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ORIGINAL ARTICLE

Rapid molecular authentication of three medicinal plant species, *Cynanchum wilfordii*, *Cynanchum auriculatum*, and *Polygonum multiflorum* (*Fallopia multiflorum*), by the development of RAPD-derived SCAR markers and multiplex-PCR

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Abstract Definitive identification of original plant species is important for standardizing herbal medicine. The herbal medicines Cynanchi Wilfordii Radix (*Baekshuoh* in Korean and *Beishuwu* in Chinese) and Polygoni Multiflori Radix (*Hashuoh* in Korean and *Heshuwu* in Chinese) are often misidentified in the Korean herbal market due to morphological similarities and similar names. Therefore, we developed a reliable molecular marker for the identification of Cynanchi Wilfordii Radix and Polygoni Multiflori Radix. We used random amplified polymorphic DNA (RAPD) analysis of three plant species, *Polygoni multiflorum*, *Cynanchum wilfordii*, and *Cynanchum auriculatum*, to obtain several species-specific RAPD amplicons. From nucleotide sequences of these RAPD amplicons, we developed six sequence characterized amplification region (SCAR) markers for distinguishing Polygoni Multiflori Radix and Cynanchi Wilfordii Radix. Furthermore, we established SCAR markers for the simultaneous discrimination of the three species within a single reaction by using multiplex-PCR. These SCAR markers can be used for efficient and rapid authentication of these closely related species, and will be useful for preventing the distribution of adulterants.

Keywords Random amplified polymorphic DNA (RAPD) · Sequence characterized amplification region (SCAR) · *Polygoni multiflorum* · *Cynanchum wilfordii* · *Cynanchum auriculatum* · Molecular authentication

Introduction

Cynanchi Wilfordii Radix (*Baekshuoh* in Korean and *Beishuwu* in Chinese) and Polygoni Multiflori Radix (*Hashuoh* in Korean and *Heshuwu* in Chinese) are important herbal drugs in oriental medicine, and are widely distributed in Korea, Japan, and China. However, these two Radixes are some of the most indiscriminately used herbal medicines because of their morphological similarity and similar names (Song et al. 2004). According to the record of Zhong Hua Ben Cao, *Baishouwu* is actually an appellative name for the root tubers of *Cynanchum wilfordii* and *Cynanchum auriculatum* from the Asclepiadaceae family (The Health Department and National Chinese Medicine Management Office 1999). Only the dried root tubers of *C. wilfordii* are prescribed in Korean Pharmacopoeia (Korea Food and Drug Administration 2008). However, *C. auriculatum* has been cultivated and distributed in its place because it grows more rapidly and is more productive than *C. wilfordii*. Unfortunately, *C. wilfordii* and *C. auriculatum* are visually similar plants and result in visually similar herbal drugs.

In addition, Polygoni Multiflori Radix is made from the dried root tubers of *Polygonum multiflorum* (Polygonaceae) in Chinese, Japanese, and Korean Pharmacopoeia (China Pharmacopoeia Committee 2005; Korea Food and Drug Administration 2008; Ministry of Health, Labour and

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Welfare 2006). However, its name, Polygoni Multiflori Radix, is often confused with Cynanchi Wilfordii Radix in the herbal market. In Korea, *Baekshuoh* and *Hashuoh* are distributed as *Baek-Hashuoh* (white *Hashuoh*) and *Jeok-Hashuoh* (red *Hashuoh*), respectively, based on their colors. Thus, *C. wilfordii* and *C. auriculatum* dried root tubers have been indiscriminately used as a substitute and/or a contaminant of Polygoni Multiflori Radix. Likewise, *C. auriculatum* dried root tubers have been misused as a substitute in both Cynanchi Wilfordii and Polygoni Multiflori Radix. Therefore, a technique to differentiate between the three species is needed.

The precise identification of crude drugs is crucial for the standardization of clinical prescriptions and chemical and pharmacological research of herbal medicines. Traditionally, subjective methods based on the morphological features such as shape, color, texture, and odor, were used for the discrimination of herbal medicines. It is often difficult to accurately identify medicinal plants from wild populations, or to differentiate species within the same genus based on this subjective evaluation. Furthermore, the use of chromatographic techniques and marker compounds to standardize herbal medicines is also limited because of variable chemical complexity, which is affected by growth, storage conditions, and harvest times, and variable sources (Joshi et al. 2004; Zhang et al. 2007).

Genetic tools that use hybridization, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for authenticating herbal medicines (Zhang et al. 2007; Shcher and Carles 2008). Recently, RAPD analysis has become one of the most effective methods for estimating genetic diversity in plant populations or cultivars because it can reveal high levels of polymorphism. However, it is less reproducible than other methods (Hosokawa et al. 2000; Agrawal et al. 2007; Shcher and Carles 2008). RAPD also has many advantages, such as its high speed, low cost, and requirement of minute amounts of plant material (Williams et al. 1990; Penner et al. 1993). RAPD analysis has been applied in herbal medicine to discriminate between species in various genera (Shcher and Carles 2008). Paran and Michelmore (1993) used RAPD analysis to develop SCAR markers, a more accurate and reliable technique**.** This technique can be used to develop markers that authenticate herbal medicines by using specific PCR primers derived from RAPD or AFLP fragments. These specific primers result in amplification products from target-containing samples, and can be used to generate unique amplification products from closely-related samples (Wang et al. 2001; Lee et al. 2006). In a previous report (Choo et al. 2009), we established SCAR markers to discriminate between *Angelica decursiva (Peucedanum decursivum), Peucedanum praeruptorum,* and *Anthricus sylvestris*, using this method and multiplex-PCR.

In this study, we analyzed RAPD patterns to develop several reliable SCAR markers that can be used for discrimination of *C. wilfordii, C. auriculatum,* and *P. multiflorum*. Furthermore, we established two rapid molecular authentication markers by combining the primers from each SCAR marker that can be used simultaneously in multiplex-PCR. These genetic markers can identify the plant species in Cynanchi Wilfordii Radix and Polygoni Multiflori Radix, to ultimately prevent indiscriminate distribution and prescription of these herbal medicines.

Materials and methods

Plant materials

Three samples each of *C. wilfordii* and *C. auriculatum*, and two samples of *P. multiflorum,* collected from native habitats and Chung-buk Agricultural Research and Extension Service Center, were used in this study (Table 1). The identification of species was performed by The Classification and Identification Committee of the Korea Institute of Oriental Medicine, composed of nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology. The identification was further confirmed by the comparison of ITS sequences against those in the NCBI GenBank: *P. multiflorum* (accession no. EF016293), *C. wilfordii* (accession no. AY548207), and *C. auriculatum* (accession no. AY548208). All plant materials were given accession numbers and preserved in the Herbarium of the Korea Institute of Oriental Medicine (Table 1).

Preparation of genomic DNA

Genomic DNA was extracted from fresh leaves and stems using the DNeasy® Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. DNA concentration and purity were determined by spectrophotometry (Nanodrop ND-1000; Nanodrop, UK) and electrophoresis in a 1.5% agarose gel with known standards. For PCR amplification, the final concentration of each DNA sample was diluted to approximately 20 ng/μl with TE buffer.

Analysis of RAPD and nucleotide sequences

Sixteen Operon 10-mer RAPD primers, RAPD Kit A (OPA 13–20) and Kit C (OPC 13–20), were used to screen the eight samples of the three medicinal plant species to determine the potential of clear polymorphisms and reproducibility (Operon Technologies, Germany). The PCRs were carried out in 30-μl reaction mixtures containing 10 mM Tris–HCl (pH 9.0), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 10 mM $(NH_4)_2SO_4$, 0.5 U *Taq*

Table 1 Plant materials

Official name	Common name	Scientific name	Specimen no.	Source	Date of collection	Lane in gel
Polygoni Multiflori Radix (*Hashuoh*)[a]	*Jeok-Hashuoh* or *Hashuoh (Red)*	*Polygonum multiflorum*	KIOM200601000080	Bonghwa, Gyeongbuk, Korea	9 May 2006	1
			KIOM200601000081	Jinahn, Chenbuk, Korea	23 May 2006	2
Cynanchi Wilfrodii Radix (*Baekshuoh*)[a]	*Baek-Hashuoh* or *Hashuoh (white)*	*Cynanchum wilfordii*	KIOM200601000192	Sancheong, Gyeongnm, Korea	28 September 2006	3
			KIOM200701000333	Hapcheon, Gyeongnam, Korea	3 July 2003	4
			KIOM200701000490	Cheongwon, Chungbuk, Korea	8 August 2007	5
–[b]	*Baek-Hashuoh* or *Hashuoh (white)*	*Cynanchum auriculatum*	KIOM200702000296	Sancheong, Gyeongnam, Korea	1 June 2007	6
			KIOM200701000494	Cheongwon, Chungbuk, Korea	8 August 2007	7
			KIOM200701000495			8

[a] Official name in Korean Pharmacopoeia

[b] No corresponding name in Korean Pharmacopoeia exists

DNA polymerase (Solgent, Korea), 30 pmole each primer, and 10–20 ng template DNA. DNA amplification was performed on a DNA Engine Dyad® PTC-0220 (Bio-Rad, USA). The parameters for RAPD analysis were 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 1 min at 42°C, 2 min at 72°C, and a final extension for 10 min at 72°C. The amplification products were separated on a 1.5% agarose gel with a 100-bp DNA ladder (Fermentas, Canada) and visualized using ethidium bromide (EtBr) staining.

The species-specific amplicons were extracted from agarose gels with a Gel Extraction Kit (Solgent, Korea) and subcloned into the pGEM-Teasy vector (Promega, USA). The nucleotide sequences of the DNA amplicons were determined from both strands by dideoxynucleotide chain termination using an automatic DNA sequencer (ABI 3730; Applied Biosystems, USA). Six amplicons were used to develop SCAR markers and were registered with the NCBI GenBank dbGSS (accession nos. HC14-2; FI857165, HC18-2; FI857166, HC15-3; FI857167, HC20-4; FI857168, HA13-5; FI857169, HC15-4; FI857170, http://www.ncbi.nlm.nih.gov/dbGSS).

Development of SCAR markers

The primers were prepared from the sequences of corresponding species-specific RAPD amplicons, excluding the 10-mer RAPD primer sequences. The melting temperature, GC contents and secondary structures of each primer were verified using CyberGene AB primer design tools (http://www.cybergene.se, Stockholm, Sweden).

Species-specific amplification and multiplex-PCR were carried out using single species-specific primer pairs, and six combinations of the three forward and three reverse primer pairs. Reactions were carried out as previously reported (Choo et al. 2009) in 30-µl volumes containing 20 pmole of each primer and 20 ng template using 1U *Taq* DNA polymerase. Amplification was performed with the following cycling conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 53°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 5 min.

Results

We used a PCR-based RAPD method to find species-specific sequences of *C. wilfordii*, *C. auriculatum*, and *P. multiflorum* at the genomic level. To control for individual variations, we examined two or three samples per species. In this study, we used 16 Operon 10-mer random primers to analyze polymorphic patterns, and identified 45 distinct species-specific DNA fragments from 12 of the primers. These polymorphic fragments, designated 1–7 DNA fragments, varied in size, ranging from 200 to 2,100 bp (Fig. 1; and data not shown). To identify SCAR markers, we analyzed the nucleotide sequences of 23 species-specific RAPD amplicons, consisting of 6 DNA fragments for *C. wilfordii*, 5 for *C. auriculatum*, and 12 for *P. multiflorum* (Fig. 1). From the resulting nucleotide sequences, two unique RAPD amplicons were registered in the NCBI GenBank dbGSS, and used to develop RAPD-derived SCAR markers (Table 2; Fig. 1).

To develop the SCAR markers for *C. wilfrodii* and *C. auriculatum*, five sets of primers were prepared based on the corresponding nucleotide sequences of four species-specific RAPD amplicons, HA13-5, HC15-3, HC15-4, and

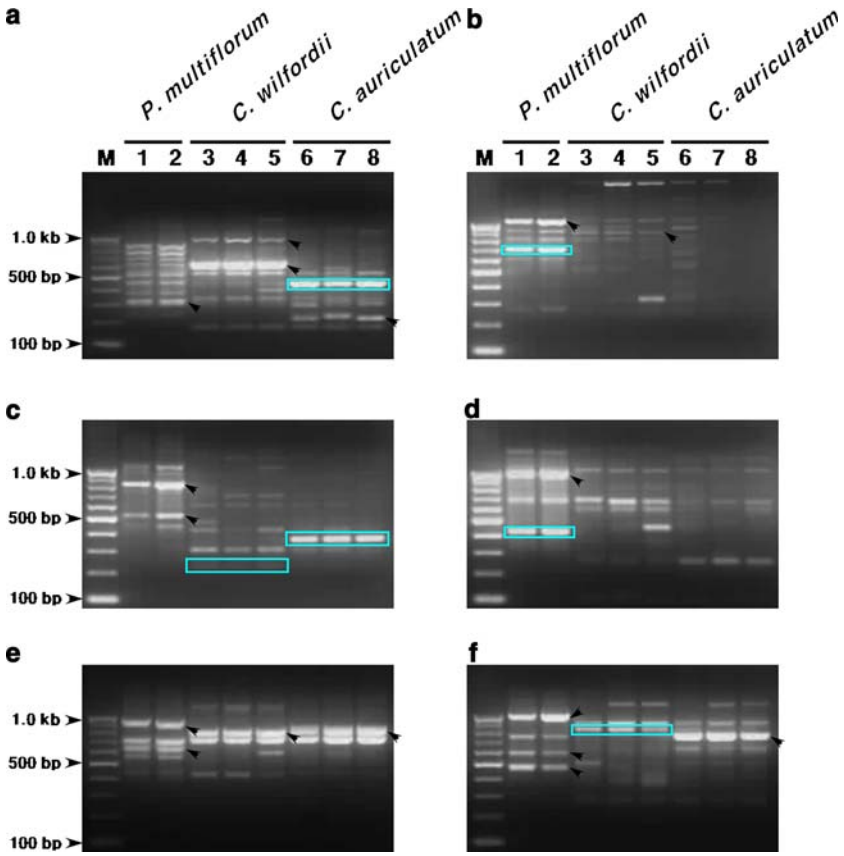

Fig. 1 RAPD profiles of *P. multiflorum*, *C. wilfordii*, and *C. auriculatum* using Operon 10-mer random primers. **a** OPA13, **b** OPC14, **c** OPC15, **d** OPC18, **e** OPC19, and **f** OPC20. The *arrowheads* and *boxes* indicate the species-specific RAPD amplicons. *Lane numbers* correspond to Table 1. *Boxes* indicate RAPD amplicons used for SCAR marker development. *M* represents a 100-bp DNA ladder

Table 2 Polymorphic RAPD amplicons and SCAR primers

RAPD primer	Polymorphic RAPD amplicons			NCBI dbGSS Accession no.	SCAR primer sequences (5′–3′)
	Name	Specificity	Size		
OPA13	HA13-5	*C. auriculatum*	439	FI857169	F (5′-GGT CAA CGA CAT GAT ATT GT-3′)
					R (5′-GTT ATT TGT GCT TAG AGT TA-3′)
OPC14	HC14-2	*P. multiflorum*	687	FI857165	F (5′-TGA GTC GTG AGT TAG AGC TC-3′)
					R1 (5′-TCA TAC ATA TCA GGG TAT GT-3′)
					R2 (5′-ATG TGT CAT TCG AGC TAA TC-3′)
OPC15	HC15-3	*C. wilfordii*	216	FI857167	F (5′-AAT CAA CAA TTA CTT GGT GT-3′)
					R (5′-TCC CTA CAC AAT CCT TTT TC-3′)
	HC15-4	*C. auriculatum*	361	FI857170	F (5′-GAT AAG GGA CTA GAC TTT AG-3′)
					R (5′-GAT AAG GCA TTA AAA GCT GA-3′)
OPC18	HC18-2	*P. multiflorum*	425	FI857166	F (5′-GCA AGT GGC AAT GGG ATT GC-3′)
					R (5′-GGA TGC CTG AGA TAA GAG GA-3′)
OPC20	HC20-4	*C. wilfordii*	760	FI857168	F (5′-GTG TAA TCC TAA TAT CTC TA-3′)
					R1 (5′-TCC GAC GGA TTG GAG TCA TA-3′)
					R2 (5′- AAC AAG TTC AAC GCT AGG AG-3′)

HC20-4. Primer specificity was evaluated using two or three samples from each species (Table 2; Fig. 2). As shown in Fig. 2a and b, amplification with the HA13-5 and HC15-4 primers yielded DNA fragments of 300 and 325 bp, respectively, in only *C. auriculatum*. Therefore, these primer sets are suitable for identification of *C. auriculatum,* and for distinguishing it from *P. multiflorum* and *C. wilfordii*. The HC15-3 and HC20-4 primer sets were derived from only *C. wilfordii* in RAPD amplification (Fig. 1c, f). However, they produced 196- and 407-bp DNA fragments, respectively, for both *C. wilfordii* and *C. auriculatum* (Fig. 2c, d). These results indicate that the

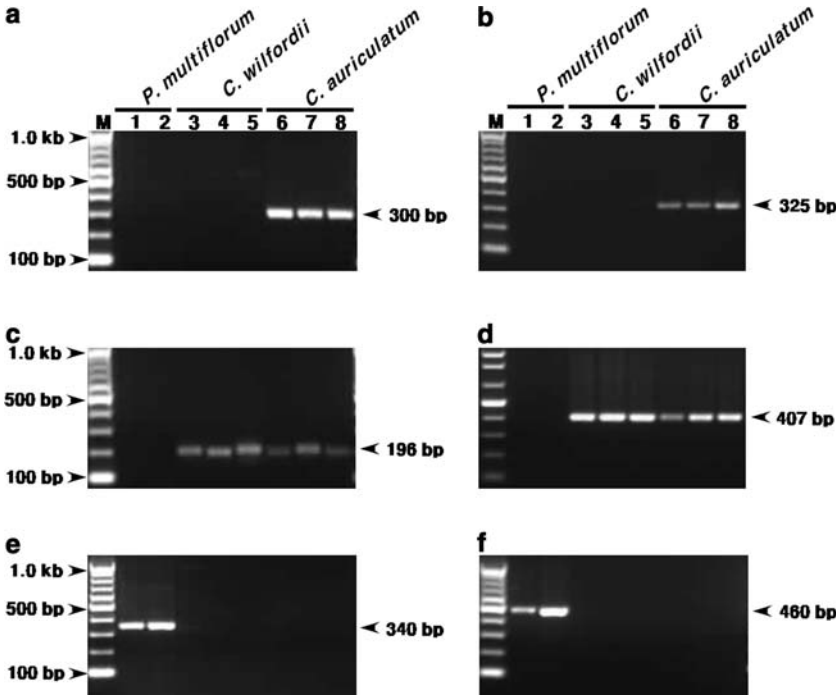

Fig. 2 Development of RAPD-derived SCAR markers for *P. multiflorum*, *C. wilfordii*, and *C. auriculatum*. PCR amplification using the following RAPD amplicon-derived SCAR primers **a** HA13-5, **b** HC15-4, **c** HC15-3, **d** HC20-4, **e** HC14-2, and **f** HC18-2. *Lane numbers* correspond to Table 1. *Arrowheads* indicate the precise size of SCAR markers. *M* represents a 100-bp DNA ladder

HC15-3 and HC20-4 SCAR markers are not enough to differentiate between *C. wilfordii* and *C. auriculatum*. However, these markers are suitable for distinguishing Cynanchi Wilfordii Radix and Polygoni Multiflori Radix. We developed a positive genetic marker for distinguishing *P. multiflorum* from *C. wilfordii* and *C. auriculatum*, which can be used to differentiate Polygoni Multiflori Radix and Cynanchi Wilfordii Radix. Three sets of primers were designed based on the corresponding sequences of two RAPD amplicons, HC14-2 and HC18-2 (Table 2; Fig. 2). As a result, we obtained two SCAR markers specific to *P. multiflorum*. Thus, only in *P. multiflorum,* PCR amplification with two primer sets, HC14-2 and HC18-2, yielded DNA fragments of 460 and 340 bp, respectively (Fig. 1e, f).

These combined results indicate that simultaneous amplification with each species-specific primer set can differentiate between all three species using the SCAR markers. Therefore, we developed two multi-species discrimination SCAR markers that can identify each species within a single reaction based on the size and number of PCR products using a multiplex-PCR method (Fig. 3). Amplification with a combination of HC18-2, HC20-4, and HA13-5 primer sets yielded three PCR products: one for *P. multiflorum* at 340 bp, one for *C. wilfordii* at 407 bp, and two for *C. auriculatum* at 407 and 300 bp (Fig. 3a). Another combination of primer sets, HC18-2, HC15-3, and HA13-5, resulted in a pattern of PCR products that could also discriminate between the three species. This combination produced a 196-bp band instead of a 407-bp band for *C. wilfordii* and *C. auriculatum* (Fig. 3b). Consequently, the amplification products from the multiplex-PCR were identical to those obtained from the individual specific primer sets, and were sufficient to discriminate among the three medicinal plant species. These multi-species discrimination SCAR markers will be effective for identifying the three species, and for the rapid authentication of Cynanchi Wilfordii Radix and Polygoni Multiflori Radix.

Discussion

This study was designed to produce a rapid genetic test for identification of '*Hashuoh*' and '*Baekshuoh*' and their potential contaminants. Thus, to prevent indiscriminative distribution and prescription of Polygoni Multiflori Radix and Cynanchi Wilfordii Radix, we used the analysis of polymorphic patterns based on RAPD to developed reliable SCAR markers for the three related species, *C. wilfordii*, *C. auriculatum*, and *P. multiflorum*. In the RAPD analysis, we detected polymorphic amplicons among *P. multiflorum*, *C. wilfordii*, and *C. auriculatum.* However, greater homology between *C. wilfordii* and *C. auriculatum* was seen, than with *P. multiflorum.* This result came up to our expectation because *P. multiflorum* is a member of the Polygonaceae family, and *C. wilfordii* and *C. auriculatum* belong to the Asclepiadaceae family. Even though some primers did not generate specific PCR products, we obtained polymorphic amplicons to distinguish between the three species. However, several polymorphic bands showed different patterns within the same species (Fig. 1). For instance, several PCR products were detected only in one sample, lane 5, but not in

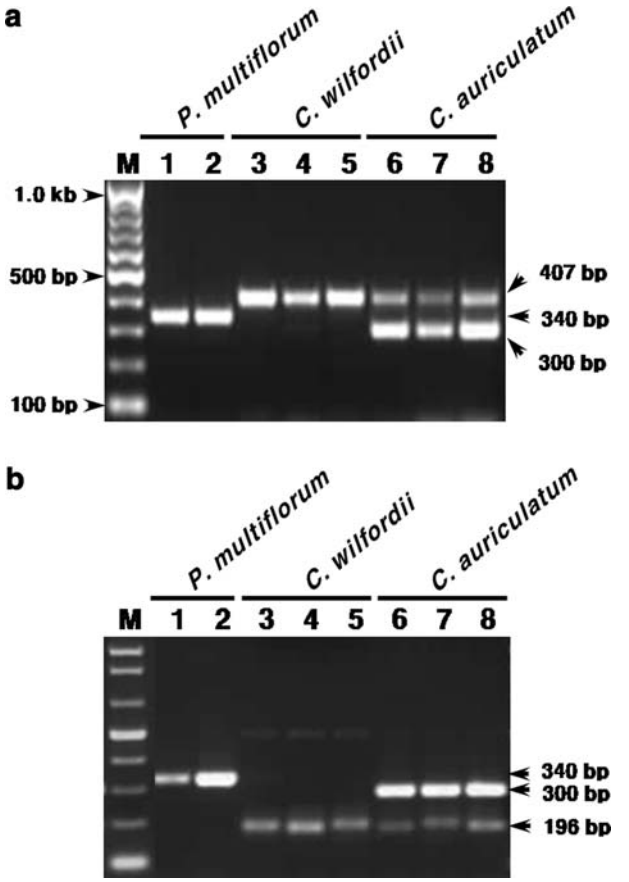

Fig. 3 Amplification of multi-species discrimination SCAR markers using multiplex-PCR. **a** Multiplex-PCR products using HC18-2, HC20-4, and HA13-5 primer pairs. **b** Multiplex-PCR products using HC18-2, HC15-3, and HA13-5 primer pairs. *Lane numbers* correspond to Table 1. *Arrowheads* indicate the precise size of SCAR markers. *M* represents a 100-bp DNA ladder

two other samples, lanes 3 and 4, in *C. wilfordii* (Fig. 1a–e). These differences were likely caused by individual variations at the genomic DNA level, such as substitutes of bases within the binding sequences of the RAPD primers (Williams et al. 1990). Technical factors, such as differences in the concentrations of template DNA and primers used for PCR amplification, can also cause these unexpected polymorphisms. We cannot exclude the possibility that non-specific RAPD products were amplified, which can be generated by the relatively short 10-mer primers used in RAPD analysis (Penner et al. 1993). To overcome these causal factors while developing SCAR markers, we tested multiple samples for each species, and used only those primers that performed similarly with all of the samples from each species (Fig. 1). Therefore, we are confident in our results and used these polymorphic RAPD amplicons in further investigations.

In the development of SCAR markers to differentiate between *C. wilfordii* and *C. auriculatum*, we prepared SCAR primer sets from nucleotide sequences of 12 RAPD polymorphic amplicons, and primer specificity was verified using multiple samples for each species. As shown in Fig. 2a and b, two primer sets designed from *C. auriculatum*-specific polymorphic amplicons, generated the expected PCR products in *C. auriculatum* samples. However, two primer sets designed from *C. wilfordii*-specific polymorphic amplicons produced specific PCR products for both *C. auriculatum* and *C. wilfordii* (Table 2; Fig. 2c, d). These results suggest that *C. wilfordii* and *C. auriculatum* have highly homologous genomic DNA sequences, and that the selection of primers is very important for developing specific SCAR markers. Even though we could not obtain a SCAR marker for *C. wilfordii*, the merged results of these SCAR markers are enough to differentiate between *C. auriculatum* and *C. wilfordii*.

Several discriminative standards based on morphological characteristics were suggested by Song et al. (2004) to distinguish Polygoni Multiflori Radix and Cynanchi Wilfordii Radix. Yan et al. (2008) analyzed the nucleotide sequences of chloroplast *matK* and 18S rRNA to differentiate *P. multiflorum* (designated as *Fallopia multiflora*) from its contaminants, *Musa basjoo* and *Reynoutria japonica.* Several sequence substitutions for their differentiation were suggested. However, a clear molecular discrimination marker was not developed to authenticate *P. multiflorum*. In this study, we investigated reliable SCAR markers for differentiating *P. multiflorum* from *C. wilfordii* and *C. auriculatum*, and developed two RAPD-derived SCAR markers from polymorphic amplicons that were specific for *P. multiflorum* (Figs. 1 and 2c). Furthermore, we established a multiplex-PCR method for rapid molecular discrimination of these three species, as previously described (Henegariu et al. 1997; Choo et al. 2009). As shown in Fig. 3, two sets of multi-species discrimination SCAR markers generated PCR products of the expected sizes, showing analytical accuracy and stability. This result suggests that our multi-species discrimination SCAR markers are applicable for authenticating each species from other closely related medicinal plants. This is the first molecular marker for determining whether official Polygoni Wilfordii Radix and Cynanchi Wilfordii Radix are pure.

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