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Selection of PCR Markers and Its Application for Distinguishing Dried Root of Three Species of *Angelica*

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ABSTRACT : An analysis of RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) was performed with three Angelica species (*A. gigas* Nakai, *A. sinensis* (Olive.) Diels and *A. acutiloba* Kitag) in an effort to distinguish between members of these three species. Two arbitrary primers (OPC02, OPD11) out of 80 primers tested, produced 17 species-specific fragments among the three species. Eight fragments were specific for *A. sinensis*, four fragments specific for *A. gigas*, five specific for *A. acutiloba*. When primers OPC02 and OPD11 were used in the polymerase chain reaction, RAPD-PCR fragments that were specific for each of the three species were generated simultaneously. Primer OPC02 produced eight species-specific fragments: four were specific for *A. sinensis*, one for *A. gigas*, and three for *A. acutiloba*. Primer OPD11 produced nine species-specific fragments: four for *A. sinensis*, three for *A. gigas*, and two for *A. acutiloba*. The RAPD-PCR markers that were generated with these two primers should rapidly identify members of the three Angelica species. The consistency of the identifications made with these species-specific RAPD-PCR markers was demonstrated by the observation that each respective marker was generated from three accessions of each species, all with different origins. We also performed the RAPD-PCR analysis with the dried Angelica root samples that randomly collected from marketed and from the OPC02 primer, obtained a *A. gigas*-specific band and the band were cloned and sequenced.

Key words : Angelica species, RAPD-PCR, dried Angelica root, species-specific fragment

INTRODUCTION

Angelica radix is belong to family of Umbelliferae, genus of Angelica. Traditionally, it has been one of the most commonly used herbs by women worldwide. It is considered a "cure-all" in Chinese medicine, it containing varying amounts of volatile oil, flavonoids, angelic acid, fluorescent bitter furanocoumarins, tannin and resins. Traditionally, Angelica radix is often combined with other herbal products and used to treat allergies, arthritis, asthma, or high blood pressure. Animal studies of Angelica radix have shown it has a slight ability to increase immune system function, so it may help to relieve allergy symptoms. In addition, laboratory studies have shown that Angelica radix has mild anti-inflammatory properties, which may make it useful in treating arthritis, asthma, and other inflammatory conditions. One of the chemicals in a related plant has been shown to promote relaxation of blood vessels, which may help to reduce blood pressure. However, no clinical evidence supports the use of Angelica radix for blood pressure control (Chen & Chen, 2004).

In genus of Angelica, there are three Angelica species used as medicine: *A. gigas* Nakai (common name: Korean Angelica), *A. sinensis* (Olive.) Diels (common name: Chinese Angelica), *A. acutiloba* Kitag (common name: Japanese Angelica). A great quantity of Angelica radix is used for chinese medicine in Korea, China and Japan. However the origin and medicinal effect is different. The Korean pharmacopoeia prescribed the *A. gigas* Nakai as a Angelica radix, the Chinese pharmacopoeia prescribed the *A. sinensis* (Olive.) Diels as a Angelica radix and the Japanese pharmacopoeia prescribed the *A. acutiloba* Kitag as a Angelica radix, respectively. Although historically these three Angelica species have been considered as same drug that have the similar components and Pharmacological activities, and used as substitution each other. But until now, there are no sufficient evidence confirm that. Moreover, recently plenty of researches reveled something differences among them (Terasawa, 1985; Han, 1991).

Because the Angelica radix were appointed as a importing regulated articles and couldn't imported as medicine, exception as a food in Korea, large amount of Angelica radix imported as a food and distributed with the domestic Danggui, mixed and used as herb medicine. Therefor couldn't expect the cor-

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rect medicinal effect and often cause the misusing. Also it is badly damaged to domestic medicinal crop farmer. For the reasons of mentioned above, it is necessary to improve the distinguish methods of the origin in Angelica species. It is very important to prevention the disorder and maintain the orders of market.

Random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990) are identified by using arbitrary primers and allow the quick construction of genetic maps for many plant species or the saturation of specific genomic regions with molecular markers (Martin *et al.*, 1991; Paran *et al.*, 1991; Michelmore *et al.*, 1991). RAPD-PCR analysis is simple and fast. It does not involve the use of radioactive isotopes and can be scaled up to analysis large numbers of samples. RAPD markers have been used for the identification of different plant species, as well as for assessing genetic diversity (Graham *et al.*, 1994; Klein-Lankhorst *et al.*, 1991; Lanham *et al.*, 1995; Moreno *et al.*, 1995). Many plants were identified species-specific RAPD markers (Mandolino *et al.*, 1999; Khan *et al.*, 2000; Bhattacharya & Ranade, 2001; Elizabeth *et al.*, 2003; Roman *et al.*, 2003; Arvind *et al.*, 2004; Li *et al.*, 2004). The objective of this study was to distinguish three Angelica species using RAPD-PCR method. It will be useful for quick and easy to identifying the origin of Angelica species.

MATERIALS AND METHODS

Plant materials

Three Angelica species of *A. sinensis* (Oliv.) Diels, *A. gigas* Nakai and *A. acutiloba* Kitagawa that already confirmed the origins by morphological observation were used in this study as a standard. The standard samples of each species were cultured on field of National Institute of Crop Science, RDA, Suwon, Korea and from the each species randomly selected 5 plants, and the DNAs were extracted from the leaves and used to screen primers suitable for distinguish from the three Angelica species. The DNAs extracted from dried Angelica root that randomly collected from market were also used. The samples used in the present study along with their species and collected site are listed in Table 1.

DNA extraction

From the leaves, total genomic DNA were extracted by CTAB (cethyltrimethy-lammonium bromide) method according to the protocols (Doyle & Doyle, 1987), with minor modifications. The young leaves were ground to a fine powder in liquid nitrogen and mixed with extraction buffer (1% CTAB, 50 mM Tris-HCI pH 8.2, 10 mM EDTA, 0.7 M NaCl, 1% 2-mercaptoethanol) until it was homogenous. After incubation at

Table 1. List of *Angelica* species used in RAPD analysis.

Entry no.	Species	Collection site	Numers of plants
1~5	*A. sinensis Diels.*	Nat. Ins. of Crop Science, RDA	5. Leaves
6~10	*A. gigas Nakai*	Nat. Ins. of Crop Science, RDA	5, Leaves
11~15	*A. acutiloba*	Nat. Ins. of Crop Science, RDA	5, Leaves
16	Unknown	Market	1, Dried root
17	Unknown	Market	1, Dried root
18	Unknown	Market	1, Dried root
19	Unknown	Market	1, Dried root
20	Unknown	Market	1, Dried root
21	Unknown	Market	1, Dried root
22	Unknown	Market	1, Dried root
23	Unknown	Market	1, Dried root
24	Unknown	Market	1, Dried root

65°C for 10~30 minutes. Following incubation, equal volume of chloroform/isoamylalcohol (24:1) was added, and the mixture was shaken vigorously. The extract was centrifuged at about 12,000 rpm for 10 min at room temperature or until the aqueous phase was clear and the supernatant transferred to a new microtube. The chloroform/isoamylalcohol extraction step was repeated twice. The DNAs were precipitated with isopropanol, and washed twice with 70% ethanol. The pellet was dissolved with TE buffer containing 0.001 $\mu g/\ell$ RNase A. In case of extraction dried root DNA, almost extraction steps same as described above except extended the incubation times at least 60min in extraction buffer. All of the DNAs extracted were quantified on 0.8% agarose gels and diluted to uniform concentration (10 ng/$\mu\ell$) for RAPD analysis and stored at −20°C until use.

RAPD-PCR analysis

Screen the primers with standard Angelica species

For selection suitable primers concurrently distinguish of the three species, 80 primers (OPA01-20, OPB01-20, OPC01-20, OPD01-20; Operon Technologies Inc., Alameda, CA) were previously screened on three standard samples. DNA was amplified following the protocol of Jin *et al.* (2003). Amplification reactions were performed in a DNA Thermal Cycler (Perkin Elmer Cetus, USA), in a reaction volume of 20 μl containing 1×PCR buffer (75 mM Tris-/HCl, pH 8.4, 50 mM KCl, 2.0 mM $MgCl_2$, and 20 mM $(NH_4)_2SO_4$), 0.2 $\mu\ell$ each of dNTP, 0.2 $\mu\ell$ primer, 1 unit of *Taq*. Polymerase (Bioneer) and 40 ng of template DNA in sterile distilled water. Amplification

reactions were carried out using the following cycle profile: Initial denaturation at 94°C for 3 min followed by 2 cycles at 94°C for 30s, 36°C for 30s, 72°C for 3 min; and 20 cycles at 94°C for 30s, 36°C for 15s, 45°C for 15s, 72°C for 90s; and 19 cycles at 94°C for 20s, 36°C for 15s, 45°C for 15s, 72°C for 120s; and a final 7 min for extension at 72°C The amplified products were separated electrophoretically on a 1.5% (w/v) agarose gel in 1×TBE buffer (80 mM Tris-borate, 2 mM EDTA, pH 8.0) and stained with ethidium bromide. A 1.0 kb ladder DNA marker (Pharmacia) was used as a size standard. The gel image was recorded using a Gel Documentation System (UVP, UK).

Test the chosen primers on dried root DNAs

To confirm the utility of the selected RAPD primers, RAPD-PCR analysis were also conducted with the dried root samples randomly collected from marketed with the same conditions as described above. The test was done to ensure amplification of the band with exact molecular weight and determine optimal annealing temperature. The primers were applied on all 9 Angelica root samples with standard samples (Table 1) to ensure the primers correctly identified their respective Angelica species.

Purification for Sequencing

The bright and highly reproducible RAPD fragment was chosen that was only amplified in *A. gigas*. The fragment was excised, cloned and sequenced following the procedure outlined in Jung *et al.* (1999). The excised PCR band was sequenced at the Bioneer Technologies Inc.

RESULTS AND DISCUSSION

Screen the primers on standard Angelica species

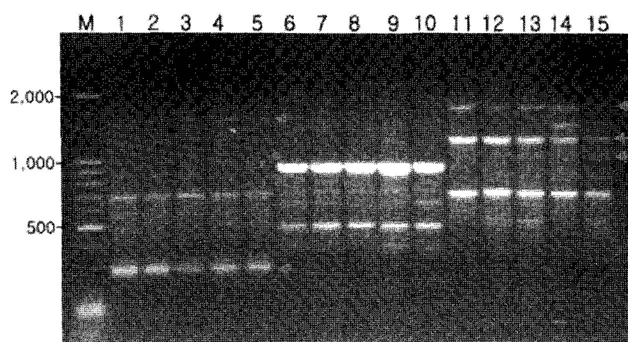

Fig. 1. Profiles of PCR products obtained from genomic DNA using the 10-based Operon C02 primer. Lane M, 1 kb DNA ladder; Lane 1~5, *A. sinensis*; Lane 6~10, *A. gigas*; Lane 11~5, *A. acutiloba*. Arrows indicate species-specific bands.

In present study, RAPD-PCR analysis were performed for distinguish of the three Angelica species. Fifteen standard plant DNAs (consist five of each species) were used to screen the primers suitable for distinguish from these three Angelica species. There are several RAPD studies in Angelica species (Lee *et al.*, 2000; Bang *et al.*, 2002). It is very important to distinguish origin of the Angelica species by morphological trait that permit us to obtain collect data from the experiment. All of plants used in the present study were distinguished by morphological traits such as shape of leaves and growth habit before DNA extraction, and the origins were already confirmed by anatomical characters (Sung *et al.*, 2004). In the RAPD-PCR analysis, the samples used in present study, produced almost identical banding patten within each species in certain primer. Indicating that the samples genetically uniform or didn't contaminated each other.

In the RAPD-PCR analysis, from the 80 primers used in this study, finally we chosen 2 primers (OPC02, OPD11) that gave distinct and reproducible bands. After RAPD-PCR analysis,

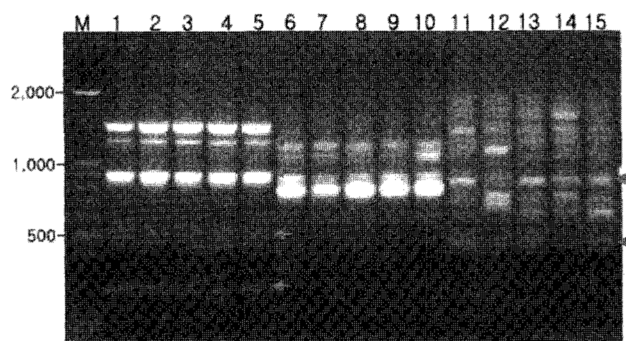

Fig. 2. Profiles of PCR products obtained from genomic DNA using the 10-based Operon D11 primer. Lane M, 1 kb DNA ladder; Lane 1~5, *A. sinensis*; Lane 6~10, *A. gigas*; Lane 11~15, *A. acutiloba*. Arrows indicate species-specific bands.

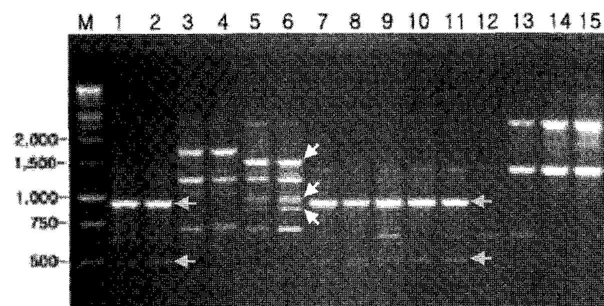

Fig. 3. Profiles of PCR products obtained from genomic DNA using the 10-based Operon CO2 primer. Lane M, 1 kb DNA ladder; Lane 1, 2: *A. gigas*; Lane 3, 4: *A sinensis*; Lane 5, 6: *A. acutiloba*; Lane 7~15 (16~24), Angelica root collected from market. Arrows indicate *A gigas*specific bands.

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  1 ccg ccc gtt ccg cat cga cat ccc ctt cat caa ctg gta aag gag ttc agc gtg tcg tgg
 61 aag ccc gcc gtc tga cgg aac caa ctc ctt cgc aga atg caa ccg tat tga cgg tca cag
121 gta tag ctg tga ctt cca cct atg gcc gca aag tcg gtg cgc tac gtg gcc caa tag gcc
181 gtc caa acg cat aac aag tgc gcc gag cga cgc cat acc gta ggg gta gct cag acg tac
241 tgc acg cat gcg gga gcc ccg gct tca cca aaa agt cgc acg aac gtg tcc gag cga tcg
301 gca agg cgc agc cac ttc gaa caa ggc cga acc ggc gta gca ccc tgc tcc tgt tgg gtg
361 ttg gcc ggt cgc gtt ctt cgt tcc agt gcc gtt ttg gna ccc ggc atc cca cat tta ctc
421 ctg ccc cgt aac cga att gga gcc ctt act tat tag gcg agt gtt cca gtc tgc ttt ccg
481 aag tcc cca agc cca att atg ggc ttt tta cta cat atc ttt ttg ggc cta cga caa ata
541 gct cgt aac atg ccc ccc gtc cgt ctc tta att tgt aga gca ttc ttt ttg tct gcg aac
601 aat ggg gac ttt act tct gag tga ggc tgt gtc tat ctg gaa acc ttt gac gag ggc tgt
661 aac cgt cga aac gtg aag cgt tgt cac tca tac gcc tgg gtc ggt acc ttc ctt ttt cgt
721 tta gag gtc atc ccg aat act gtt act ctt att taa gac ctg ggg gaa aga tag ggg agg
781 agg gcc gcc ggt cgg ttg act aag tcc cga gga gag gta att ctt ttt aga agg cgg gga
841 aat agg ttg ggg cgt gag tcg tag cga tac acg tca gcg gat ttg tcg gct tgt atg tcg
901 cgt tat cag cca cc
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Fig. 4. The DNA sequences obtained from specific to *A. gigas* Nakai band.

from the OPC02 primer, we observed fore bands specific to *A. sinensis*, about 1,600 bp, 1,000 bp, 860 bp, 300 bp; one band specific to *A. gigas*, about 950 bp; three bands specific to *A. acutiloba*, about 1,700 bp, 1,300 bp, 1,100 bp, respectively (Fig. 1). From the OPD11 primer, observed fore bands specific to *A. sinensis*, about 1,300 bp, 900 bp, 500 bp and 250 bp; three bands specific to *A. gigas*, about 1,250 bp, 1,100 bp and 750 bp; two bands specific to *A. acutiloba*, about 800 bp and 450 bp, respectively (Fig. 2). Using these two primer, we successfully distinguished the three species by presence or absence of specie-specific bands, concurrently.

Test the chosen primers on dried root DNAs

Many medicinal plant, especially the Angelica species, the dried root were used as medicine. To confirm the utility of the selected RAPD primers, RAPD-PCR analysis were also conducted with the Angelica root samples randomly collected from market with the same conditions as described above. The test was done to ensure amplification of the band with exact molecular weight and determine optimal annealing temperature. The two primers (OPC02, OPD11) were applied on all 9 dried Angelica root samples that randomly collected from marked with standard Angelica leaf samples (Table 1) to ensure the primers correctly identified their respective Angelica species. From the OPC02 primer, we obtained one of bright and reproducible band, approximately 900 bp that specific to *A. gigas* (Fig. 3). From the RAPD patterns produced from OPC02 primer, we distinguished the 5 dried root samples were belong to *A. gigas*.

In present study, we successfully distinguished the three Angelica species using RAPD-PCR method, and also distinguished the dried Angelica root samples that randomly collected from market by RAPD-PCR method. Also the *A. gigas*-specific RAPD-PCR marker were sequenced (Fig. 4) for developing a speces-specific SCAR markers. It will be useful for quick and easy to identifying the origin of Angelica species.

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