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SCAR markers for discriminating species of two genera of medicinal plants, *Liriope* and *Ophiopogon*

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ABSTRACT. The development of DNA markers that can closely discriminate between *Liriope* and *Ophiopogon* species is vital for efficient and accurate identification of these species, and to ensure the quality, safety, and efficacy of medicines made from these plants. We developed species-specific molecular markers for these two genera. Forty RAPD primers were tested to detect polymorphism; species-specific RAPD bands were gel-purified, cloned, and sequenced. Primers for sequence-characterized amplified regions (SCARs) were then designed, based on nucleotide sequences of specific RAPD primers. SCAR markers SA06 and SB05, specific to *Ophiopogon japonicus*, amplified 460- and 553-bp DNA fragments, respectively. The marker SA12 amplified a 485-bp fragment specific to *Liriope platyphylla*. This is the first report of a species-specific SCAR marker for this group. These markers will be useful for rapid identification of closely related *Liriope* and *Ophiopogon* species.

Key words: *Liriope*; *Ophiopogon*; RAPD; SCAR; Species discrimination

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INTRODUCTION

The genus *Liriope* includes about eight species and *Ophiopogon* includes about 54 species, both of which are distributed in China, Korea, Pakistan, Japan, Indochina (Vietnam, Laos), and the Philippines. Steroidal glycosides in the tubers of *Liriope* are widely used to treat many diseases, including myocardial ischemia and thrombosis, hypoxia, senility, and to help regulate blood sugar (Kako et al., 1995; Wang et al., 2011). In Korea, *Liriope spicata*, *L. platyphylla*, and *Ophiopogon japonicus* have been used in traditional medicine for hundreds of years to treat respiratory diseases, including cough and sputum (Kim et al., 2002). In addition, *L. spicata* and *L. platyphylla* are commonly used for landscaping; they show strong resistance to environmental pollution, and have become popular and economically important ground covers and borders. The two species are closely related and vegetatively similar. To the untrained eye, the tubers appear similar, making authentication difficult and the use of one as an adulterant in the production of another easy; this can compromise the quality of a given medicine (Liu J et al., 2010). Traditional identification and evaluation is based on agronomic, morphological and physiological traits such as leaf size, maturity time and seed shape, which can be substantially influenced by environmental conditions, making it difficult to accurately discriminate among medicinal plants in wild populations or among species of certain genera (Lin et al., 2011). As commercial preparations may consist of one or more variants, rapid and simple assays for identifying variants, both for commercial production and quality control, are needed.

Molecular biology is becoming increasingly popular as a powerful tool for unambiguous raw-drug authentication. It can reveal genetic differences rapidly in detail, and the results are generally not confounded by environmental effects (Arif et al., 2010; Oliveira et al., 2010). Polymerase chain reaction (PCR)-based methods, including random amplified polymorphic DNA (RAPD), make it possible to compare organisms directly at the molecular level, and have been used to authenticate medicinal plant materials (Claros et al., 2000; Park et al., 2009). RAPD requires less DNA template and is relatively easy to conduct compared to other methods, but it has poor reproducibility and stability, and has some limitations in practical application. The reproducibility of RAPD is affected by the quality of DNA, the concentrations of primer and template, and even different sources of DNA polymerase (Techen et al., 2004). However, converting RAPD markers into sequence-characterized amplified region (SCAR) markers can greatly improve specificity and stability, making the system more convenient and efficient for testing different alleles.

Because it is rapid, simple and relatively inexpensive, particularly to analyze numerous samples, the SCAR method has been used to identify a wide range of medicinal plant species, including *Panax ginseng* (Wang et al., 2001), *Artenisia princeps* and *A. argyi* (Lee et al., 2006), *Phyllanthus emblica* (Dnyaneshwar et al., 2006), *Anthricus sylvestris* (Choo et al., 2009), *Bambusa balcooa* (Das et al., 2005) and *Atractylodes* (Chen et al., 2001).

Many pharmacological studies of *Liriope* and *Ophiopogon* have been conducted; however, the identification of species-specific DNA markers in these two genera has moved slowly. Although several molecular genotyping techniques exist, including the use of target region amplification polymorphisms (Zhang and Chen, 2010), inter-simple sequence repeats (Liu X et al., 2010), RAPD (Huang et al., 2009), and EST-SSR (Li et al., 2011), a simple method for identifying closely related species at the molecular level is still needed. In the present study, we chose two species of *Liriope* and *Ophiopogon* each, that are commercially and therapeutically important and widely used in traditional medicine for treating various ailments

in Korea, to develop SCAR markers using RAPD primers. To the best of our knowledge, this is the first report of the successful identification of *L. platyphylla* and related species using species-specific SCAR markers developed from RAPD markers.

MATERIAL AND METHODS

Plant material and DNA extraction

Six lines of *Liriope* and *Ophiopogon* were collected from different geographical regions across Korea and China. Plant samples were authenticated by the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science (RDA, Suwon, Republic of Korea). Two species of each genus were used: *L. platyphylla* and *L. spicata*, and *O. japonicus* and *O. jaburan*. The details of the plant samples used are presented in Table 1.

Table 1. Details of the plant samples used in this study.

Name of the plant species		Specimen No.	Place of collection	Lane in gel
Liriope	*L. platyphylla*	KULP017	RDA	1
	L. spicata	KULP018	Taebaek, Kangwon-do	2
	L. spicata	KULP070	Hangzhou, China	3
Ophiopogon	*O. japonicus*	KULP023	RDA	4
	O. japonicus	KULP071	Shandong, China	5
	O. jaburan	KULP062	Seogwipo, Jeju-do	6

To establish a rapid and efficacious molecular method for identifying these closely related species, we extracted DNA from fresh leaves and dried raw tubers. DNA was extracted from fresh leaves using a Qiagen DNA Extraction kit (Qiagen, Hilden, Germany). In addition, we used the extraction buffer hexadecyltrimethylammonium bromide (CTAB) to improve DNA isolation. Briefly, 200 mg tuber was chopped into small species, powdered using liquid nitrogen, and then transferred to tubes containing 2 mL CTAB/PVPP (polyvinylpolypyrrolidone) extraction buffer consisting of 0.1 M Tris-HCl, 1 M NaCl, 20 mM EDTA, 1% CTAB, and 1% PVPP (w/v). The mixture was incubated at 65°C for 20 min and extracted with an equal volume of chloroform/isoamyl alcohol (24:1). After centrifugation (12,000 *g*) for 5 min, the supernatant was transferred to a clean tube and precipitated with two volumes of precipitation buffer (50 mM Tris HCl, 4 mM NaCl, 10 mM EDTA and 1% CTAB) at 12,000 *g* for 20 min. After extraction with an equal volume of chloroform/isoamyl alcohol (24:1), the DNA pellet was re-precipitated with ice-cold ethanol, washed with 70% ethanol, and dissolved in 200 µL 1X TE buffer. The extracted DNA was treated with 5 µL RNase A (10 mg/mL) to remove any contaminating RNA. The relative purity and concentration of the extracted DNA was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The final concentration of each DNA sample was adjusted to 20 ng/µL.

RAPD analysis and PCR conditions

PCR mixtures (25 µL) (Rafalski and Tingey, 1993) contained 2.5 µL 10X PCR buffer, 0.4 µL dNTP mixture (0.2 mM each of dATP, dCTP, dGTP, and dTTP), 2 µL 50 ng template

DNA, and 0.5 µL 1.25 U Taq DNA polymerase, in a final volume of 25 µL with distilled water. Amplifications were performed in a DNA thermal cycler (PTC-200) using the following parameters: 94°C for 5 min; 35 cycles at 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 5 min. The amplified PCR products were resolved by electrophoresis on 1.5 % agarose gels and 1X TBE buffer. DNA was stained with RedSafe TM Nucleic Acid Staining Solution (iNtRON Biotechnology, Korea) and photographed under ultraviolet light. RAPD was performed with 40 random decamer primers (OPA 1-20 and OPB 1-20) obtained from Operon Technologies (Alameda, CA, USA). Each experiment was repeated at least twice to confirm the species-specific fingerprinting pattern and reproducibility.

Cloning and sequencing of species-specific RAPD amplicons

The species-specific RAPD amplicons were separated by gel electrophoresis and excised from the agarose gel using the Solgent Gel and PCR Purification kit (Solgent, Korea). These eluted fragments were then ligated into the PUC-19 Vector (Solgent) following supplier instructions and transformed into the competent stain DH 5α, and the recombinant plasmid DNA was purified from the white colonies using the Solgent Plasmid Mini-prep kit (Solgent). Three distinct white colonies were chosen from the Luria-Bertani (LB)/ampicillin/X-gal/isopropy1-β-D-1-thiogalactopyranoside (IPTG) plate and the recombinant plasmid was isolated from each overnight colony. The inserted fragments were sequenced on an Applied Biosystems ABI 3500 capillary sequencer. DNA sequence analysis was performed using the BLAST sequence analysis programs at the National Center for Biotechnology Information (NCBI).

Development of SCAR markers

Four clear and reproducible RAPD bands, putative markers, were selected to develop species-specific SCAR markers. Based on the sequenced RAPD fragment, the nucleotide sequence of each of the cloned RAPD fragments was used to design pairs of SCAR primers. The SCAR primer pairs were used for PCR amplification of genomic DNA from the six samples (four species, Table 1). The reaction mixture contained 50 ng plant DNA, 0.4 mM dNTP mix, and 1.25 U Taq DNA polymerase in a final volume to 25 µL .Thermal cycling conditions for amplification using SCAR primers were optimized as follows: 94°C for 5 min, 35 cycles at 94°C for 30 s, at 60°C for 40 s, and at 72°C for 2 min, and a final extension at 72°C for 5 min. Homology searches were performed using GenBank's non-redundant Viridiplantae database at http://www.ncbi.nlm.nih.gov.

RESULTS

Isolation of genomic DNA from different organs

High-quality genomic DNA was isolated from the fresh leaves as well as dried tuber samples. An absorbance (OD260/OD280) ratio of 1.8-2.0 indicated that the quality of extracted DNA met the requirements of subsequent experiments. Extracted DNA samples from the root and fresh leaves for contrastive experiments were analyzed at the same time (data not shown). Regarding RAPD, no obvious difference was found between the two types of

template DNA by electrophoresis. The quantity and quality of DNA showed that both of these DNA extraction methods were suitable for RAPD PCR amplification.

Identification of species-specific RAPD markers in *Liriope*

Of the 40 RAPD primers screened, 23 produced clear, reproducible, and polymorphic profiles. The approximate size range of the RAPD products was 200 bp to 2.5 kbp. Reproducibility of the amplification pattern was checked by repeating each reaction twice under the same conditions. Although a number of species-diagnostic RAPD bands were noted, most of them were either rather faint or not repeatedly found in all of the representative individuals of the four species. Thus, some potentially species-specific, clear RAPD bands were eliminated from consideration. In contrast, the electrophoresis results showed that primer OPA-06 amplified a single, bright band of approximately 460 bp from *O. japonicus* but not from other three species (Figure 1). Similarly, the primer OPA-08 amplified a distinct, reproducible band from two species of *Ophiopogon*, but not from the two *Liriop*e species (Figure 2). The primer OPA-12 produced a 485-bp band only in *L. platyphylla* (Figure 3). The primer OPB-05 amplified a distinct band from all *Liriope* samples and from *O. japonicus* (No. 5, China) but not from *O. japonicus* (No. 4) or *O. jaburan* (No. 6) (Figure 4). These four bands, produced by OPA-06, OPA-08, OPA-12, and OPB-05, were selected as putative species-specific markers.

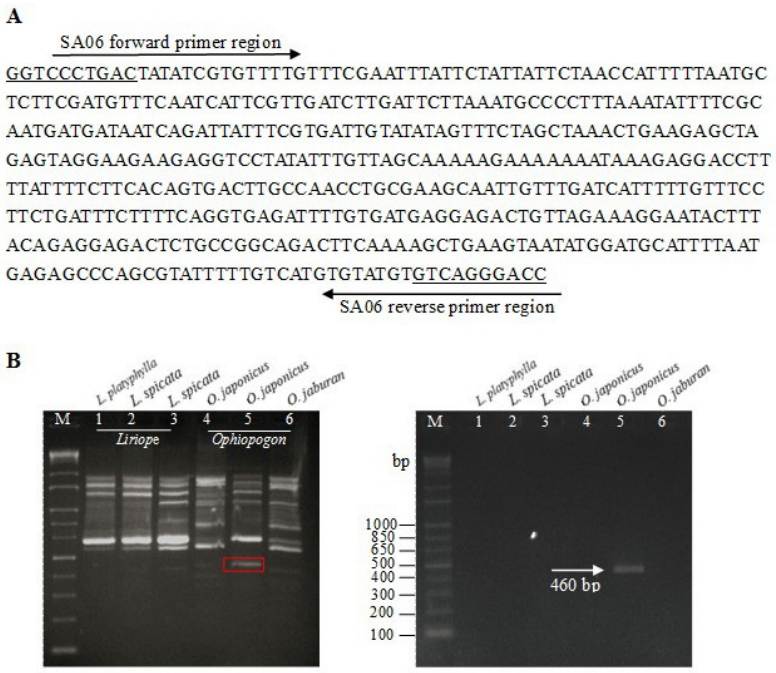

Figure 1. Nucleotide sequence of an *Ophiopogon japonicus*-specific DNA band using the OPA-06 primer (**A**). Agarose gel electropherogram of six accessions of *Liriope* and *Ophiopogon* after RAPD analysis with OPA-06. The results confirm the primer specificity for the SA06 marker (**B**). The numbers (1-6) correspond to the lane numbers in Table 1. The precise size of the SA06 marker is indicated on the left of the panel and with an arrow. *Lane M* represents a 2-kb DNA ladder.

Conversion of RAPD markers into SCAR markers based on cloning and sequencing of the four species-specific markers

Polymorphic DNA fragments produced by the RAPD primers were cloned and sequenced for conversion into SCAR markers. Based on the sequences of the cloned RAPD fragments, several SCAR primer sets were designed and tested. Among them, four primer sets successfully generated the anticipated polymorphism.

The four putative species-specific RAPD markers were successfully cloned and sequenced. The sizes of the inserted DNA fragments were confirmed by PCR analysis. The first 10 nucleotides of the sequences completely matched the corresponding RAPD primers used.

These four primers were easily visualized by electrophoresis, and the amplification showed a dominant presence/absence pattern. As shown in Figure 1, the SA06 primer amplified a 460-bp fragment only from *O. japonicus*. The SA08 primer generated a 441-bp fragment only from the genus *Ophiopogon* (lanes 4-6; Figure 2). The SA12 primer generated a 485-bp fragment only from *L. platyphylla* (Figure 3). Finally, SB05 amplified fragments from all *Liriope* species (lanes 1-3) and *O. japonicus* (lane 5) (Figure 4).

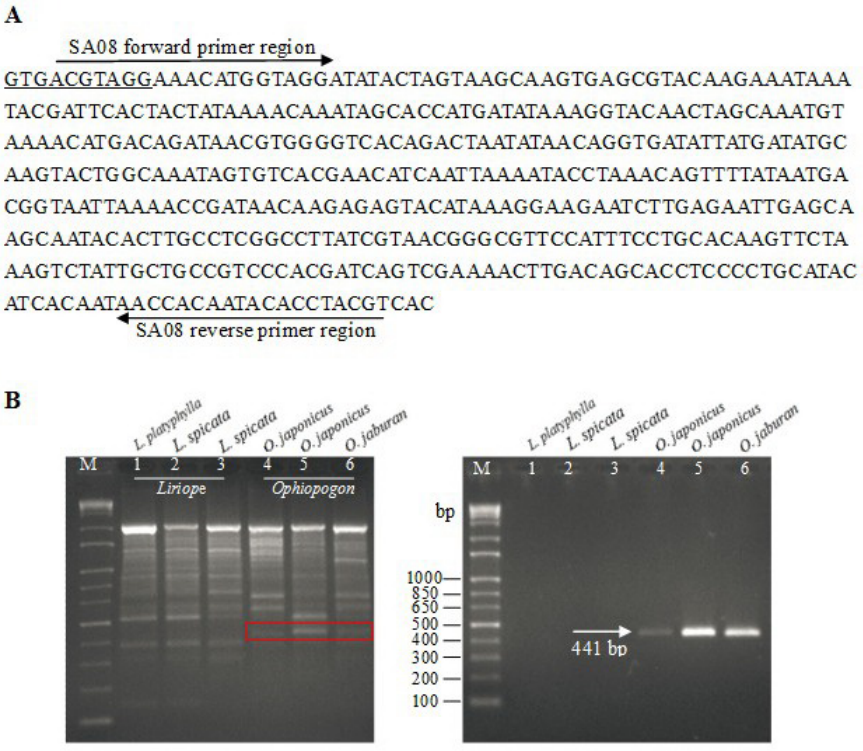

Figure 2. Nucleotide sequence of an *Ophiopogon* DNA band with the OPA-08 primer (**A**). Agarose gel electropherogram of six accessions of *Liriope* and *Ophiopogon* after RAPD analysis with OPA-08. The results confirm the primer specificity for the SA08 marker (**B**). The numbers (1-6) correspond to the lane numbers in Table 1. The precise size of the SA08 marker is indicated on the left of the panel and with an arrow. *Lane M* represents a 2-kb DNA ladder.

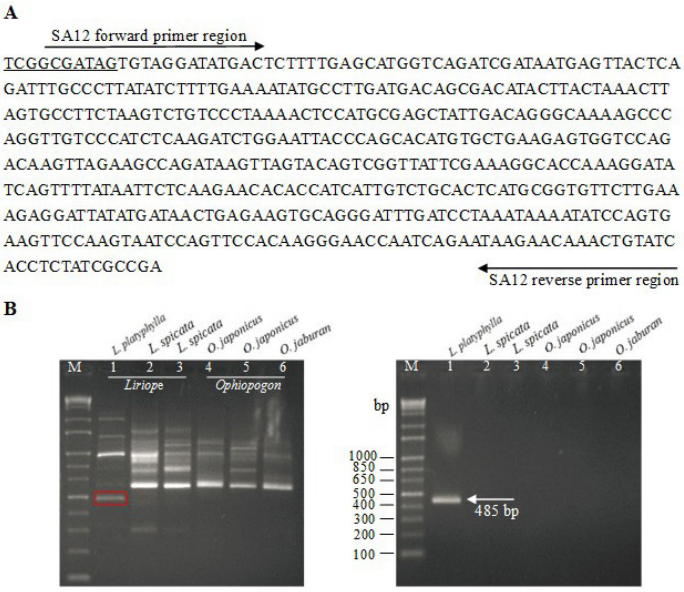

Figure 3. Nucleotide sequence of a *Liriope platyphylla*-specific DNA band using the OPA-12 primer (**A**). Agarose gel electropherogram of six accessions of *Liriope* and *Ophiopogon* after RAPD analysis with OPA-12. The results confirm the primer specificity for the SA12 marker (**B**). The numbers (1-6) correspond to the lane numbers in Table 1. The precise size of the SA12 marker is indicated on the left of the panel and with an arrow. *Lane M* represents a 2-kb DNA ladder.

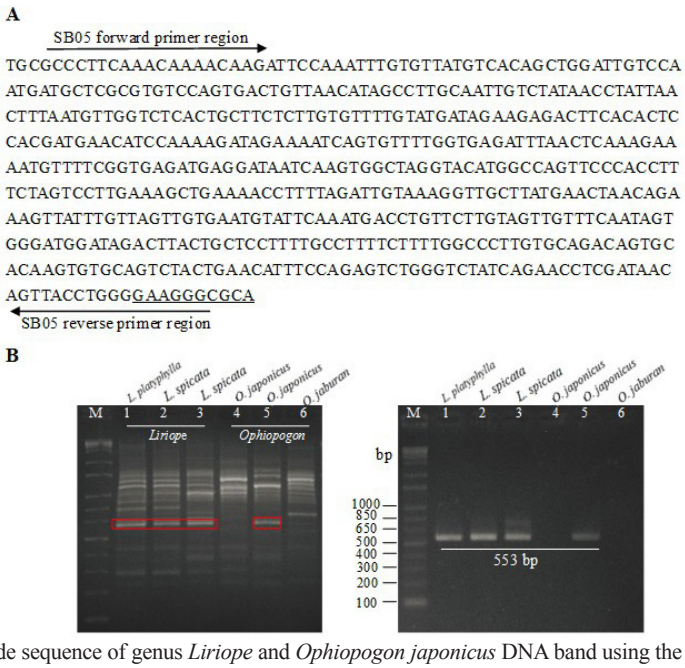

Figure 4. Nucleotide sequence of genus *Liriope* and *Ophiopogon japonicus* DNA band using the OPB-05 primer (**A**). Agarose gel electropherogram of six accessions of *Liriope* and *Ophiopogon* after RAPD analysis with OPB-05. The results confirm the primer specificity for the SB05 marker (**B**). The numbers (1-6) correspond to the lane numbers in Table 1. The precise size of the SA06 marker is indicated on the left of the panel. *Lane M* represents a 2-kb DNA ladder.

Homology searches were performed within GenBank's non-redundant database at NCBI using the BLASTN program. However, the BLAST search of the OPA-06, OPA-12, and OPB-05 sequences (Figures 1, 2, and 4) using the NCBI nucleotide database showed no significant match with any known nucleotide sequence. Conversely, OPA-08 showed a significant match with cobyrinic acid a,c-diamide synthase (Table 2).

Table 2. Species-specific SCAR primer sequences derived from cloned RAPD fragments and optional annealing temperature (Tm) for each set of reactions.

RAPD primer	SCAR primer	Sequence (5'-3')	Tm (°C)	PCR product (bp)	BLAST	GenBank No.
OPA-06	SA06F	GGTCCCTGACTATATCGTGTT	62	460	Predicted protein	XP_002322179
	SA06R	GGTCCCTGACACATACACATG	64			
OPA-08	SA08F	GTGACGTAGGAAACATGGTAG	62	441	Cobyrinic acid ac-diamide synthase	ZP_06369075.1
	SA08R	GTGACGTAGGTGTATTGTGGT	62			
OPA-12	SA12F	TCGGCGATAGTGTAGGATATG	62	485	Hypothetical protein	XP_002280042.1
	SA12R	TCGGCGATAGAGGTGATACA	60			
OPB-05	SB05F	TGCGCCCTTCAAACAAAACAAG	64	553	Hypothetical protein	CAJ00237.1
	SB05R	TGCGCCCTTCCCCAGGTAAC	66			

The nucleotides underlined represent the sequence of the RAPD primers used.

DISCUSSION

The use of herbs in medicine is growing (Devaiah et al., 2011). Thus, discrimination among genuine species has become a key issue in the standardization of herbal drugs. According to the World Health Organization's general guidelines on methods of research and evaluation of traditional medicines, the first step in assuring the quality, safety, and efficacy of herbal medicines is the correct identification of constituent species (Anonymous, 2010). Molecular marker technology has proven to be a valuable tool for identifying adulterants and substitutions in herbal medicines (Devaiah and Venkatasubramanian, 2008).

In the present investigation, we used SCAR markers from sequenced, species-specific RAPD bands to overcome the reproducibility problem associated with the RAPD technique, in attempt to discriminate different species of *Liriope* and *Ophiopogon* objectively. Because *Liriope* herbs are used in the dried root form, species-specific primers are needed. The high-quality genomic DNA extracted from different parts of the plant also proved to be considerably effective.

Using 40 random decanucleotide primers, several RAPD markers specific to different species were recognized. However, only clear, strong, and reproducible RAPD bands of appropriate size, from two repeated PCRs for each primer were selected for cloning and sequencing. Based on sequence analysis of the specific RAPD amplicons, we prepared primers and tested their specific application. Finally, as shown in Figures 1-4, four SCAR primers designed from four specific RAPD sequences for different species were obtained. In our RAPD analysis, significant polymorphisms were observed among *Liriope* and *Ophiopogon*. The SA08 primer generated a 441-bp fragment only from the genus *Ophiopogon* (lanes 4-6), indicating that it can be used to discriminate *Ophiopogon* from the closely related genus *Liriope*. In addition, this marker, which binds to cobyrinic acid a,c-diamide synthase, involved in the cobalamin biosynthetic process (Raux et al., 1998), would be valuable for marker-assisted selection. The SB05 primer generated a 553-bp fragment from the genus *Liriope* and *O. japonicus* (lane 5),

showing that it can be used to discriminate among *Liriope* as well as between *O. jaburan* and *O. japonicus* (Figure 4). Finally, the SA06 marker and SA12 markers were found to be specific for *O. japonicus* and *L. phatyphylla*, respectively.

Some RAPD primers showed different patterns in the same species. For example, SA06 produced a 460-bp fragment from *O. japonicus* (No. 5, from China) but not from *O. japonicus* (No. 4, from RDA, Korea). These differences were probably caused by individual variation and/or artificial multi-generations of selection.

Species-specific marker development for plant taxa require reliable, reproducible, amplified genomic sequences, particularly when they are to be used in germplasm patent disclosures or in legal issues regarding varietal infringement (Das et al., 2005). PCR-based identification is particularly useful when botanical identification based on morphology is difficult, such as when samples are incomplete, damaged, and/or dried (Ray and Roy, 2009). Choo et al. (2009) used multiplex-PCR, which is rapid and highly specific for DNA of closely related species, to successfully amplify specific markers from mixed DNA samples. Such efficient, precise and sensitive techniques are required for rapid identification of variants at the inter- and/or intraspecies level (Weder, 2002).

The SCAR markers reported here are the first for Korean *Liriope* cultivars. They proved to be reliable and efficient for distinguishing closely related species. Moreover, this technique offers rapid identification at low cost, among other attributes. The markers reported here will be useful for the identification of Korean *Liriope* cultivars and the management of herbal medicines, and also in similar studies in the future.

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