Article

Differentiating Authentic Adenophorae Radix from Its Adulterants in Commercially-Processed Samples Using Multiplexed ITS Sequence-Based SCAR Markers

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Abstract: Determining the precise botanical origin of a traditional herbal medicine is important for basic quality control. In both the Chinese and Korean herbal pharmacopoeia, authentic Adenophorae Radix is defined as the roots of *Adenophora stricta* and *Adenophora triphylla*. However, the roots of *Codonopsis lanceolata*, *Codonopsis pilosula*, and *Glehnia littoralis* are frequently distributed as Adenophorae Radix in Korean herbal markets. Unfortunately, correctly identifying dried roots is difficult using conventional methods because the roots of those species are morphologically similar. Therefore, we developed DNA-based markers for the identification of authentic Adenophorae Radix and its common adulterants in commercially-processed samples. To develop a reliable method to discriminate between Adenophorae Radix and its adulterants, we sequenced the nuclear ribosomal DNA internal transcribed spacers (nrDNA-ITS) and designed sequence-characterized amplified region (SCAR) primers specific to the authentic and adulterant species. Using these primers, we developed SCAR markers for each species and established a multiplex-PCR method that can authenticate the four herbal medicines in a single PCR reaction. Furthermore, we confirmed that commercially-processed herbal medicines, which often have degraded DNA, could be assessed with our method. Therefore, our method is a reliable genetic tool to protect against adulteration and to standardize the quality of Adenophorae Radix.

Keywords: Adenophorae Radix; internal transcribed spacer (ITS); sequence characterized amplification region (SCAR) marker; multiplex PCR; molecular identification

1. Introduction

A common problem in herbal medicines is the adulteration and contamination by related and/or allied species, as well as those from unrelated genera [1]. It is difficult to identify authentic plant materials from adulterants and substitutes because of their morphological similarity, and confusion arises from the vernacular names of herbal medicines between provinces and countries [1–3]. Adenophorae Radix (*Sa-Sam* in Korean and *Nan-Sha-Shen* in Chinese) is an important herbal medicine that is prescribed as an antitussive and expectorant and has a wide range of immune-altering effects [4]. Adenophorae Radix is a representative herbal medicine that has been adulterated by both closely-related plant species and unrelated genera in both Korea and China [3–5]. In China, the roots of many *Adenophora* species belong to the Campanulaceae and related plants have been added to

Adenophorae Radix, including *Adenophora polyantha* Nakai and *Adenophora hunanensis* Nannf. (defined as a synonym of *Adenophora petiolata* subsp. *hunanensis* [Nannf.] by D. Y. Hong and S. Ge), although it is defined as the roots of only *Adenophora stricta* Miq. and *Adenophora tetraphylla* (Thunb.) Fisch. (defined as a synonym of *Adenophora triphylla* [Thun. A. DC.]) [6]. The morphological characteristics of dried roots of these inauthentic species are similar to those of authentic *A. stricata* and *A. tetraphylla* [4,7]. In addition, the roots of *Glehnia littoralis* F. Schmidt ex Miq. belong to the Apiaceae, described as Glehniae Radix in the Chinese pharmacopoeia, are also used as Adenophorae Radix because of a similar medicinal name (*Bei-sha-shen*) in China [3,7]. Even though Adenophorae Radix is described as the roots of *A. stricta* and *A. triphylla* var. *japonica* (Regel.) Hara in the Korean herbal pharmacopoeia, the roots of diverse *Adenophora* species and those of *Codonopsis lanceolata* (Siebold and Zucc.) Benth. And Hook. f. ex Trautv. (Campanulaceae) and Codonopsis Lanceolatae Radix have also been used as Adenophorae Radix in Korean herbal markets because of the morphological similarity of the species in the same genus and the incorrect or imprecise definition of the botanical origin in ancient medical literature (http://boncho.kiom.re.kr/herbarium/codex.php) [8,9]. Furthermore, because of the similar morphological features of processed herbal medicines, the roots of *Codonopsis pilosula* (Franch.) Nannf. (Campanulaceae), which is defined as Codonopsis Pilosulae Radix, may also be distributed as Adenophorae Radix in Korea [9].

In traditional medicine, Adenophorae Radix, Glehniae Radix, and Codonopsis Lanceolatae Radix have different medical properties and efficacies. In detail, Adenophorae Radix has been used to nourish the yin of the lung and stomach and generate clear heat and fluids, whereas Codonopsis Pilosulae Radix has been used to tonify the middle burner (digestive system) and to augment the spleen and lung qi. Glehniae Radix also has been used to nourish the yin and generate fluids, but has been characterized for clearing the lungs and augmenting the stomach. By contrast, Cdonopsis Lanceolatae Radix has been traditionally used to detoxify and expel pus and promote milk secretion [10]. As such, the pharmaceutical prescriptions for authentic herbal medicines require quality control of herbal medicines [1]. In previous studies, identifying the botanical origins and quality of the medicines was carried out through morphological and genetic approaches, as well as chemical analysis [3–5,9,11]. However, these approaches have limited utility for authenticating commercially-processed Adenophorae Radix in Korean herbal markets due to degradation of DNA and the removal of morphologic features during processing. Therefore, we developed additional simple and reliable molecular genetic tools for detecting the adulterants and contaminants of Adenophorae Radix.

DNA barcoding is a reliable genetic tool used to identify species and is frequently employed to study phylogenetic relationships [12–14]. This method also has been used to identify the botanical origins of herbal medicines and for discriminating between authentic product and inauthentic adulterants [13,15,16]. Over 17 DNA barcode regions can be used to identify the species of Plantae and to differentiate authentic herbal medicines [1], with the genes RuBisCO (*rbc*L) and maturase-K (*mat*K) in the chloroplast genome being commonly used [17,18]. The internal transcribed spacer (ITS) sequences within the nuclear ribosomal RNA genes (nrDNA) may be used to distinguish plants at the species level [19]. However, the nrDNA-ITS regions have been of limited use because the available primers for these sequences amplify DNA from fungi, other endophytic microorganisms, and multiple copies of the ITS regions. Recently, ITS2 has been proposed as a universal DNA barcode for identifying plant species, herbal medicines, and herbaceous dietary supplements, but it has some disadvantages, such as the complexity of the experiment, the difficulty of interpreting the results, the limitation of sample numbers, and the detection of contaminants [16,20,21]. A sequence-characterized amplified region (SCAR) marker assay can reliably and reproducibly distinguish species based on sequence information obtained from DNA fingerprinting or barcoding in diverse plants and herbal medicines. This method amplifies only target-containing samples using specific primers and differentiates positive or negative amplification of target regions, as well as length polymorphisms of target regions by gel electrophoresis of closely related samples [22–24]. Therefore, the SCAR method is simple to carry out, its results are simple to interpret, and it produces fewer errors in PCR amplification and

sequencing [21]. Furthermore, the efficiency and ability of this assay improved with the combination of individual SCAR markers and multiplex PCR, namely, multiplexed SCAR marker assays [22,25,26]. These have been abundantly and successfully applied to authenticate medicinal plant species and commercially-processed herbal medicines [22].

A genetic assay has already been developed to discriminate between authentic Adenophorae Radix species based on 28S and 5S rDNA intergenic spacer region sequences of *A. stricta*, *A. triphylla*, *A. hunanensis*, and *G. littoralis* [4]. Another molecular genetic tool can identify authentic Glehniae Radix from common herbal adulterants, including *A. triphylla* and *A. stricta*, using ITS2 DNA barcoding [3]. However, there are differences in the range of plant species that adulterate Adenophorae Radix between Korea and China. Therefore, an additional marker is needed to authenticate Adenophorae Radix and to simplify analysis. In this study, we analyzed the entire ITS sequence of six medicinal plant species, *A. stricta*, *A. triphylla*, *A. triphylla* var. *japonica*, *C. lanceolata*, *C. pilosula*, and *G. littoralis*, and developed SCAR markers to differentiate the four herbal medicines using the ITS1 region. In addition, we established a multiplexed SCAR assay for the simultaneous authentication of these herbal medicines in a single PCR reaction. Furthermore, we confirmed that commercial herbal medicines could be successfully used in the assay. Therefore, our method could be used to reliably detect adulteration and to standardize the quality of Adenophorae Radix.

2. Materials and Methods

2.1. Plant and Herbal Materials

Twenty-four plant samples were used: four samples each of *A. stricta*, *A. triphylla*, *A. triphylla* var. *japonica*, and *G. littoralis*, three samples of *C. lanceolata*, and five samples of *C. pilosula* (Table 1). Fresh leaves were collected from native habitats and plantations in different geographical locations in Korea and China, and stored at −70 °C after freezing in liquid nitrogen. Herbal medicines were purchased from different herbal markets across Korea and China. All specimens of plants and herbal medicines were deposited in the Korean Herbarium of Standard Herbal Resources (IH code KIOM) after being given voucher numbers, as shown in Table 1 and Table 4. Morphological identification was performed using guidelines from the Classification and Identification Committee of the KIOM, which has nine experts in the fields of plant taxonomy, botany, pharmacognosy, and herbology.

Table 1. Plant materials used in this study.

Name		Habitat Information	Voucher Number	Abbreviation	Lane in Gel
Scientific Name (Family)	Herbal Name				
Adenophora stricta Miq. (Campanulaceae)	Adenophorae Radix	Caoping, Li, Longnan, Gansu, China	KIOM200701000519	AS-CP	1
		Jisan, Jindo, Jeonnam, Korea	KIOM200701000873	AS-JS	2
		Guyuan, Ningxia, China	KIOM201201005303	AS-GA	3
		Sangju, Gyeongbuk, Korea	KIOM201301006488	AS-SJ	4
Adenophora triphylla (Thunb.) A. DC. (Campanulaceae)	Adenophorae Radix	Cheoncheon, Jangsu, Jeonbuk, Korea	KIOM200701000682	AT-CC	5
		Yaksan, Wando, Jeonnam, Korea	KIOM200701000741	AT-YS	6
		Daegang, Danyang, Chungbuk, Korea	KIOM201101004148	AT-DG	7
		Longjing, Yanbian, Jilin, China	KIOM201201005563	AT-LJ	8
Adenophora triphylla var. *japonica* (Regel) Hara (Campanulaceae)	Adenophorae Radix	Sancheong, Gyeongnam, Korea	KIOM201201004951	ATJ-SC	9
		Beichuan, Mianyang, Sichuan, China	KIOM201201005359	ATJ-BC	10
		Sangju, Gyeongbuk, Korea	KIOM201301006312	ATJ-SJ	11
		Gandong, Hwacheon, Gangwon, Korea	KIOM201401009347	ATJ-GD	12
Codonopsis lanceolata (Siebold & Zucc.) Benth. & Hook. f. ex Trautv. (Campanulaceae)	Codonopsis Lanceolatae Radix	Sangju, Namhae, Gyeongnam, Korea	KIOM201001002725	CL-SJ	13
		Sinan, Jeonnam, Korea	KIOM201101003615	CL-SN	14
		Geochang, Gyeongnam, Korea	KIOM201201004752	CL-GC	15
Codonopsis pilosula (Franch.) Nannf. (Campanulaceae)	Codonopsis Pilosulae Radix	Ya'an, Sichuan, China	KIOM201101004238	CP-YA	16
		Helong, Yanbian, Jilin, China	KIOM201101004335	CP-HL	17
		Girin, Inje, Gangwon, Korea	KIOM200701000339	CP-GR	18
		Jinbu, Pyeongchang, Gangwon, Korea	KIOM200801001104	CP-JB	19
		Wangqing, Yanbian, Jilin, China	KIOM201101004270	CP-WQ	20
Glehnia littoralis F. Schmidt ex Miq. (Apiaceae)	Glehniae Radix	Gujwa, Jeju, Jeju, Korea	KIOM200601000041	GL-JJ	21
		Anmyeon, Taean, Chungnam, Korea	KIOM200701000230	GL-AM	22
		Geojin, Goseong, Gangwon, Korea	KIOM200701000231	GL-GJ	23
		Jukbyeon, Uljin, Gyeongbuk, Korea	KIOM200601000062	GL-JB	24

2.2. Preparation of Genomic DNA and PCR Amplification

Genomic DNA was extracted from the herbal medicines and the fresh leaves stored at −70 °C using DNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA). The DNA concentration and purity was determined using a Nanodrop ND-1000 (Nanodrop, Wilmington, DE, USA) and electrophoresis through 1.5% agarose gel with known standards. The final concentration of the DNA samples was adjusted to approximately 15 ng/μL with TE buffer and stored at −20 °C pending PCR amplification.

The nrDNA-ITS regions were amplified using the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) with previously-reported PCR parameters [27,28]. PCR reactions were performed in 50 μL mixtures consisting of 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 DNA polymerase (SolGent, Daejeon, Korea), 0.5 μM of each primer, and 15 ng of template DNA. PCR amplification was carried out using a Proflex PCR system (Applied Biosystems, Foster City, CA, USA) with the following parameters: 95 °C for 5 min (pre-denaturation); 35 cycles of 30 s at 95 °C (denaturation), 30 s at 55 °C (annealing), and 2 min at 72 °C (extension); and a final extension for 5 min at 72 °C. PCR products were separated on 1.5% agarose gels with a 100 bp DNA ladder (SolGent, Daejeon, Korea).

2.3. Analysis of Nucleotide Sequences and Phylogenetic Relationships

The nrDNA-ITS amplicons (approximately 800 bp long) were extracted from the agarose gels using a Gel Extraction Kit (SolGent, Daejeon, Korea) and subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) for sequencing. The cloning of inserts was confirmed by colony PCR using the vector primer set, T7 and SP6, and the sequences of cloned amplicons were determined on both strands by Sanger sequencing using ITS1 and ITS4 primers and an automatic DNA sequence analyzer (ABI 3730, Applied Biosystems Inc., Foster City, CA, USA). To check the sequence quality of the amplified products for each sample, such as PCR errors, misreading, and geographical sequence variation, we obtained nucleotide sequences from five inserted DNA fragments per sample and compared their nucleotide sequences after alignment of the entire sequences using the multiple alignment tool (version 7.2.5, BioEdit, North Carolina State University, Raleigh, NC, USA) [29]. In addition, all sequences were subjected to a similarity search against the GenBank database to confirm the origins of the individual nrDNA-ITS sequences using a basic local alignment search tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast). The resulting representative nrDNA-ITS sequences of each sample were registered in the NCBI GenBank (accession numbers: *A. stricta*, KY829514-KY829517; *A. triphylla*, KY829518-KY829521; *A. triphylla* var. *japonica*, KY829522-KY829525; *C. lanceolata*, KY829526-KY829528; *C. pilosula*, KY829529-KY829533; and *G. littoralis*, KY829534-KY829537).

The final sequences of the ~800 bp nrDNA-ITS amplicons were analyzed and manually curated using the BioEdit program, version 7.2.5 [29]. The inter- and intra-species variability of all sequences was analyzed using the MEGA6 program, version 6.06, with the Kimura-2-parameter (K2P) model [30]. To analyze the phylogenetic relationships of the species targeted in this study, we inferred a phylogenetic tree using the neighbor joining (NJ) method with the K2P model, pairwise deletion for gaps/missing data treatment, and 1000 replications for bootstrapping with *Cisium japonicum* (KM051436) as an outgroup reference.

2.4. Development of the Multiplexed SCAR Marker Assay and Monitoring of Commercial Herbal Medicines

Primers specific for the four herbal medicines were designed after aligning the 24 sequences with ClustalW in BioEdit [29]. Several candidate SCAR forward primers specific to the four herbal medicines were designed, along with one common reverse primer, based on the unique indels and/or base substitutions of the aligned ITS1 region sequences. For verifying primer specificity, PCR amplification was carried out in 20 μL of reaction mix (a mix similar to the nrDNA-ITS reaction, but with 0.5 μM of each SCAR primer instead of the ITS1 and ITS4 primers). PCR amplification conditions were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 65 °C for 30 s, and 72 °C for 1 min; and a final

extension at 72 °C for 5 min. The PCR products were visualized using a 1.5% agarose gel to check the size and number of DNA fragments. To confirm the primer specificities, the DNA fragments were extracted and those sequences were compared with the original nrDNA-ITS sequences.

For establishing a multiplex assay method, four forward SCAR primers and one reverse primer, which together amplify differently sized DNA fragments for each herbal medicine, were combined in a single PCR reaction, and optimal conditions were determined by altering PCR parameters such as annealing time and temperature, and the concentration of the template and primers. The amplified PCR products were analyzed using a 1.5% agarose gel to check the number and size of amplicons. To validate the multiplex-PCR method and adulteration of Adenophorae Radix in the markets, 37 commercially-processed Adenophorae Radix samples and adulterants were purchased, and their botanical origin was verified. Approximately 15 ng of total genomic DNA was used in 20 μL multiplex-PCR assay mixtures consisting of 10 mM Tris-HCl (pH 9.0), 2.5 mM MgCl$_2$, 200 μM of each dNTP, 10 mM (NH$_4$)$_2$SO$_4$, 0.5 U Taq DNA polymerase (SolGent, Daejeon, Korea), and 0.5 μM of each primer. PCR amplification parameters were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products were visualized using 1.5% agarose gel electrophoresis to check the size and number of DNA fragments.

3. Results

3.1. Analysis of nrDNA-ITS Sequences

To analyze the variable sequences of the medicinal plants used in Adenophorae Radix and the related adulterants, we first amplified the nrDNA-ITS regions of the 24 original plant samples listed in Table 1, successfully amplifying the ~800 bp fragments. The sequences of all the PCR amplicons were determined using both T7 and SP6 primers after cloning into the pGEM-T Easy Vector system, and they were registered in GenBank with the accession numbers indicated in Materials and Methods. The length of the entire nrDNA-ITS region was 793–795 in three *Adenophora* species, 743 bp in *C. lanceolata* and *C. pilosula*, and 689 bp in *G. littoralis*. They aligned to a length of 799 bp (Table 2). The sequence variability and species-specific sequence variants of the two ITS regions were analyzed separately. The intra- and inter-species variability of the ITS1 region was 0.0000–0.0205% ± 0.0104% and 0.2760% ± 0.2279% to 0.5810% ± 0.0122%, respectively, and that of ITS2 was 0.0000–0.0198% ± 0.0114% and 0.2657% ± 0.2132% to 0.5717% ± 0.0133%, respectively (Table 2). *A. stricta* had the most diverged ITS1 and ITS2 sequences within the species (intra-specific), whereas *G. littoralis* had the most variability among the species (inter-specific) (Table 2). The intra- and inter-species variability was similar between the ITS1 and ITS2 regions, but the species-specific nucleotide substitutions, which are useful for SCAR primer design, were more numerous in ITS1, especially between *C. lanceolata* and *C. pilosula* (Figure S1 and data S1). Therefore, we used the ITS1 region for the further development of SCAR markers for Adenophorae Radix and its adulterants.

To analyze the phylogenetic relationships among the four herbal medicines and related plant species, we inferred a phylogenetic tree based on the NJ method using full-length nrDNA-ITS sequences. As a result, twenty-four samples were clustered into four groups with 100% bootstrap values consisting of monophyletic Adenophorae Radix, Cdonopsis Lanceolatae Radix, Codonopsis Pilosulae Radix, and Glehniae Radix groups (Figure S2). At the species levels, *C. lanceolata* was genetically closer to *C. pilosula* than to the other species and *G. littoralis* was most distant from the other species. Furthermore, *Adenophora* species, *A. stricta*, *A. triphylla*, and *A. triphylla* var. *japonica*, did not cluster depending on species (Figure S2). These phylogenetic results confirmed that the four herbal medicines can be distinguished using the sequence variability of nrDNA-ITS regions.

Table 2. Summary of the characteristics of nrDNA-ITS barcode sequences.

Species	Sequence Length (bp)		Intra-Species Variability (%)		Inter-Species Variability (%)	
	Constant	Aligned	ITS1	ITS2	ITS1	ITS2
A. stricta	793–795	799	0.0205 ± 0.0104	0.0198 ± 0.0114	0.2840 ± 0.2322	0.2657 ± 0.2132
A. triphylla	793–795	799	0.0111 ± 0.0062	0.0198 ± 0.0101	0.2760 ± 0.2279	0.2661 ± 0.2169
A. triphylla var. *japonica*	793–795	799	0.0092 ± 0.0061	0.0192 ± 0.0094	0.2778 ± 0.2292	0.2686 ± 0.2184
C. lanceolata	743	799	0.0000 ± 0.0000	0.0029 ± 0.0025	0.3566 ± 0.1824	0.3233 ± 0.1893
C. pilosula	743	799	0.0047 ± 0.0061	0.0035 ± 0.0045	0.3480 ± 0.1560	0.3375 ± 0.1678
G. littoralis	689	799	0.0023 ± 0.0025	0.0000 ± 0.0000	0.5810 ± 0.0122	0.5717 ± 0.0133

3.2. Development of SCAR Markers for Distinguishing Herbal Medicines

To screen the candidate sequences for locations for species-specific SCAR primers, we analyzed the entire sequence and identified several variable regions for each herbal medicine (Figure S1). We designed several forward primers matching the diverged sequences in ITS1 and one common reverse primer (ITS1 SCAR R) to the conserved 5.8S gene (Figure 1). We verified the specificity of the PCR amplification with the 24 plant samples listed in Table 1. The four SCAR primers, when combined with ITS SCAR R, amplified products with the expected sizes only in their respective samples (Table 3, Figures 1 and 2). The Adenophorae Radix–specific forward primer, AD ITS1 SCAR F, and the reverse primer, ITS1 SCAR R, produced a 231 bp amplicon only with the 12 samples from the three *Adenophora* species, whereas no PCR products were observed with the other 12 Adenophorae Radix–related plant samples (Figure 2). Therefore, this primer set differentiates Adenophorae Radix from its related herbal medicines. The other three specific forward primers, CL ITS1 ACAR F for *C. lanceolata*, CP ITS1 SCAR F for *C. pilosula*, and GL ITS1 SCAR F for *G. littoralis*, also amplified their expected 267, 167, and 124 bp products only in their respective target species, with no products detected using the other samples (Table 3 and Figure 2). Thus, these three SCAR markers distinguish individual herbal medicines from Adenophorae Radix and identify plant material at the species level.
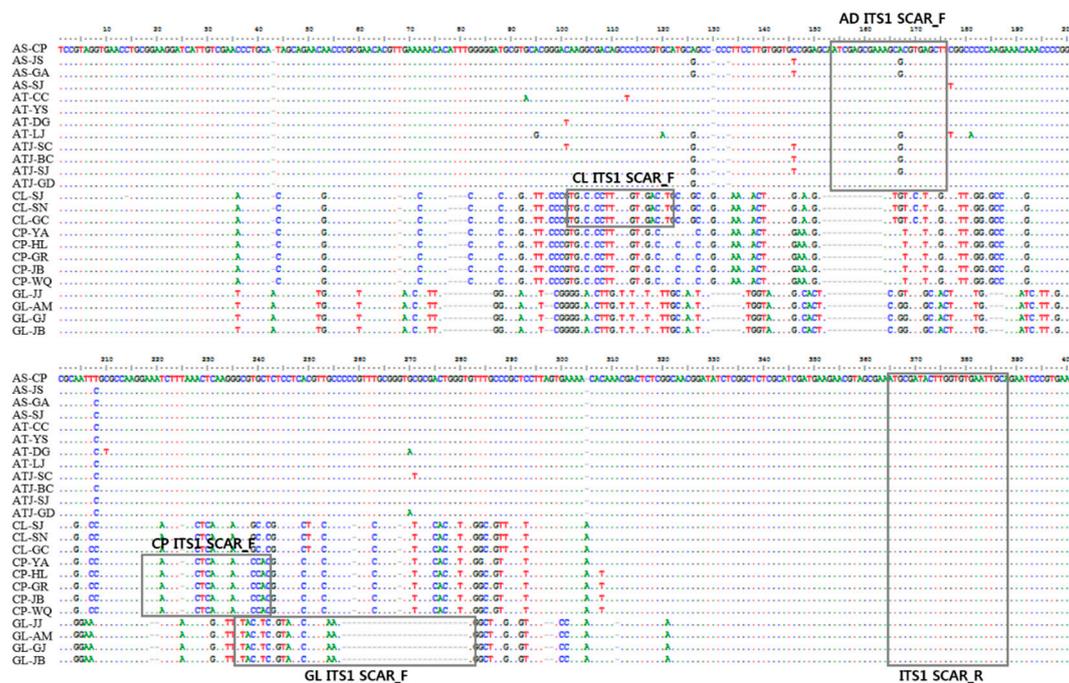

Figure 1. Comparative analysis of ITS1 region sequences and the positions of species-specific primers. Sequences were aligned using ClustalW in BioEdit (version 7.2.6). Boxes indicate the sequence of SCAR (sequence-characterized amplified region) primers used in the multiplex SCAR assay. Primer names are indicated under or above the boxes.

Table 3. Sequences of the ITS1-SCAR primers and their specificity, amplicon sequence, and amplicon size.

Primer Direction	Primer Name	Primer Sequence (5′ → 3′)	Specificity	Amplicon Size (bp)
Forward	AD ITS1 SCAR F	ATC GAG CGA AAG CGC GTG AGC T	*Adenophora* sp.	231
	CL ITS1 SCAR F	TGG CCC CTT GCC GTC GAC CT	*C. lanceolata*	267
	CP ITS1 SCAR F	AAA ACT TAA CTC AAA GAG CGC GA	*C. pilosula*	167
	GL ITS1 SCAR F	GTA CGT CCG TAT CCC GTT AAG G	*G. littoralis*	124
Reverse	ITS1 SCAR R	GCA ATT CAC ACC AAG TAT CGC AT	All samples	-

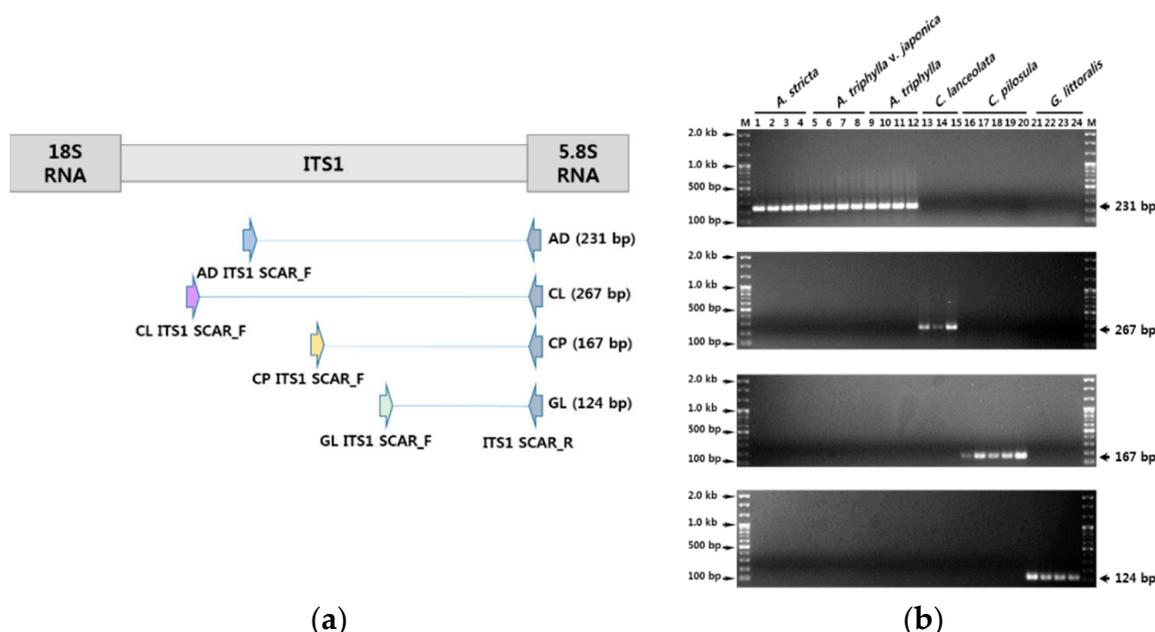

(**a**)　　　　　　　　　　　　　　　　　　　　(**b**)

Figure 2. Development of species-specific SCAR markers based on inter-species sequence variability. (**a**) Schematic diagram of primer position and SCAR amplicon sizes; and (**b**) the verification of primer specificity for Adenophorae Radix, Codonopsis Lanceolatae Radix, Codonopsis Pilosulae Radix, and Glehniae Radix. The numbers 1–24 correspond to those listed in Table 1, in the 'Lane in gel' column. The precise lengths of DNA fragments are indicated to the right side of the gel images. M represents the 100 bp DNA ladder.

3.3. Establishment of a Multiplex SCAR Amplification Method and Monitoring of Commercially-Processed Herbal Medicines

The four individual SCAR markers produced mutually uniquely-sized amplicons that could be used to differentiate the four herbal medicines in a single gel electrophoresis. Therefore, we used a multiplex-PCR assay to concurrently amplify the SCAR amplicons. The combination of five primers, namely one common reverse primer (ITS1 SCAR R) and four forward primers (AD ITS1 SCAR F, CL ITS1 SCAR F, CP ITS1 SCAR F, and GL ITS1 SCAR F), was used in a single PCR reaction. We optimized the reaction conditions and PCR parameters, obtaining the same SCAR amplicons as those from the individual SCAR primer sets (Figure 3), suggesting that this multiplex-PCR assay can distinguish the four herbal medicines in a single reaction.
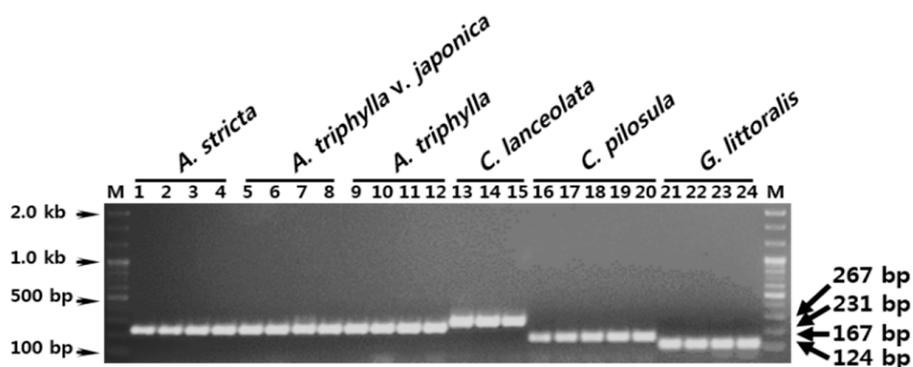
Figure 3. Establishment of a multiplex-PCR assay using species-specific SCAR markers. The numbers 1–24 correspond to those listed in Table 1. The precise lengths of DNA fragments are indicated to the right side of the gel images. M represents the 100 bp DNA ladder.

To verify the reproducibility and investigate the present state of Adenophorae Radix distribution, we assessed 37 commercial herbal medicines using the multiplex SCAR assay (Figure 4 and Table 4). As shown in Table 4, we purchased 17, nine, eight, and three samples that were listed as Adenophorae Radix, Codonopsis Lanceolatae Radix, Glehniae Radix, and Codonopsis Pilosulae Radix, respectively. Of the 37 commercial herbal medicines, eight samples of Adenophorae Radix purchased in Korean herbal markets were identified as Codonopsis Lanceolatae Radix (the roots of *C. lanceolata*) (Figure 4 and Table 4). Thus, our multiplexed SCAR assay distinguished Adenophorae Radix from adulterants in commercially-processed herbal medicines in a single reaction. Our results indicate that our ITS1-based multiplexed SCAR marker assay could be used as a reliable genetic tool to protect against adulteration and to standardize the quality of Adenophorae Radix.
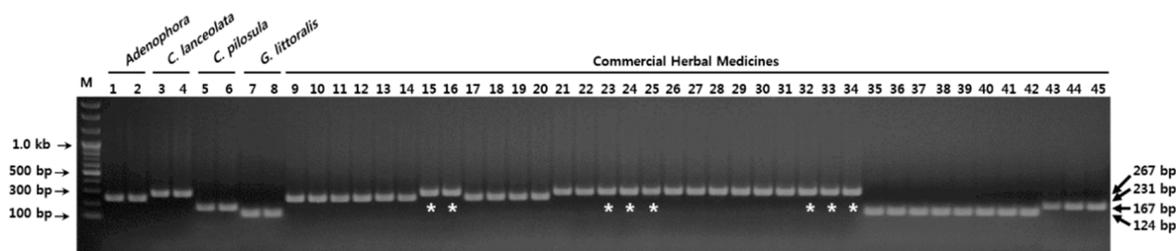

Figure 4. Monitoring of commercially processed herbal medicines using the multiplexed SCAR assay. Lanes 1–8: control plant materials used to develop SCAR markers. Lanes 9–45 correspond to the numbers 1–37 listed in Table 4, which are commercially-processed herbal medicines purchased from Chinese and Korean herbal markets. The precise lengths of DNA fragments are indicated to the right side of the gel images. M represents the 100 bp DNA ladder. Asterisks (*) mark the inauthentic commercial herbal medicines.

Table 4. Sample information and monitoring results of the 37 commercial herbal medicines assessed using our multiplexed SCAR assay.

No.	Voucher No.	Product Name	Multiplex-SCAR Result	Country
1	2-15-0678	Adenophorae Radix	Adenophorae Radix	Korea
2	2-15-0679	Adenophorae Radix	Adenophorae Radix	Korea
3	2-15-0680	Adenophorae Radix	Adenophorae Radix	Korea
4	2-15-0681	Adenophorae Radix	Adenophorae Radix	Korea
5	2-15-0682	Adenophorae Radix	Adenophorae Radix	Korea
6	2-15-0683	Adenophorae Radix	Adenophorae Radix	Korea
7	2-15-0684	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
8	2-15-0685	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
9	2-15-0705	Adenophorae Radix	Adenophorae Radix	China
10	2-15-0706	Adenophorae Radix	Adenophorae Radix	China
11	2-15-0707	Adenophorae Radix	Adenophorae Radix	China
12	2-15-0708	Adenophorae Radix	Adenophorae Radix	China
13	2-15-0711	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
14	2-15-0712	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
15	2-15-0713	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
16	2-15-0714	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
17	2-15-0715	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
18	2-15-0716	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
19	2-15-0717	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
20	2-15-0718	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
21	2-15-0719	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
22	2-15-0720	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
23	2-15-0721	Codonopsis Lanceolatae Radix	Codonopsis Lanceolatae Radix	Korea
24	2-15-0722	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
25	2-15-0723	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
26	2-16-0302	Adenophorae Radix	Codonopsis Lanceolatae Radix	Korea
27	2-15-0730	Glehniae Radix	Glehniae Radix	Korea
28	2-15-0731	Glehniae Radix	Glehniae Radix	Korea
29	2-15-0732	Glehniae Radix	Glehniae Radix	Korea
30	2-15-0733	Glehniae Radix	Glehniae Radix	Korea
31	2-15-0734	Glehniae Radix	Glehniae Radix	Korea
32	2-15-0735	Glehniae Radix	Glehniae Radix	Korea
33	2-15-0736	Glehniae Radix	Glehniae Radix	Korea
34	2-15-0737	Glehniae Radix	Glehniae Radix	Korea
35	2-17-0017	Codonopsis Pilosulae Radix	Codonopsis Pilosulae Radix	China
36	2-16-0025	Codonopsis Pilosulae Radix	Codonopsis Pilosulae Radix	China
37	2-15-0025	Codonopsis Pilosulae Radix	Codonopsis Pilosulae Radix	China

4. Discussion

Conventional identification of plants has usually relied on morphology. However, morphological features, such as the shapes of leaves, inflorescences, and flowers, are removed during commercial processing. Since accurate identification of herbal medicine based on morphological taxonomic keys is difficult, a DNA barcode system was established for accurate and reliable discrimination of plant species and botanical origins for herbal medicines [14,15,25,31]. Plant DNA barcoding led to remarkable improvements in the identification of the botanical origins of herbal materials and in the quality control of herbal medicines, but it still cannot be applied to many plant taxa because of failed PCR amplification, insufficient sequence variability between species, and incomplete sequence information [1,16,19]. To overcome these limits, the comparative analysis of genomic polymorphisms, using methods such as RAPD and AFLP, and the investigation of new DNA barcode regions using plastid genome sequencing have been conducted for a large number of medicinal plant species [15,24,32]. SCAR markers and multiplexed SCAR marker assays, developed using sequence information from genomic analyses, as well as from DNA barcodes, have become popular because the assays are simple to perform and have low analytical cost. To identify the botanical

origins of herbal medicine based on the DNA barcoding method, many experimental procedures are needed, such as DNA extraction, PCR amplification, gel electrophoresis and rescue, sub-cloning into the pGEM-T Easy vector, *E. coli* transformation and incubation, plasmid DNA purification and restriction enzyme digestion (or colony PCR), sequencing of inserted amplicons, and sequence comparison or BLAST search. These experimental processes require approximately three or four days of work. However, the SCAR-based method does not require PCR product extraction from the agarose gel, sub-cloning into the vector and transformation, plasmid DNA purification, sequencing, or even sequence analysis. Thus, this method needs only DNA extraction, PCR amplification, gel electrophoresis, and species identification, and can identify the botanical origin of herbal medicines within a few hours. Moreover, the DNA barcoding method involves additional costs for sub-cloning, transformation into the competent cell, and sequencing, which are not necessary in the SCAR method [21]. Judging from our laboratory experience, these additional costs amount to at least $300–$400 USD for the analysis of 10 herbal medicine samples. Therefore, the SCAR method is much more convenient and less expensive for the authentication of herbal medicines and for the identification of the botanical origins of herbal medicines than the DNA barcoding method [22,24,26]. In spite of these disadvantages, DNA barcoding is mightily important for plant species identification with high resolution and for obtaining primary sequence information in species-specific marker development.

The roots of several *Adenophora* species and of other related medicinal plant species, including *G. littoralis*, *C. lanceolata*, and *C. pilosula*, have different chemical properties and biological activities [3,4,11]. However, the roots of these three species are consistently mislabeled in herbal markets and used for inappropriate clinical purposes in both Korea and China. Therefore, we analyzed the plant species that have been used as authentic herbal medicines and inauthentic adulterants of Adenophorae Radix in Korea and China. Of note, *A. tetraphylla* was defined as a synonym of *A. triphylla* in a recent reconsideration of the taxonomic system for *Adenophora* Fischer, although the original plant material for Adenophorae Radix was described as the roots of *A. tetraphylla* and *A. stricta* in Chinese pharmacopoeia [6,7]. Therefore, we considered *A. tetraphylla* as *A. triphylla* in our taxonomic identification (Table 1).

Since DNA is usually degraded in commercial herbal medicines, genetic analysis must be amenable to short DNA fragments [33]. We considered this point for developing SCAR markers and designed diverse SCAR primers that amplify variable ranges of DNA fragments. We designed SCAR markers in the ITS1 region based on aligned nrDNA-ITS sequences. Sequence substitutions and indels were more abundant in ITS1 than in ITS2 (Figure S1). In fact, differences between *C. lanceolata* and *C. pilosula* were rarely identified in the ITS2 region. The SCAR primers were, therefore, designed only for the ITS1 region and selected for different amplicon sizes depending on the herbal medicine, allowing for optical discriminability and stable amplificability by the multiplexed SCAR assay (Figure 1). To assess and increase the reproducibility of single and multiplexed SCAR markers, we optimized the PCR conditions by adjusting the annealing temperature, time, and concentration of primers and templates as described in Materials and Methods. Finally, we confirmed the discriminability and amplificability of our assay by assessing the adulteration of commercially-processed Adenophorae Radix, Codonopsis Lanceolatae Radix, Glehniae Radix, and Codonopsis Pilosulae Radix samples. We successfully assessed 37 commercially-processed herbal medicines and identified eight cases of adulteration (Figure 4 and Table 4). To confirm the botanical origin and sequence identity of the PCR amplicons obtained using the multiplexed SCAR assay, PCR products were sub-cloned into the pGEM-T Easy Vector and sequenced. The resulting sequences were aligned with the entire nrDNA-ITS sequences obtained from control plant materials listed in Table 1 and were confirmed against GenBank data using BLAST searches. All the sequences were identical to the corresponding sequences of control plants listed in Table 1. These results demonstrate that our multiplexed SCAR marker assay is a useful genetic tool for differentiating between Adenophorae Radix and its commercial adulterants.

In this study, we could not identify the species used in Adenophorae Radix because the genus *Adenophora* cannot be well resolved at the species-level using universal DNA barcodes (Figures S1

and S2). In a previous study, we analyzed the nrDNA-ITS regions and the chloroplast *mat*K and *rbc*L gene sequences using several *Adenophora* species, but could not identify useful species-specific variability [34]. In addition, another study using the ITS2 sequence was unable to discriminate between species of the genus *Adenophora* [3]. We also could not differentiate three *Adenophora* species using nrDNA-ITS sequences in this study. These results confirm the low level of ribosomal sequence variation among species of the genus *Adenophora*. Therefore, to verify the accurate botanical origins of the roots of *Adenophora* species, further studies are needed to develop genetic markers for use in genomic fingerprinting analyses, such as RAPD, and comparative analyses of organellar or whole genome sequences are needed to obtain intra-species sequence information.

Supplementary Materials: The following are available online at www.mdpi.com/2076-3417/7/7/660/s1: Figure S1, Comparative analysis of nrDNA-ITS sequences used in this study; Data S1: FASTA file of nucleotide sequences of nrDNA-ITS used in this study; Figure S2: Phylogenetic relationships of medicinal plant species based on the nrDNA-ITS sequences.

Author Contributions: Byeong Cheol Moon designed and performed the experiments and wrote the manuscript; Wook Jin Kim, Kyeong Suk Han and Inkyu Park performed the experiments; and Sungyu Yang, Young Min Kang and Renzhe Piao collected and identified the plant samples.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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