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Original Article

Molecular authentication of Pinelliae Tuber and its common adulterants using RAPD-derived multiplex sequence characterized amplified region (multiplex-SCAR) markers

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Abstract: The identification of authentic plant species is important for the quality control of traditional herbal medicines. The Pinelliae Tuber described as the tuber of only *Pinellia ternata* in national pharmacopoeia of Korea, China, and Japan. However, the tubers and herbal medicines derived from *Pinellia ternata* are similar to those derived from *P. tripartita*, *P. pedatisecta*, and *Typhonium flagelliforme*, and the correct identification of species is very difficult using conventional methods. Therefore, this study was carried out to develop DNA based markers for the identification authentic Pinelliae Tuber and its common adulterants in species levels. To develop a reliable method to discriminate Pinelliae Tuber and its closely related three common adulterants, we introduced sequence characterized amplification region (SCAR) markers using randomly amplified polymorphic DNA (RAPD)-based SCAR methods. Several distinct SCAR markers were developed that amplified unique single DNA fragments in *P. ternata*, *P. tripartita*, *P. pedatisecta*, and *T. flagelliforme* based on the verification of species-specific RAPD amplicons. Furthermore, a useful molecular marker was established for multiplex PCR, enabling the four species to be distinguished concurrently in a single PCR reaction. These genetic markers enable the efficient and rapid discrimination of authentic Pinelliae Tuber, the herbal medicine derived from the tuber of *P. ternata*, from closely related adulterant species and will be useful for the standardization of this herbal medicine.

Keywords: Pinelliae Tuber, *Pinellia ternata*, random amplified polymorphic DNA (RAPD), sequence characterized amplification region (SCAR), multiplex PCR, molecular authentication marker

Introduction

Pinellia ternata (Thunb) Makino (Araceae) is an important medicinal plant distributed throughout most East Asian countries including Korea, China, and Japan. The herbal drug from the tuber of *P. ternata* (Thunb) Makino, *BanHa* (*BanXia* in Chinese), has been a well-known and widely used expectorant, antitussive, and antivomiting ingredient in traditional Asian herbal medicine for approximately 1,500 years [1]. The crude extract of the tuber, called Pinelliae Tuber in herbal medicine, is toxic, and processing with alunite and ginger is required for internal use [2]. However, the dried tuber of *P. ternata* is a good example of the complications can arise in herbal medicine because inauthentic substitutions of closely related species and adulterants are common. Since the morphological similarity of the aerial parts and the dried tuber, *P. ternata* has been adulterated with medicinal materials from members of the genera *Pinellia* and *Typhonium*. *Pinellia*, a genus of Araceae, is recognized by the present plant taxonomist as having 9 species and distributed in mainland China, Korea, and Japan [3, 4]. The genus *Typhonium* suggested having about 50 species from India to Polynesia and distributed 9 species in China [3]. Among these, several plant species such as *P. pedatisecta* Schott, *T. flagelliforme* (Lodd.) Blume, and *T. divaricatum* Blume (a synonym of *T. roxburghii* Schott) have been used as adulterants of Pinellae Tuber including two *Arisaema* species, *A. erubescense* (Wall) Schott and *A. yunnanense* Buchet, since ancient time in China [5,

6]. In the literatures, it has been reported that some additional plant species belongs to these genera [*P. tripartita* (Blume) Schott, *T. trilobatum* (L.) Schott, *A. franchetianum* Engl., *A. intermedium* Blume etc.] could also be used Pinelliae Tuber [5]. These inauthentic medicinal ingredients were decreased in the producing areas and herbal markets because the production of Pinelliae Tuber was increasing depend on the cultivation than wild collection in response to the depletion of natural resources [7]. However, some of adulterants including the tuber of *P. pedatisecta* Schott, *P. tripartita* (Blume) Schott, and *T. flagelliforme* (Lodd.) Blume were still adulterated due to the short growing period and high productivity against *P. ternata* in cultivation stage [8, 9]. In particular, the dried tubers of *P. pedatisecta* and *T. flagelliforme* are frequently cultivated as *P. ternata* and sold in mixed form with *P. ternata* in Korea and China. Unfortunately, because of the morphological similarities in the tubers and the lack of visual differences among the processed herbal drugs, it is very difficult to determine the species and adulterants used. Therefore, accurate and reliable methods are required to differentiate between genuine Pinelliae Tuber and inauthentic herbal materials.

DNA fingerprinting has been used for plant phylogenetic studies and species identification, and this method has been applied to herbal medicines [10-12]. In particular, the developments of sequencing techniques and polymerase chain reaction (PCR) enabled medicinal plant materials to be distinguished at the inter- and intra-specific levels [13-17]. Random amplified polymorphic DNA (RAPD) markers are also used for taxonomic analysis because they are fast, inexpensive, require a minute amount of plant material, and reveal high levels of polymorphism; however this method has disadvantages. RAPD is less reproducible than other methods and require the analysis of various polymorphic amplicons [18]. Therefore, to identify accurate and reproducible genetic markers, sequence characterized amplified region (SCAR) marker analysis was developed based on DNA barcoding and RAPD markers in various plant genera [19-22]. These methods have improved the accuracy of species discrimination and are substantially more effective than subjective methods based on morphological features or analytical methods using chromatographic techniques and marker compounds. In recent years, molecular genetic tools have been applied to standardize herbal medicines derived from *P. ternata* by distinguishing between genuine herbal materials and substitutes or adulterants. For instance, the sequence of the mannose-binding lectin gene was analyzed and a pair of specific primers was suggested to distinguish *P. ternata* from its adulterants, *P. pedatisecta* and *Arisaema heterophyllum* [8]. The chloroplast *atp*B-*rbc*L intergenic spacer was also sequenced and analyzed to distinguish between *P. ternata* and *Arisaema yunnanense* [6]; however, these studies do not consider the use of the common adulterants, the tubers of *T. flagelliforme* and *P. tripartite*. Furthermore, precise and efficient authentication tools for distinguishing *P. ternata* from its adulterants at the species level are not available.

In this study, we analyzed the RAPD patterns for four important medicinal plant species, namely, *P. ternata, P. pedatisecta, P. tripartita,* and *T. flagelliforme*, and developed several SCAR markers to identify the species by comparative sequence analysis of species-specific RAPD amplicons. In addition, we established a combination of SCAR marker primers for the simultaneous authentication of these four species by a single multiplex PCR. These tools will be useful for distinguishing the four medicinal plants species and differentiating between authentic herbal medicines and those within appropriate substitutes and adulterants.

Materials and methods

Plant materials

Nineteen total genotypes, including five from *P. ternata,* four from *P. tripartita,* four from *T. flagelliforme*, and six from *P. pedatisecta*, were used in the analysis (**Table 1**). Samples were collected from native habitats in Korea and China and stored at -70°C after freezing in liquid nitrogen. All plant materials were given accession numbers and specimens were preserved in the Korean Herbarium of Standard Herbal Resources (KHSHR) at the Korea Institute of Oriental Medicine. Species identification was performed by the Classification and Identification Committee of the KIOM, which comprises nine experts in the fields of plant

Table 1. Summary of information regarding plant materials

Plant name		Source	Sample name	Lane in gel
Scientific name	Herbal medicine			
Pinellia ternata (Thunb.) Makino	Pinelliae Tuber	Chengdu, Sichuan, China	PT1	1
	(Ban xia, Ban Ha)[b]	Seogwipo, Jeju, Korea	PT2	2
		Sacheon, Gyeongnam, Korea	PT3	3
		Sanmenxia, Henan, China	PT4	4
		Quiyang, Guizhou, China	PT5	5
Pinellia tripartita (Blume) Schott	-[a]	Geoje, Gyeongnam, Korea	PTP1	6
	(San Lie Ban xia, Dae Ban Ha)[b]	Sejong, Chungnam, Korea	PTP2	7
		Jeonju, Jeonbuk, Korea	PTP3	8
		Tongyeong, Gyeongnam, Korea	PTP4	9
Pinellia pedatisecta Schott	-[a]	Dingxi, Gansu, China	PP1	10
	(Hu Zhang, Ho Jang Ban Ha)[b]	Changsha, Hunan, China	PP2	11
		Quiyang, Guizhou, China	PP3	12
		Kaili, Guizhou, China	PP4	13
		Anguo, Hebei, China	PP5	14
		Harbin, Heilongjiang, China	PP6	15
Typhonium flagelliforme (Lodd.) Blume (= Arum falgelliforme Lodd.)	-[a]	Pingnan, Guangxi, China	TF1	16
	(Shui Ban Xia, Su Ban Ha)[b]	Pingnan, Guangxi, China	TF2	17
		Baoxing, Sichuan, China	TF3	18
		Baoxing, Sichuan, China	TF4	19

[a]There is no appropriate official herbal name. [b]Common inauthentic herbal names in China and Korea, respectively.

taxonomy, botany, pharmacognosy, and herbology.

Preparation of genomic DNA

Genomic DNA was extracted from fresh leaves stored at -70°C and from herbal medicines using DNeasy® Plant Mini Kits (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. DNA concentrations and purities were determined by spectrophotometry (Nanodrop ND-1000, Nanodrop, Wilmington, 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/ul in TE buffer.

Amplification of DNA barcodes and RAPD markers

Forty-eight 10-mer RAPD primers (Operon Technologies Inc., Alameda, CA, USA) were used to determine genomic profiles and to obtain species-specific RAPD amplicons. PCR reactions were carried out in 30 ul 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, Daejeon, Korea), 30 pmol primer and 10-20 ng template DNA. DNA amplification was performed on a DNA Engine Dyad® PTC-0220 (Bio-Rad, Foster City, CA, USA). The parameters for the RAPD analysis were 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 1 min at 42°C, and 2 min at 72°C, and a final extension for 10 min at 72°C. PCR 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 light.

Analysis of nucleotide sequences

The amplified species-specific RAPD amplicons were recovered from agarose gels with a Gel Extraction Kit (Solgent, Daejeon, Korea), and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Nucleotide sequences of the inserts were determined from both strands via dideoxynucleotide chain termination using an automatic DNA sequence analyzer (ABI 3730, Applied Biosystems Inc., Foster City, CA, USA). The nucleotide sequences of four amplicons used to develop the SCAR markers were registered in NCBI GenBank (accession nos. BH2-900, KF424997; DH4-600, KF424998; HD2-300, KF424999; and SE2-300, KF425000).

Development of SCAR markers

Specific primers for the SCAR markers were designed from the sequences of the corresponding polymorphic RAPD amplicons using PRIMERBLAST 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 templates were used. The amplification conditions were as follows: predenaturation at 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. 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°C), number of PCR cycles (23-38 cycles), amount of primers (5-20 pmol), and combination of SCAR primers.

Results

Analysis of RAPD genomic profile

The RAPD fingerprinting method was employed to analyze genomic polymorphism and to find species-specific sequences for *P. ternata, P. pedatisecta, P. tripartita,* and *T. flagelliforme.* To control for individual and geographical variation, more than four samples for each species were collected individually from different habitats and analyzed. Forty-eight random primers were used, of which 40 produced distinct and reproducible amplification profiles. These primers revealed diverse polymorphic DNA fragments, which were sufficient for differentiating the four medicinal plants species. The polymorphic DNA fragments varied in size and number, with 1-8 amplicons ranging in length between 150 and 2,300 bp (**Figure 1** and some data not shown). These polymorphic amplicons were observed in all of the species and were most abundant in *T. flagelliforme* (**Figure 1**). These

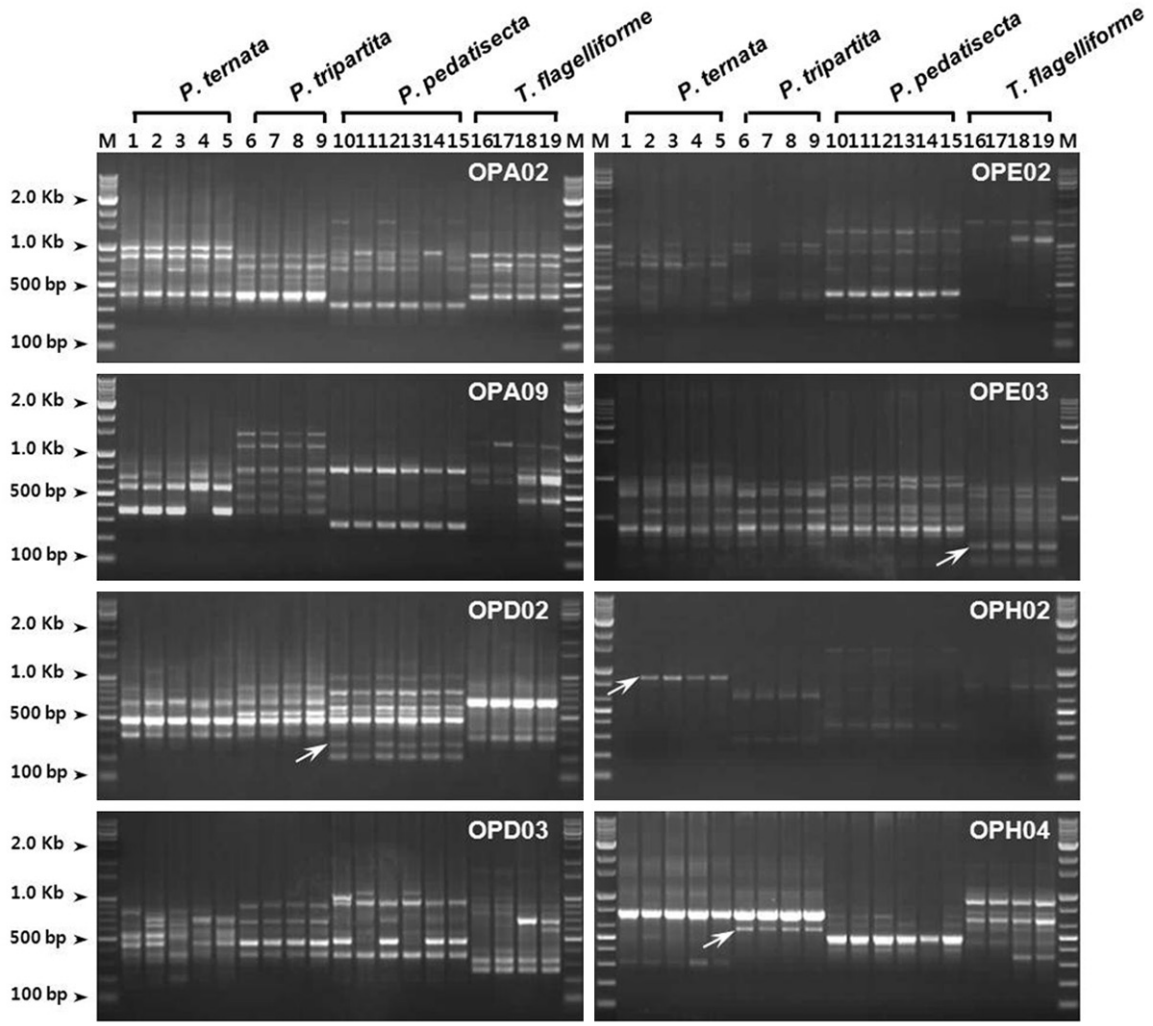

Figure 1. Randomly amplified polymorphic DNA (RAPD) profiles of *Pinellia ternata*, *P. tripartita*, *P. pedatisecta* and *Typhonium flagelliforme* using Operon 10-mer random primers. The RAPD primer names are indicated in the gel images. Arrows indicate species-specific RAPD amplicons used in this study. Lanes 1-19 correspond to those listed in **Table 1**. M represents a 1.0 kb plus DNA ladder.

results indicated that the species that belonged to the same genus had more similar genomic fingerprinting profiles to each other than they did with the other genus, *Typhonium*, and suggested that polymorphic amplicons (individually or in combinations) among the different plant species can be used as molecular markers to identify each of the four species.

Development of SCAR markers for the identification of species

The identification of a species by DNA markers largely depends on the genomic region targeted. To obtain species-specific genomic sequence information for authenticating Pinelliae Tuber, we analyzed RAPD genomic markers and screened polymorphic amplicons using the 19 accessions of *P. ternata* and three closely related species. Forty species-specific RAPD amplicons, including nine from *P. ternata*, eight from *P. pedatisecta*, ten from *P. tripartita*, and twelve from *T. flagelliforme*, were sequenced (**Figure 1**). Candidate SCAR primers were designed from nucleotide sequences of each RAPD amplicon to develop SCAR markers. Primer specificity and the utility for the detection or differentiation of each species were then confirmed.

P. ternata must be clearly distinguished from other species because only its tuber is used as

A

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1   TCGGACGTGACCCTAATTACCCAATAATTTGGACATTAAATCAAGTTTAAACACAATTAGGCCACTAAACCTAACCTAAA   80
81  CCCTAGCCCACAAGGTGAGAGCATGGAGAACTCCTCTTCTAACCAAGGAAGGGTGCTGGAGGCAGCCCCAAAGCCGGCAG  160
161 GGACGAGCTGGGCGCGGATCCCCCTTGATTGGGGCAATAAACACATGGATCTCTCTTCCCCCTCTATATATACATGGTGA  240
241 TACCCCTCATGGCCGAACCCTAGGCGTGCTGGGCGGCTTGGAGGCATGGGGAGAGCAGCTAGGAGGCGATCCAACTCGTG  320
    BH2-900 F2
321 GAGGCTCGTCATCCTCTTCTTCCTCTTCTTCCTTTCACGCTCAAACCGAGCTCTCTCTCTCCGTGAACGAGGTAGGTGAT  400
401 CCCCGGGCGCAAACATCATGAGTCCTATACCGTTTTGTAGATCTCCATGTTAGCAAACTAAGGGCGCAAGAATGCCTAAA  480
481 TTTCCTGTTGTATCCATCGCGAATAAGGCATTTTGCCTGCGTCGTGCGTTCTAGGGCTCCAAGAGCCAAACCGTCGATTT  560
561 CTCGTGATACGTGCAATGGGAATCCAATTCCTTATGAATGTTCAAAGATCTCGACGAGATCTACAAGTTTCATGACTACC  640
641 ACTTTTGCTGATTCGGCCGTTTAGACCATCGTTTATTGTGTGTAAGGTTTTGCCCGAAACTCGCATTTTTCTGGAAGAAC  720
        BH2-900 R3
721 ATTGGAAGTGGCGCCAGATGCAGATGGCGCGACTTCCCAGGACTTACGGAGTGGCGTCACACCCTATTGACGCTACTTCC  800
            BH2-900 R2
801 AGTTAAGTTATTTGTCAGACCTGACACCCGTCACGTCCGA                                          840
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B

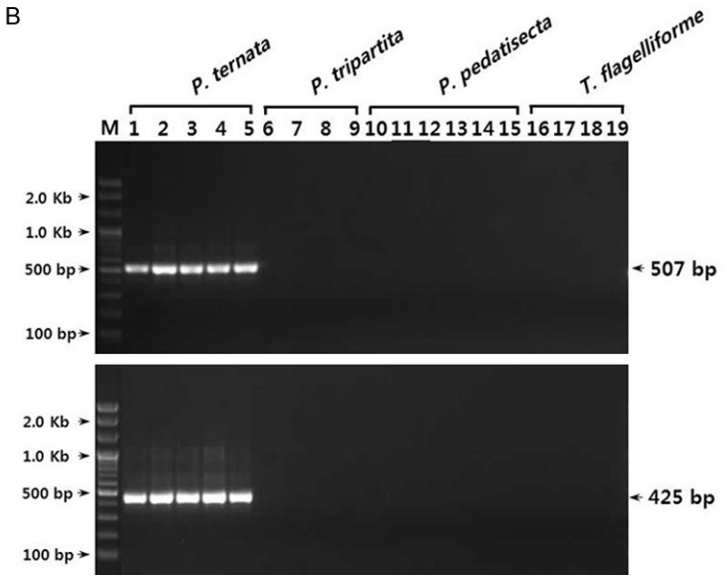

Figure 2. Nucleotide sequence of BH2-900 *P. ternata*-specific randomly amplified polymorphic DNA (RAPD) amplicon and the development of sequence characterized amplification region (SCAR) markers. A. The nucleotide sequences of the BH2-900 RAPD amplicon and primers. B. Confirmation of primer specificity for the *P. ternata*-specific SCAR markers. M represents a 1.0 kb DNA ladder. The precise size of each SCAR marker is indicated to the right of the panel, with the arrowhead. Lanes 1-19 correspond to those listed in the columns of **Table 1**.

the herbal medicine Pinelliae Tuber in Korea and China. To obtain species-specific SCAR markers for identifying *P. ternata*, 14 sets of oligonucleotide primers were prepared based on the 840 bp nucleotide sequence of BH2-900, which is the *P. ternata*-specific RAPD amplicon from the OPH02 random primer, and the specificity of each primer set was confirmed for the 19 accessions (**Figures 1** and **2**). In all five of the *P. ternata* samples, the BH2-900 F2/R2 and F2/R3 primer sets produced a single distinct DNA fragment at the expected sizes of 507 bp and 425 bp, respectively (**Figure 2**). No non-specific PCR products were observed for the other 14 accessions of three species (**Figure 2B**). These results indicate that these two primer sets are suitable for the identification of *P. ternata* and for distinguishing it from *P. pedatisecta, P. tripartita*, and *T. flagelliforme*.

To develop SCAR markers for the identification of *P. tripartita,* the sequences of ten *P. tripartita*-specific RAPD amplicons were analyzed. Among these, DH4-600 had suitable sequence regions for use as SCAR markers of *P. tripartita*. Based on the 561 bp nucleotide sequence of DH4-600, 32 sets of primers were prepared and their specificities were evaluated. In inauthentic *P. tripartita*, PCR amplification with three sets of primers, DH4-600 F4/R1, F4/R2, and F6/R4, yielded the expected DNA fragments of 190 bp, 133 bp, and 356 bp, respectively, but no amplification was observed in the other species (**Figure 3**).

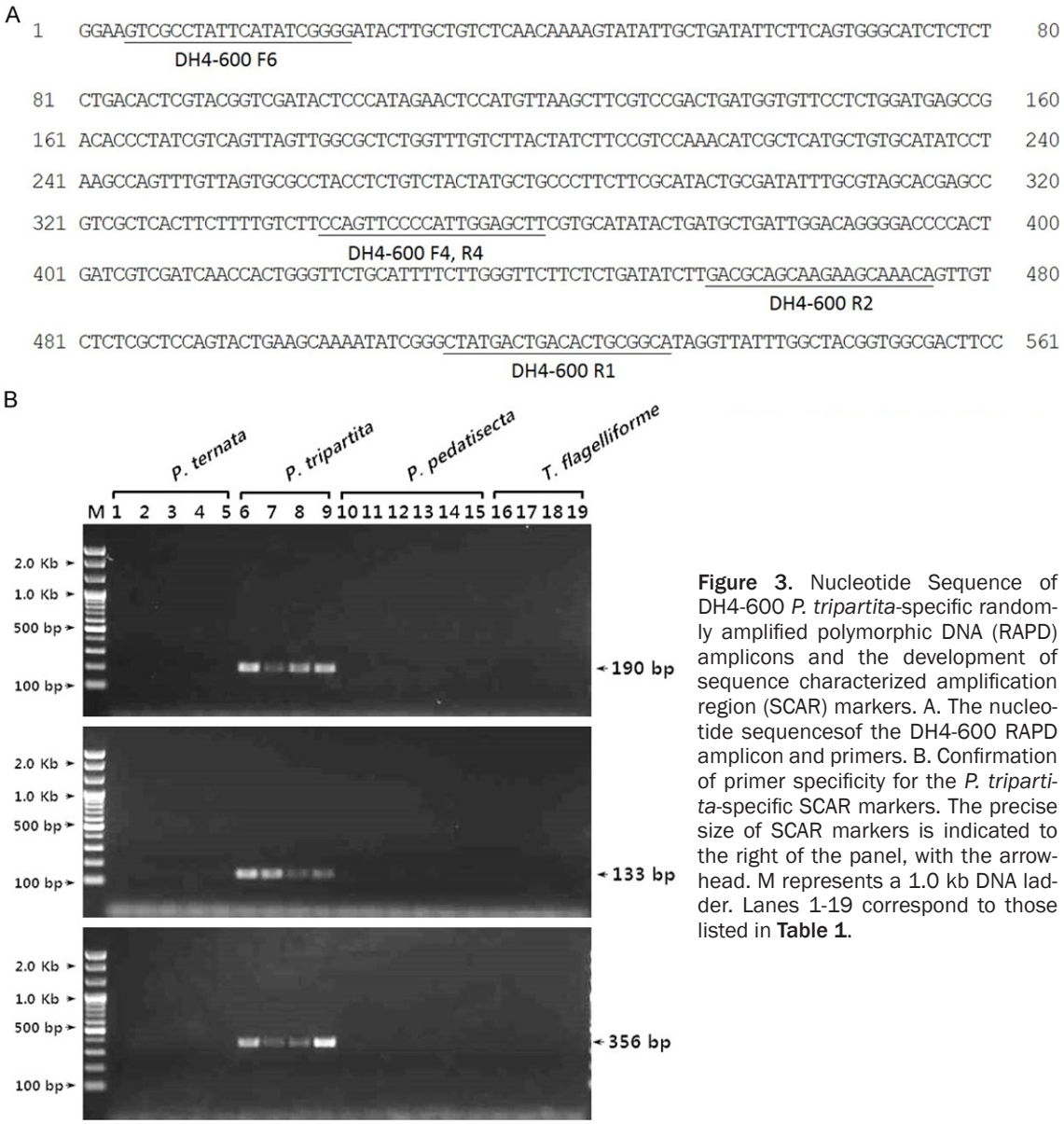

Figure 3. Nucleotide Sequence of DH4-600 *P. tripartita*-specific randomly amplified polymorphic DNA (RAPD) amplicons and the development of sequence characterized amplification region (SCAR) markers. A. The nucleotide sequencesof the DH4-600 RAPD amplicon and primers. B. Confirmation of primer specificity for the *P. tripartita*-specific SCAR markers. The precise size of SCAR markers is indicated to the right of the panel, with the arrowhead. M represents a 1.0 kb DNA ladder. Lanes 1-19 correspond to those listed in **Table 1**.

The identification of *P. pedatisecta* is also necessary because it is an inauthentic adulterant species of Pinelliae Tuber, which usually causes contamination at the cultivation stage. To discriminate whether the tuber of *P. pedatisecta* was contaminated, we evaluated eight polymorphic RAPD amplicons specific to *P. pedatisecta* and obtained two sets of SCAR primers. As shown in **Figure 4**, HD2-300 F1/R2 and F2/R1 primer sets amplified unique and abundant DNA fragments from *P. pedatisecta* at the expected 105 bp and 100 bp, respectively, but not from the other species (**Figure 4**).

Owing to its productivity and advantages related to agricultural cultivation, the tuber of *T. flagelliforme*is cultivated and distributed as Pinelliae Tuber in the herbal market. To develop a reliable SCAR marker for identifying this major adulterant species, we evaluated 12 *T. flagelliforme*-specific RAPD amplicons and obtained two primer sets. Among the four sets of SCAR primers based on the 275 bp nucleotide sequence of the SD3-300 amplicon amplified from the OPE03 Operon primer, only the SE3-300 F1/R1 and F2/R1 primer sets produced distinct 237 bp and 178 bp DNA fragments in only four accessions of *T. flagelliforme* (**Figure 5**).

These combined results indicate that species-specific SCAR markers can be used to distin-

A

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1   GGACCCAACCTTATCAAGGGTGAGTAGTCTCTTACAGGGAAAAGGTAAGATTGAAATATTATTAAAAATCGCATTCTCAC   80
                HD2-300 F1                                              HD2-300 F2

81  CCTCCATACAAGCATGAGAGGCTAAGCAGCCAGCCACTTTATTAGCTTCCCGATAAATATGAACAATAGACAGTCTCTGT   160
                HD2-300 R2                                              HD2-300 R1

161 TAGGGGTGTCAAAAACACAATAACCCAAACTATAAGAGTGGTTTAAAATGGGTAAAACTCTCAGTGTTTACCTATTATTG   240

241 AACTAGTGGTTGGGTCC                                                                  257
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B

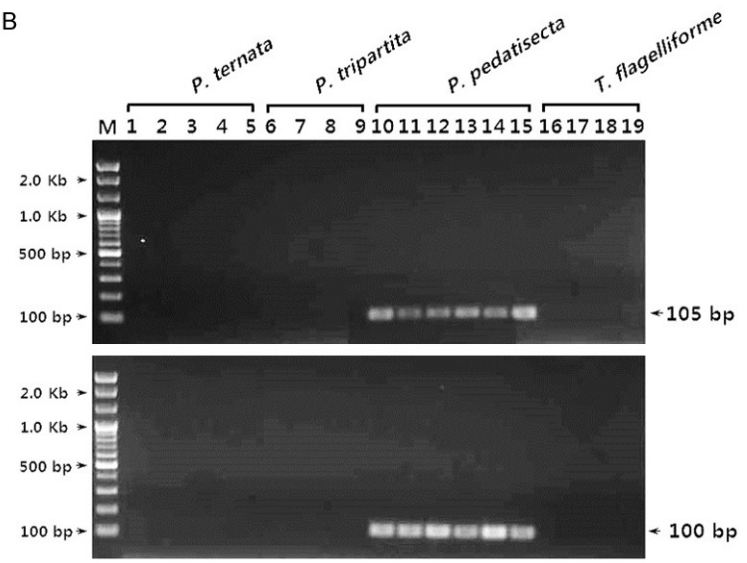

Figure 4. Nucleotide Sequence of HD2-300 *P. pedatisecta*-specific randomly amplified polymorphic DNA (RAPD) amplicons and the development of sequence characterized amplification region (SCAR) markers. A. The nucleotide sequences of the HD2-300 RAPD amplicon and primers. B. Confirmation of primer specificity for the *P. pedatisecta*-specific SCAR markers. M represents a 1.0 kb DNA ladder. The precise size of SCAR markers is indicated to the right of the panel, with the arrowhead. Lanes 1-19 correspond to those listed **Table 1**.

A

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1   CCAGATGCACTCCTTCGATCATTGTAATGGGAGGTATGTGCCCGATAATGTTCTAGAGAACAAATTGTGTTAGAGAAAGT   80
                                SE3-300 F1

81  GACTTTGGAGGGACCTGTAAGGTAATTTAGCAATTCGCTTTTAAAGTAATATCCATGGTAAGAAATTGTAGTTTAAGTTC   160
                SE3-300 F2

161 TTGGGGACAAGTACTGAGGACGACCTTCAGAATTAGAGAAACATTAGTAAGATGATAAGGCCATTTGATCATCGAACAAT   240

241 GGGAAGGTATTAAACTGGCGGGACGGTGCATCTGG                                               275
                SE3-300 R1
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B

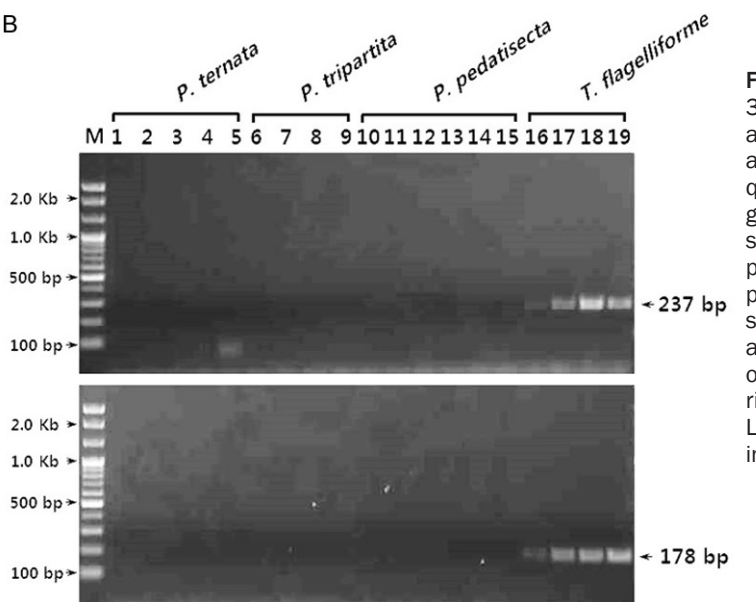

Figure 5. Nucleotide Sequence of SE3-300 *T. flagelliforme*-specific randomly amplified polymorphic DNA (RAPD) amplicons and the development of sequence characterized amplification region (SCAR) markers. A. The nucleotide sequences of the HD2-300 RAPD amplicon and primers. B. Confirmation of primer specificity for the *P. pedatisecta*-specific SCAR markers. M represents a 1.0 kb DNA ladder. The precise size of SCAR markers is indicated to the right of the panel, with the arrowhead. Lanes 1-19 correspond to those listed in **Table 1**.

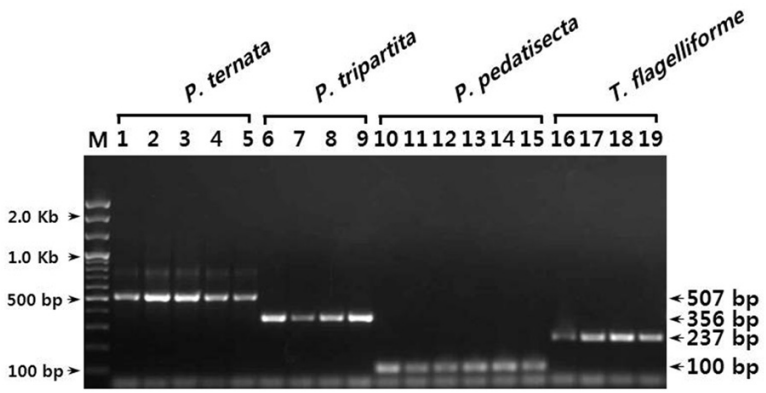

Figure 6. Establishment of a multiplex sequence characterized amplification region (SCAR) marker using a combination of species-specific primers and multiplex PCR. Confirmation of multiplex PCR products from the combination of the primers BH2-900 F2/R2, DH4-600 F6/R4, HD2-300 F2/R1, and SE3-300 F1/R1. M represents a 1.0 kb DNA ladder. Lanes 1-19 correspond to those listed in **Table 1**. Arrowheads to the right of the panels indicate the precise sizes of the SCAR markers.

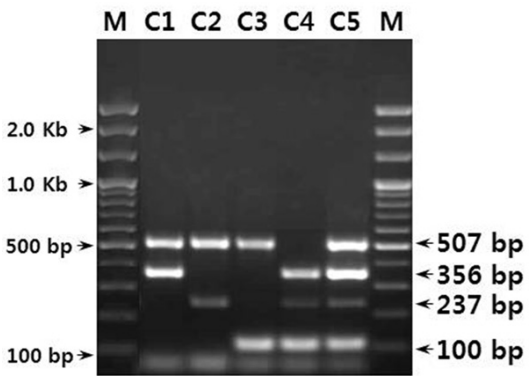

Figure 7. Verification of the efficiency and ability of the multiplex sequence characterized amplification region (SCAR) marker for the authentication of Pinelliae Tuber at the species levels. Amplification of multiplex PCR products and verification of the authentication ability using diverse mixtures of Pinelliae Tuber and the dried tuber of its adulterant plant species with a multiplex-SCAR primer combination. C1 is the mixture of *P. ternata* and *P. tripartita*; C2 is the mixture of *P. ternata* and *T. flagelliforme*; C3 is the mixture of *P. ternata* and *P. pedatisecta*; C4 is the mixture of *P. tripartita*, *P. pedatisecta*, and *T. flagelliforme*; and C5 is the mixture of *P. ternata*, *P. tripartite*, *P. pedatisecta*, and *T. flagelliforme*. M represents a 1.0 kb DNA ladder. Arrowheads to the right of the panels indicate the precise sizes of the SCAR markers.

guish all four medicinal plants, and thus these markers provide a reliable method for the authentication of official herbal materials and the identification of substitutes and/or adulterants.

Establishment of multiplex-SCAR marker

The SCAR markers developed in this work were used simultaneously in a multiplex PCR amplification to broaden the application of the markers. A multispecies SCAR marker was developed based on the size difference of PCR products. To establish the optimal primer combination and PCR parameters, we obtained multiplex-PCR conditions for several simultaneous amplification accessions. Amplification with the primer combination BH2-900 F2/R2, DH4-600 F6/R4, HD2-300 F2/R1, and SE3-300 F1/R1 yielded four abundant PCR products that showed distinct differences in size between the four species. Therefore, the multiplex PCR amplification products were identical to those obtained using the specific primer sets for each species, and provided a rapid method for authentication of the four species in a single reaction (**Figure 6**). To evaluate the sensitivity and utility of multiplex-SCAR markers, we verified this method using genomic DNA prepared from diverse mixtures of these four species. As shown in **Figure 7**, the resulting multiplex PCR products were sufficient to discriminate each of the adulterants in contaminated Pinelliae Tuber. These results demonstrate that the multispecies SCAR markers established in this work are effective for identifying species and standardizing traditional herbal medicines containing Pinelliae Tuber.

Discussion

Morphological features such as shape, color, and texture are traditionally used for the identification of plants used in herbal medicine; however, these methods are often inaccurate when differentiating between herbal materials because environmental conditions influence morphological characteristics. Therefore, it is necessary to develop reliable tools for the accurate identification of closely related species used for herbal medicines. DNA analyses including DNA barcoding, genomic fingerprinting, SCAR markers, and SSR are reliable meth-

ods for identifying plant species because the genetic composition of each species is unique, irrespective of the physical form, storage, and processing of the samples [23-26].

Initially, we examined the internal transcribed spacer (ITS) region to determine markers for *P. ternata*, *P. pedatisecta*, *P. tripartita*, and *T. flagelliforme* because it is highly variable. In this study, the ITS region amplified by the ITS1 and ITS4 primers produced incongruent nrDNA-ITS PCR products in all of the samples analyzed for the sequence comparison and identification of nucleotide substitutions (data not shown) because it revealed multi-fragment PCR products. Therefore, we adopted a strategy to develop RAPD-derived SCAR markers and multiplex PCR. First, RAPD was used to obtain potential species-specific nucleotides, because 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 to design stable species-specific primers in a short period of time [27]. In RAPD genomic profiling, there is little variation within species, but intra-specific variation was detected in one or two samples of a species (**Figure 1**). Such differences reflect individual variations at the genomic level, including base substitutions within the binding sequences for the RAPD primers [13, 18]. Technical issues, such as the comparatively short RAPD primers and high annealing temperatures could also have caused these unexpected polymorphisms. To overcome such variability, RAPD fragments were generated from 48 random 10-mer primers and a total of 40 potential species-specific PCR products were collected. Furthermore, more than eight species-specific RAPD amplicons were analyzed and nucleotide sequences were determined for fragments amplified in all samples of each species (**Figure 1**). This RAPD analysis made it possible to identify candidate SCAR markers to identify each species (**Figures 2**-**5**).

In this study, we developed a reliable SCAR markers enough to discriminate between official Pinelliae Tuber (the dried tuber of *P. ternata*), and its common adulterants (the dried tuber of *P. pedatisecta*, *P. tripartita*, and *T. flagelliforme*) based on species-specific RAPD amplicons. We also established a molecular diagnostic method suitable to distinguish each four species simultaneously in a single multiplex PCR. These genetic tools enable the efficient and rapid authentication of inauthentic herbal materials and will be useful for the standardization of herbal medicines.

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Disclosure of conflict of interest

None.

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