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RAPD 분석과 multiplex-PCR을 이용한 석창포 감별용 SCAR 마커 개발

문병철* · 지윤의* · 이영미* · 천진미* · 이아영* · 추병길** · 김호경*†
*한국한의학연구원 한약자원연구센터, **전북대학교 농업생명과학대학

Development of SCAR Markers for the Authentication of Acori Rhizoma Based on the Analysis of RAPD and Multiplex-PCR

Byeong Cheol Moon*, Yunui Ji*, Young Mi Lee*, Jin Mi Chun*, A Yeong Lee*, Byung Kil Choo and Ho Kyoung Kim*†**

**Center of Herbal Resources Research, Korea Institute of Oriental Medicine, Daejeon 305-811, Korea.*
***College of Agriculture and Life Science, Chonbuk National University, Jeonju 561-756, Korea.*

ABSTRACT : The rhizomes and herbal medicines originating from *Acorus gramineus*, *A. calamus*, *A. tatarinowii*, and *A. gramineus* var. *pusilus*, show significant similarity, and the correct identification of species is very difficult. Random Amplified Polymorphic DNA (RAPD) and Sequence Characterized Amplified Region (SCAR) were used to develop a reliable method for identification of these four species. Several distinct SCAR markers were developed from species-specific RAPD amplicons for each species. Furthermore, a useful molecular marker was established for multiplex-PCR, in order to the four species could be distinguished concurrently. These markers allow efficient and rapid identification of closely-related *Acorus* species and will be useful for standardization of herbal medicines.

Key Words : *Acorus* Species, Random Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplification Region (SCAR), Molecular Marker, Multiplex-PCR

INTRODUCTION

Traditional means of species authentication rely on observation of morphological features such as shape, color, texture and odor of organs, as well as histological characteristics. However, these features are greatly influenced by environmental conditions and identification rely, to a great extent, mainly on the experience level of the individual performing the assessment (World Health Organization, 2005; Kalpana *et al*., 2004; Zhang *et al*., 2007). Medicinal materials that originate from members of the genus *Acorus*, family of Araceae, including *Acorus gramineus* $S_{OLAND.}$, *A. calamus* L., *A. tatarinowii* $S_{CHOTT.}$, and *A. gramineus* $S_{OLAND.}$ var. *pusilus* $E_{NGL.}$, represent a good example of the complications that can arise in herbal medicine. The aerial parts and rhizomes of these plants show similar morphological characteristics and the national pharmacopoeia differently prescribes the original plant species of the herbal medicine in Asian traditional medicines. In Korea and Japan, traditional medicines that contain dried rhizomes of *A. gramineus* are used for medicinal purposes, while *A. calamus* is found mostly as a component of bath liquid (Sugimoto *et al*., 1999; Korea Food & Administration, 2009; Ministry of Health, Labour and Welfare, 2011). By contrast, the Indian Ayurvedic system uses *A. calamus* rhizomes in medicinal materials and both *A. calamus* and *A. tatarinowii* are employed for clinical purposes in traditional Chinese medicines (Dan *et al*., 2004; China Pharmacopoeia Committee, 2010). The presence of rhizome material from the *A. gramineus* subspecies, *A. gramineus* var. *pusillus*, has sometimes adulterated these herbal medicines. Unfortunately, due to morphological similarities in the rhizomes and few visual differences in the processed herbal drugs, it is very difficult to discriminate correctly between *A. gramineus*, *A. tatarinowii, A. calamus* and *A. gramineus* var. *pusilus*.

Molecular biological tools, such as DNA sequencing and DNA fingerprinting, have been used for plant phylogenetic studies and species identification in herbal medicines (Zhang *et al*., 2007; Techen *et al*., 2004; Welsh and McClelland, 1991; Fushimi *et al*.,

†Corresponding author: (Phone) +82-42-868-9502 (E-mail) hkkim@kiom.re.kr
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1997; Vos *et al*., 1995; Kang *et al*., 2002; In *et al*., 2005). In particular, the development of polymerase chain reaction (PCR) has enabled medicinal plant materials to be distinguished at inter- and/or intra-species levels via random amplified polymorphic DNA (RAPD) of genetic polymorphisms (Zhang *et al*., 2007; Williams *et al*., 1990; Shucher and Carles, 2008; Weder, 2002). RAPD has many advantages, since it is fast and inexpensive, and only requires a minute amount of plant material; however, it does need high levels of polymorphism and can be less reproducible than other methods (Penner *et al*., 1993). To develop specific, accurate and reproducible authentication markers, sequence characterized amplified region (SCAR) marker analysis has been developed and employed in various plant genera (Paran and Michelmore, 1993; Shucher and Carles, 2008; Bang *et al*., 2004; Park *et al*., 2007; Kiran *et al*., 2010). This method has improved the precise discrimination and is substantially more effective than subjective methods based on morphological features or analytical methods using chromatographic techniques and marker compounds. SCAR markers have been used for the authentication of medicinal plant species in *Panax*, *Angelica*, *Phyllanthus*, *Artemisia* and other important herbal medicines (Wang *et al*., 2001; Choo *et al*., 2009; Dnyaneshwar *et al*., 2006; Lee *et al*., 2008; Moon *et al*., 2010; Lim *et al*., 2007). In previous research, the phylogenetic relationship between *A. gramineus* and chemotypes of *A. calamus*, was analyzed by the comparing the 5S-rRNA region (Sugimoto *et al*., 1999; Bertea *et al*., 2005). The correlation between the β-asarone content and ploidy level was determined using a RAPD marker for *A. calamus* (Ahlawat *et al*., 2010). However, there are no precise identification tools for distinguishing *Acorus* plants and herbal materials at the species level.

In this study, the RAPD patterns of four medicinally-important *Acorus* plant species, *A. gramineus*, *A. tatarinowii*, *A. calamus* and *A. gramineus* var. *pusillus*, were analyzed and several SCAR markers were developed to distinguish each species by the comparative sequence analysis of species-specific RAPD amplicons. In addition, a combination of SCAR marker primers allowed simultaneous discrimination of these four species by a single multiplex-PCR. These tools will be useful for distinguishing the four *Acorus* plants species and elucidating authentic herbal materials from inappropriate substitutes and adulterants.

MATERIALS AND METHODS

1. Plant materials

Sixteen *Acorus* genotypes were used from *A. gramineus, A. tatarinowii, A. calamus* and *A. gramineus* var. *pusillus,* with four representative samples of each (Table 1). These samples were collected from different native habitats in Korea and China. Species identification was performed by the 'Classification and Identification Committee of the Korea Institute of Oriental Medicine (KIOM)', which comprises nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. Fourteen additional *Acorus* plant samples were collected at the Jeju Agricultural Research and Extension Service Center (Jeju

Table 1. Plant materials and specimen information.

Scientific name	Herbal Name	Source	Date of collection	Lane in gel
Acorus gramineus Soland.	Acori Gramineri Rhizoma	Hallim, Jeju, Korea	09. 4. 14	1
		Hwabuk, Jeju, Korea	09. 4. 15	2
		Hawon, Seogwipo, Korea	09. 4. 15	3
		Chengdu, Sichuan, China	09. 9. 12	4
Acorus tatarinowii Schott.	Acori Tatarinowii Rhizoma	Wuhan, Hubei, China	08. 8. 13	5
		Chengdu, Sichuan, China	09. 9. 12	6
			09. 9. 12	7
		Chengdu, Sichuan, China	09. 9. 16	8
Acorus calamus L.	Acori Calami Rhizoma	Anui, Gyeongnam, Korea	09. 6. 26	9
			09. 6. 26	10
		Gosan, Chunbuk, Korea	09. 6. 12	11
		Daea Arboretum, Chunbuk, Korea	08. 5. 15	12
Acorus gramineus Soland. var. *pusillus* Engl.	–[†]	Gwanpo, Chungnam, Korea	09. 9. 23	13
			09. 9. 23	14
		Hallim, Jeju, Korea	09. 4. 14	15
		Chengdu, Sichuan, China	09. 9. 12	16

[†]There is no appropriate name.

ARESC, Korea). Three commercial herbal medicines, one Acori Calami Rhizoma and two Acori Gramineri Rhizoma, were purchased from herbal markets. All plant materials were given accession numbers and specimens were preserved in the Herbarium of the Korea Institute of Oriental Medicine (Table 1).

2. Preparation of genomic DNA

Genomic DNA was extracted from fresh leaves and herbal medicines using DNeasy® Plant Mini Kits (QIAGEN, CA, USA), according to the manufacturer's protocol. DNA concentrations and purities were determined by spectrophotometer (Nanodrop ND-1000, Nanodrop, DE, USA) and 1.5% agarose gel electrophoresis with known standards. For PCR amplification, the final concentration of each DNA sample was approximately 20 ng/㎖ in TE buffer.

3. Analysis of RAPD and nucleotide sequences

Thirty four 10-mer (Operon Technologies Inc., CA, USA) and six 20-mer RAPD primers (Seolin Bioscience, Seoul, Korea) were used to analyze genomic profiles and to obtain species-specific RAPD amplicons. PCR reactions were carried out in 30 ㎕ reaction mixtures containing 10 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 200 mM each dNTP, 10 mM $(NH_4)_2SO_4$, 0.5 U *Taq* DNA polymerase (Solgent, Korea), 30 pmol primer and 10~20 ng template DNA. DNA amplification was performed on a DNA Engine Dyad® PTC-0220 (Bio-Rad, CA, USA). The parameters for RAPD analysis were 95℃ for 5 min, followed by 35 cycles of 30 s at 95℃, 1 min at 42℃, and 2 min at 72℃, and a final extension for 10 min at 72℃. The amplification products were separated on 1.5% agarose gels with a 1 kb plus DNA ladder (Solgent, Daejeon, Korea) and visualized with ethidium bromide (EtBr) staining under ultraviolet (UV) light.

Species-specific amplicons were recovered from agarose gels with a Gel Extraction Kit (Solgent, Daejeon, Korea) and subcloned into the pGEM-T Easy vector (Promega, WI, USA). Nucleotide sequences of the inserts were determined from both strands via dideoxynucleotide chain termination using an automatic DNA sequence analyzer (ABI3730, Applied Biosystems Inc., CA, USA). The nucleotide sequences of the six amplicons used to develop SCAR markers were registered in the NCBI GenBank dbGSS (accession nos. CPA15-1, GS927766; CPA18-1, GS927767; CPD2-1, GS927768; CPD2-2, GS927769; CPE3-1, GS927770; and CPE6-1, GS927771; http://www.ncbi.nlm.nih.gov/dbGSS).

4. Development of SCAR markers

Specific primers were designed from the sequences of corresponding polymorphic RAPD amplicons using PRIMER BLAST in the GenBank database (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The melting temperatures, GC contents and secondary structures of each primer were verified using CyberGene AB primer design tools (http://www.cybergene.se, Stockholm, Sweden). The confirmation of amplification specificity for each of the primer pairs was performed in the same reaction mixture as described above, except that 20 pmol of each primer and 20 ng template were used. The amplification conditions were: pre-denaturation at 95℃ for 5 min, followed by 30 cycles of 95℃ for 1 min, 53℃ for 30 s and 72℃ for 2 min, and a final extension at 72℃ for 5 min (Choo *et al*., 2009). Four forward and four reverse SCAR primers were used for multiplex-PCR and optimal conditions were determined by altering the following parameters: the annealing time (20~60 s), annealing temperature (51~61℃), number of PCR cycles (23~38 cycles), amounts of primers (5~20 pmol), and combination of SCAR primers.

RESULTS

1. RAPD analysis of genomic profile

The RAPD fingerprinting method was employed to analyze genomic polymorphic profiles and to find species-specific sequences for *A. gramineus*, *A. tatarinowii*, *A. calamus* and *A. gramineus* var. *pusillus*. To control for individual and geographical variation, four samples of each species were analyzed and these were collected from different habitats. Forty random primers were used and 30 of these produced distinct and reproducible amplification profiles. Twenty-seven primers revealed diverse polymorphic DNA fragments, which were sufficient for differentiating the four *Acorus* species. The polymorphic DNA fragments varied in size and number, with 1~6 amplicons ranging in length between 260~2,000 bp (Fig. 1). These polymorphic amplicons were observed in all of the species and were most abundant in *A. calamus* (Fig. 1). These results suggest that the individual or combined distinct polymorphic amplicons among the different *Acorus* species can be used as molecular markers to identify each species.

2. Development of SCAR markers for the identification of *Acorus* species

Twenty five species-specific RAPD amplicons, five from *A. gramineus*, seven from *A. tatarinowii*, eight from *A. calamus*,

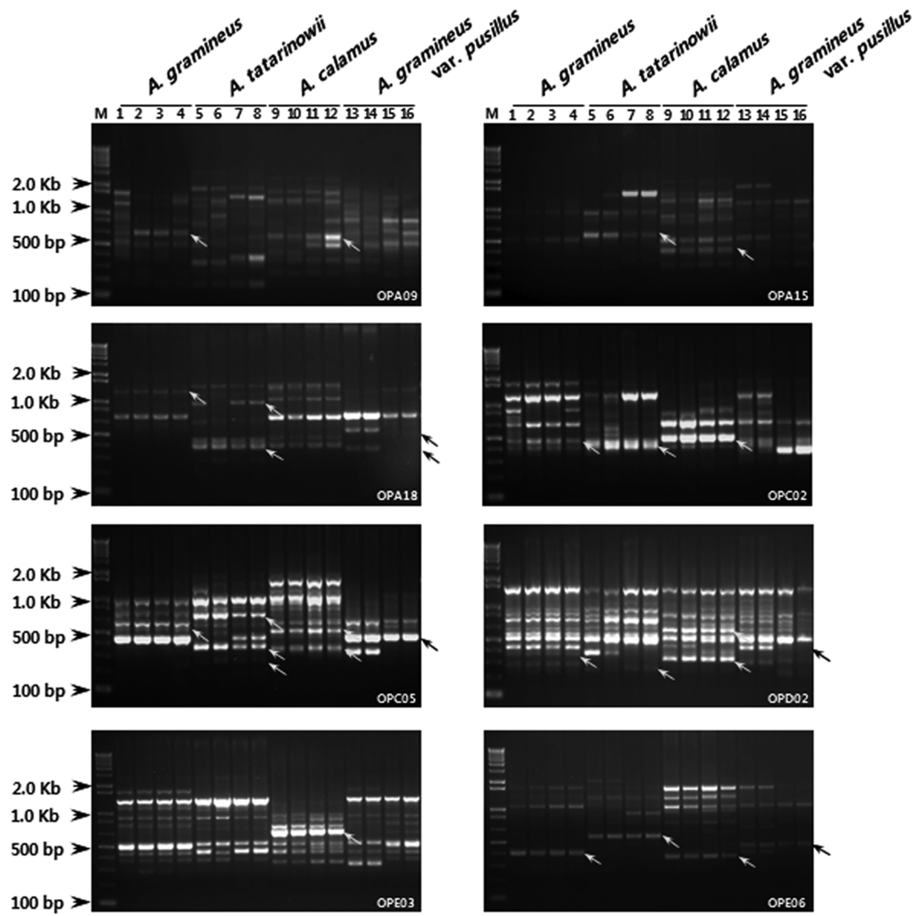

Fig. 1. RAPD profiles of *A. gramineus*, *A. tatarinowii*, *A. calamus* and *A. gramineus* var. *pusillus* using Operon 10-mer random primers. Arrows indicate species-specific RAPD amplicons. Lanes 1∼16 correspond to Table 1. M represents a 100 bp DNA ladder.

Table 2. Species-specific amplicons and primers used to develop SCAR markers in this work.

RAPD Primer	Polymorphic RAPD amplicons			NCBI dbGSS Accession No.	SCAR primer		
	Name	Specificity	Size		Name	Nucleotide sequences (5′-3′)	SCAR size
OPA15	CPA15-1	*A. tatarinowii*	578	GS927766	CPA15-1 F1	TCCCTAAAGATGCCACGTCTAACC	309 bp
					CPA15-1 R2	ACGGGTTGAGGTTGAGTCAGGT	
					CPA15-1 F4	GTGGGTCCCACTACCTTTTGC	240 bp
					CPA15-1 R2	ACGGGTTGAGGTTGAGTCAGGT	
OPA18	CPA18-1	*A. gramineus var.pusillus*	541	GS927767	CPA18-1 F3	TGTAAAGGCGAGCCCAAAGG	199 bp
					CPA18-1 R4	TTGAGAGTCAATACCTCCCC	
OPD2	CPD2-1	*A. gramineus*	351	GS927768	CPD2-1 F1	ACCAACCTGCTACAACTTGGGG	274 bp
					CPD2-1 R5	ACGCTTGGGCTCCTTGATTA	
					CPD2-1 F3	GTATTATATGCTTTCACA	145 bp
					CPD2-1 R5	ACGCTTGGGCTCCTTGATTA	
	CPD2-2	*A. calamus*	621	GS927769	CPD2-2 F3	GTCACCCAGCAATTAAGGCAG	173 bp
					CPD2-2 R3	TCACTATGGTTGTGTCATGGG	
OPE3	CPE3-1	*A. calamus*	656	GS927770	CPE3-1 F1	AAACACACAGTCACACCAATCAGACTT	271 bp
					CPE3-1 R1	GCAAACAAGGAAGTGACAACGC	
OPE6	CPE6-1	*A. calamus*	362	GS927771	CPE6-1 F2	TCCGCACAGAGGACCTTTCACC	222 bp
					CPE6-1 R2	CCTCATGTGGGTGCAGTCTGA	

and five from *A. gramineus* var. *pusillus*, were recovered from agarose gels and then sequenced (Fig. 1). Candidate SCAR primers were designed from the resulting nucleotide sequences. Primer specificity and utility for detection or differentiation of each *Acorus* species, was then confirmed.

To obtain species-specific SCAR primers for identifying *A. gramineus*, five pairs of oligonucleotides were prepared using the 351 bp nucleotide sequence of CPD2-1, which was amplified *A. graminus*-specific RAPD amplicon in OPD2 primer, and the specificity of each primer pair was confirmed with the four *Acorus* species (Table 2 and Fig. 2A). In all four of the *A. gramineus* samples, the CPD2-1 F1/R5 and F3/R5 primer pairs produced single distinct DNA fragments at the expected sizes of 274 and 145 bp, respectively (Fig. 2A). No non-specific PCR products were observed in the other three *Acorus* species (Table 2 and Fig. 2A). These results indicate that these two primer pairs are suitable for identification of *A. gramineus* and for distinguishing it from *A. tatarinowii*, *A. calamus*, and *A. gramineus* var. *pusillus*.

A. tatarinowii must be clearly distinguished from other *Acorus* species because it is prescribed separately from *A. calamus* only in the Chinese pharmacopoeia (China Pharmacopoeia Committee, 2010). Therefore, a SCAR marker was developed for distinguishing *A. tatarinowii* from other species. Based on the 578 bp nucleotide sequence of the *A. tatarinowii*-specific amplicon CPA15-1, four primer pairs were prepared and their specificity was evaluated. Two primer pairs, CPA15-1 F1/R2 and CDP15-1 F4/R2, amplified unique and abundant fragments from *A. tatarinowii* (309 and 240 bp, respectively), but not the other *Acorus* species (Fig. 2B and Table 2).

To discriminate *A. calamus*, three pairs of primers were prepared from nucleotide sequences corresponding to *A. calamus*-specific RAPD amplicons, 621 bp CPD2-2, 656 bp CPE3-1 and 362 bp CPE6-1 (Fig. 1 and Table 2). In authentic *A. calamus*, PCR amplification with the primer pairs CPD2-2 F3/R3, CPE3-1 F1/R1, and CPE6-1 F2/R2 yielded the expected DNA fragments of 173 bp, 271 bp, and 222 bp, respectively, but no amplification was observed in the other species (Fig. 2C).

Among the four pairs of SCAR primers based on the 541 bp nucleotide sequence of the CPA18-1 RAPD amplicon, only the F3/R4 primer pair amplified a unique and abundant 199 bp DNA fragment from *A. gramineus* var. *pusillus* (Fig. 1, Fig. 2D and Table 2).

Taken together, our results indicate that these SCAR markers can be used to distinguish between all four medicinal *Acorus* plants, as well as detecting their presence in medicinal materials. Thus, these markers provide a reliable method for the authentication of materials and identification of substitutes and adulterants.

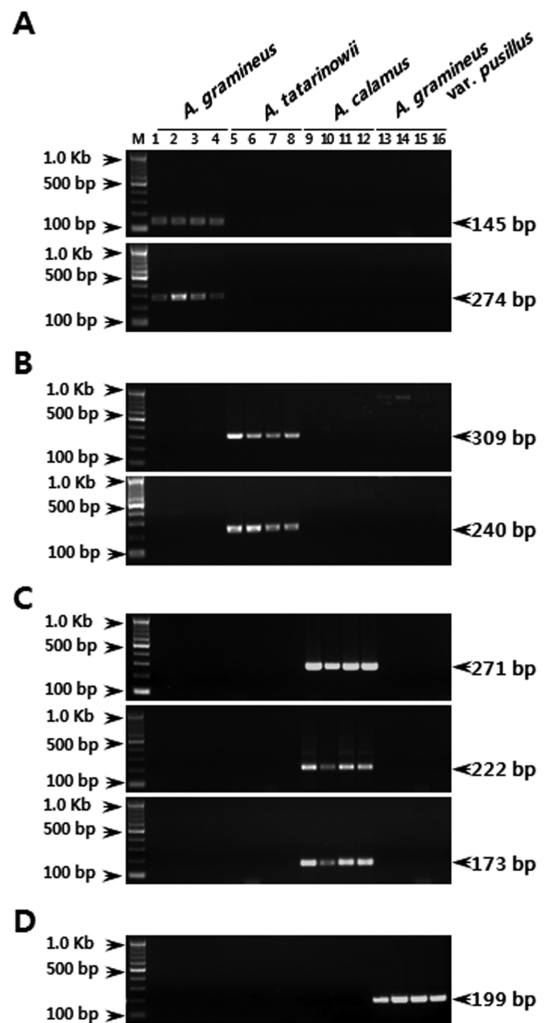

Fig. 2. Development of RAPD-derived SCAR markers for *A. gramineus*, *A. tatarinowii*, *A. calamus* and *A. gramineus* var. *pusillus*. PCR amplification using species-specific SCAR primers originating from the following species-specific amplicons: *A. gramineus*, CPD2-1 (A); *A. tatarinowii*, CPA15-1 (B); *A. calamus*, CPD2-2, CPE3-1 and CPE6-1 (C); and *A. gramineus* var. *pusillus*, CPA18-1 (D). Lanes 1∼16 correspond to Table1. Arrow heads to the right of the panels indicate the precise sizes of the SCAR markers. M represents a 100 bp DNA ladder.

3. Establishment of rapid authentication SCAR markers by multiplex-PCR

Species-specific primers developed in this work were simultaneously used in multiplex-PCR amplification to improve

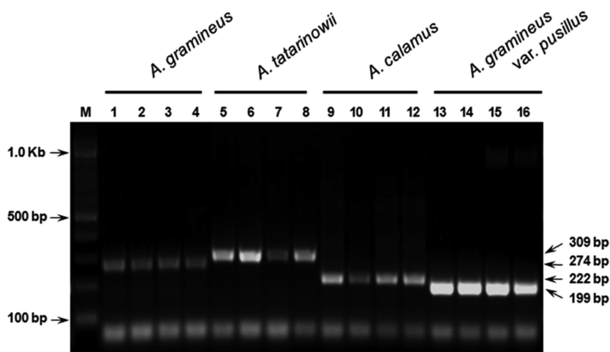

Fig. 3. Amplification of a rapid authentication SCAR marker using a combination of SCAR primers in a single multiplex-PCR. Multiplex-PCR products were generated from a combination of the following SCAR primers: CPD2-1 F1/R5, CPA15-1 F1/R2, CPE6-1 F2/R2, and CPA18-1 F3/R4. Lanes 1~16 correspond to Table 1. Arrowheads to the right of the panels indicate the precise sizes of the SCAR markers. M represents a 100 bp DNA ladder.

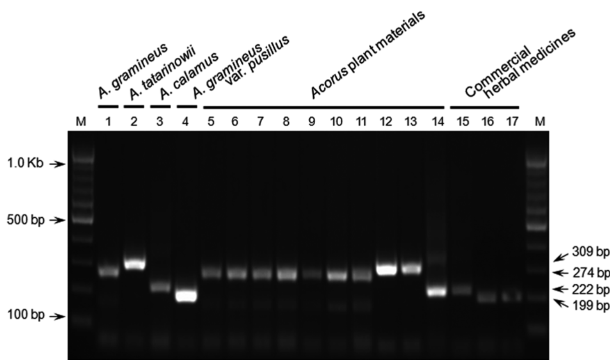

Fig. 4. Verification of rapid authentication SCAR markers using additional *Acorus* medicinal materials. Multiplex-PCR products were generated from the following combination of SCAR primers: CPD2-1 F1/R5, CPA15-1 F1/R2, CPE6-1 F2/R2, and CPA18-1 F3/R4. Lanes 1~4, 5~14 and 15~17 indicate PCR products from control *Acorus* plants, additional *Acorus* plant materials and commercial herbal medicines, respectively. Arrow heads to the right of the panels indicate the expected sizes of amplification products. M represents a 100 bp DNA ladder.

the application of the markers and a multi-species authentication SCAR marker was developed based on the numbers and sizes of PCR products. The amplification with the combination of primer CPD2-1 F1/R5, CPA15-1 F1/R2, CPE6-1 F2/R2, and CPA18-1 F3/R4 yielded four abundant PCR products that showed distinct patterns for every species. Thus, the multiplex-PCR amplification products were identical to those obtained with the specific primer pairs and provided rapid authentication and differentiation of the four *Acorus* plant species in a single reaction (Fig. 3). To evaluate the sensitivity of this method, an additional 17 samples of *Acorus* plant materials and herbal medicines were tested. As shown in Fig. 4, the resulting multiplex-PCR products were sufficient for discriminating between the four plant species and components of herbal medicines. Importantly, two commercial herbal medicines that purported to be Acori Gramineri Rhizoma, were shown to be dried rhizomes of *A. gramineus* var. *pusillus* (Fig. 4; lanes 16 and 17). These results demonstrate that concurrent multi-species authentication SCAR markers are effective for identifying species and standardizing traditional herbal medicines containing material from *Acorus* plants.

DISCUSSION

Environmental conditions and subjective evaluation of morphological characteristics influence the identification of plant species. Thus, it is necessary to develop reliable tools for accurate species identification and for the standardization of herbal medicines. DNA analysis is one of the most reliable methods for identifying plant species, since the genetic composition is unique to each individual species, irrespective of the physical form of the sample, and DNA is less affected than morphology by age, physiological conditions, environmental factors, harvest, storage and processing of the samples (Ma *et al*., 2002; de Feo *et al*., 2003; de Oliveira *et al*., 1996; Chnag *et al*., 2006).

Initially, internal transcribed spacer sequences (ITS) were examined to find suitable markers for discrimination between the four *Acorus* species (data not shown); however, the marker nucleotides reported as in Rhei Rhizoma were not identified (Yang *et al*., 2004). RAPD was used to obtain potential species-specific nucleotide sequences, since it allows identification of species level differences without requiring prior knowledge of the DNA sequence, and it can be used to screen large numbers of loci in a short period of time (Yip *et al*., 2007). RAPD patterns indicated the following order of homology between *A. gramineus* and the other *Acorus* species: *A. gramineus* var. *pusillus*, *A. tatarinowii*, and *A. calamus* (Fig. 1). This result provides indirect evidence that *A. gramineus* var. *pusillus* belongs as a subspecies with *A. gramineus*. By contrast, some of the RAPD products showed different patterns within the same species and several PCR products were detected in only one or two samples of a species (Fig. 1). In *A. tatarinowii*, the OPA15 primer yielded a 1.2 kb PCR product and the OPC5 primer amplified a 450 bp PCR product (lanes 7 and 8), but these products were not observed in lanes 5 and 6. However, the abnormal products were

observed more frequently in *A. gramineus* var. *pusillus* (Fig. 1). Such differences were likely caused by individual variations at the genomic level, including base substitutions within the binding sequences for the RAPD primers (Williams *et al*., 1990; Penner *et al*., 1993). Technical factors, such as comparatively short RAPD primers and high annealing temperatures could also have caused these unexpected polymorphisms (Penner *et al*., 1993). To overcome such variability, RAPD fragments were generated from 30 random 10-mer primers and 25 potential species-specific PCR products were collected. Furthermore, more than each five species-specific RAPD amplicons were analyzed and nucleotide sequences were determined for fragments amplified in all samples of the same species (Fig. 1). This RAPD analysis made it possible to identify candidate SCAR markers for distinguishing each species.

In developing SCAR markers, six of the 25 amplicons generated clear species-specific PCR products (Fig. 2). Thus, eight pairs of SCAR primers amplified single PCR products and these were suitable for discrimination between the *Acorus* species (Table 2, Fig. 2). However, fourteen of the other primer pairs generated from the six amplicons mentioned in Table 2, and 58 primer pairs prepared from the remaining 19 amplicons, were not species-specific and amplified one or two abundant DNA fragments in at least two species (data not shown). In verifying specificity of the SCAR primers, most of the *A. gramineus*-specific primers amplified the same PCR products in both *A. gramineus* and *A. gramineus* var. *pusillus* (data not shown). The polymorphic RAPD patterns also demonstrated a high degree of homology between the genomic DNA sequences of all four *Acorus* species, with the greatest homology between *A. gramineus* and *A. gramineus* var. *pusillus*, as stated above.

Through comprehensive analysis of the eight SCAR markers, four SCAR markers were identified that could discriminate between *A. gramineus*, *A. tatarinowii*, *A. calamus* and *A. gramineus* var. *pusillus*, when combined into a single multiplex-PCR. The amplification reaction conditions were optimized, as previously described (Choo *et al*., 2009). As shown in Fig. 3, the multiplex-PCR products were amplified at the expected sizes, showing analytical accuracy and stability. An additional 17 plants and medicinal samples were examined to determine the wider applicability of the multiplex-PCR method (Fig. 4). These results indicate that the multi-species authentication SCAR markers established in this work are able to authenticate each species from other closely-related Acorus species in both plant materials and herbal medicines.

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