variation (22.7 %). The AJ-8 showed the highest (31.1 %). The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*H*) within populations. The mean *H* of *A. japonica* (1.606) was higher than that of *A. macrocephala*.

SCAR analysis

Eighteen species-specific RAPD bands were obtained from 52 OPERON and URP primer sets. Five bands were found specific to *A. japonica*, whereas three bands were specific to *A. macrocephala*. The distinct two primer pairs, *AjR1* and *AmR1*, were designed from the sequences of RAPD fragments. The primer pairs, *AjR1* and *AmR1*, were the amplified genomic DNAs of *A. japonica* and *A. macrocephala*.

The DNA sequence *AjR1* consists of 1,117 bp nucleotides and was specific to *A. japonica*. The fragment amplified the expected 1,200 bp DNA bands in individuals from *A. macrocephala*, whereas no products were detected in individuals from *A. japonica*. The DNA sequences of *AmR1* consist of 1,325 bp nucleotides and were specific in A. macrocephala (*Fig. 2*).

Searching (in) GenBank revealed that the 1,117 bp (868–1,010 bp) DNA fragment from marker *AjR1* share 78 % nucleotides in the complete sequence of the *Medicago truncatula* clone mth1-8o8. However, the
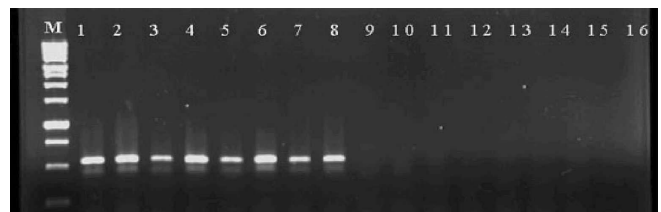

Figure 1. – Ideogram of species-specific DNA fragments obtained by RAPD analysis and RAPD *AjR1* sequences. M, molecular weight of polymorphic DNA; Lane 1–8, 8 different accessions of *A. japonica*; Lane 9–16, *A. macrocephala*.
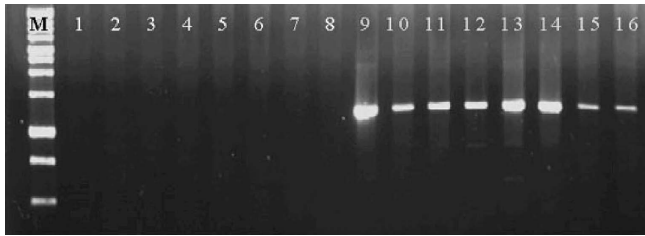
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Figure 2. – Ideogram of species-specific DNA fragments obtained by RAPD analysis and RAPD *AmR1* sequences. M, molecular weight of polymorphic DNA; Lane 1–8, *A. japonica*; Lane 9–16, *A. macrocephala.*

sequences of *AmR1* marker were not homologous in the GenBank search and *AmR1* marker was revealed as unique sequences based on the GenBank search.

Two SCAR primer pairs (*AjR1* and *AmR1*) were designed according to the nucleotide sequences obtained by cloned RAPD fragments. The SCAR primers were then used to amplify the genomic DNA of both species. A single, distinct, and easily identifiable band specific to each species is shown in *Figs. 1* and *2*.

Discussion

A recent demand for the health-promoting medicinal herbal plants has been increasing. In parallel with this trend, Chinese medicines are second only to licorice in herb medicine imports. The active components of *A. japonica* and *A. macrocephala* are volatile oils. Sesquiterpenoids such as atractylon, atractylenolide III and 3β-acetoxyatractylon are the major oils, but the content levels are different between these two species (SAKAMOTO et al., 1996). Thus, it is an (indispensably) important issue to establish an assay system for the discrimination of the herbal medicines and provide a quality control. The first step in this process is to develop efficient markers.

Primers for SCAR marker can be selected on the basis of the nucleotide sequence of RAPD and is used to understand the existence of specific nucleotide sequence by PCR. For example, BEHURA et al. (1999) applied to SCAR marker for efficiently discriminating varieties of rice. SCAR markers have been practically applied to various fields such as identifying resistance gene (DEDRYVER et al., 1996) and thermo sensitive genes (LANG et al., 1999).

To develop a fast, cheap, and reliable PCR-based assay, NEGI et al. (2000) employed PCR-walking technology to isolate sequences adjacent to the linked RAPD. Based on the sequence information of the cloned flanking sequence of marker, new primers can be designed. In this study, the two pairs of primers designed from nucleotide sequences of the RAPD bands could produce specific SCAR markers (*AjR1* and *AmR1*), and could be used to easily discriminate between *A. japonica* and *A. macrocephala.* With these two SCAR markers, Korean and Chinese herbal medicines in the Korean herbal market could be discriminated.

Consequently, the SCAR markers identified in this study will confer an efficient method for discriminating of their origins and unique reproductive modes in *Atractylodes* species. The development of discriminative method for herbal medicines using SCAR marker is helpful to protect the unique national resources, to set up distribution system for Korean herbal market, protect home industries, and provide reliable medicines to consumers. Moreover, this study can assist in future selective breeding programs as reliable and reproducible molecular markers.

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Genetic Diversity of Two Evergreen Oaks [*Quercus suber* (L.) and *Quercus ilex* subsp. *rotundifolia* (Lam.)] in Portugal using AFLP Markers

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Abstract

The genetic variability of cork oak (*Quercus suber*, L.)
in Portugal was evaluated by AFLP using five primer
combinations. Three hundred and thirteen trees from
three geographically contrasting regions exhibited a
high level of genetic variation. The genetic profile of
each individual is composed of 291 loci, randomly posi-
tioned in the genome and consists of monomorphic and
polymorphic fragments. Similarities and dissimilarities
among the individuals were quantitatively evaluated by
numerical taxonomy. The overall sample shows a pro-
portion of AFLP polymorphic markers of 71%, denoting
a high level of variability. Ninety percent of the polymor-
phic markers identified in cork oak genotypes are uni-
formly distributed throughout the cork oak populations
of Algarve, Alentejo and Trás-os-Montes regions. The
coefficients of genetic similarity vary from 0.61 to 0.88
implying that 60% of fragments found are common. A
sample of 52 holm oak [*Quercus ilex* subsp. *rotundifolia*
(Lam.)] trees from overlapping areas was also analysed
by AFLP with the same five primer combinations. How-
ever the codification of markers together with those
selected on cork oak profiles was feasible with only one
primer combination due to an apparent much higher
polymorphism. AFLP and numerical taxonomy analysis
enabled to differentiate the taxa and showed that the
level of similarity observed between the profiles of the
individuals from holm oak species was lower than that
observed in cork oak, implying that apparently the
degree of polymorphism is higher in *Q. ilex* subsp. *rotun-
difolia* than that quantified in *Q. suber*.

A Bayesian approach was used to assess *Q. suber* total
genetic diversity (Ht = 0.2534, P < 0.001) of which 1.7%
(Fst = 0.0172, P < 0.001) was assigned to differences
among populations. Analysis of molecular variance
(AMOVA) showed that most genetic variation is com-
prised within populations (96%) while 3.6% is among
populations (Φst = 0.036, P < 0.001). Differences among
populations within geographic regions account for 2.6%
(Φsc = 0.026, P < 0.001) of the total variation and only
1.3% (Φct = 0.013, P = 0.007) is attributed to variation
among regions denoting little differentiation of popula-
tions over a range of 700 km.

Introduction

Cork oak (*Quercus suber* L.), an evergreen species, is
mainly distributed in the western Mediterranean Basin.
The largest stands, covering about 700.000 ha are locat-
ed in Portugal and correspond to 21% of the forest area
in Portugal and to 30% of the world cork producing
area. Cork oak plantations are very important for the
economy and constitute a social and environmental
issue that has to be taken into consideration as the
unparalleled decline occurring in the Iberian Peninsula

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