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Original Article

Identification of *Acorus gramineus*, *A. calamus*, and *A. tatarinowii* using sequence characterized amplified regions (SCAR) primers for monitoring of Acori graminei rhizoma in Korean markets

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Abstract: Acori Graminei Rhizoma (AGR), widely used in traditional herbal medicine, is composed of the roots of *Acorus gramineus* Soland. The family Acoraceae includes *A. gramineus*, *A. calamus*, and *A. tatarinowii*, among others. We compared genomic DNA sequences of AGR for polymorphisms. The sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA, the *rbcL* region of chloroplast DNA from *A. gramineus*, *A. calamus*, and *A. tatarinowii* were compared. We designed primers specific to the ITS region of *A. calamus* and *A. tatarinowii* (A. cataF4/R4) and the internal primer Araceae Radix (IntAcoF2/R4). Random amplification of polymorphic DNA (RAPD) analysis showed a difference in *A. calamus* using the UBC 681 primer. A specific primer (Aca681-F/R) amplified 138 base pairs of *A. calamus*. The primers designed for this study (A. cataF4/R4, Aca681-F/R, and IntAcoF2/R2) can be used for multiplex PCR to distinguish the three species of *Acorus*. An allelic discrimination assay was conducted using commercially available AGR. We used sequence-characterized amplified region (SCAR) markers to confirm whether AGR purchased at a market was *A. gramineus*. Our study indicated the SCAR markers could be used as molecular evidence to distinguish Araceae Radix.

Keywords: Acori graminei rhizoma, Acorus calamus, Acorus gramineus, Acorus tatarinowii, monitoring, SCAR marker

Introduction

Owing to the recent import liberalization of agricultural products caused by Korea's entry into the World Trade Organization and International Union for the Protection of New Varieties of Plants, it is necessary to differentiate various species of domestic and foreign agricultural products. In particular, it is very difficult to differentiate between similar species of medicinal herbs and their places of origin by morphological observation alone because medicinal herbs are typically dried products.

Acori graminei rhizoma (AGR) originates from the root of *Acorus gramineus* Soland (Araceae) and distributed throughout Korea, China, Japan, Siberia, and Europe [1]. *Acorus gramineus* is a medicinal herb that has been referenced more than 1200 times in "Bojebang", the most comprehensive oriental prescription book [2]. Its root has been prescribed for the treatment of convulsions and stomach aches and as a sedative in oriental medicine. In addition, *Acorus gramineus* is effective against oxidation, to activate suppressive neuroceptors, to show antibacterial activity, and to act against Alzheimer's disease [3].

Acori plants include two species: *A. gramineus* and *A. calamus* L. *A. gramineus* Soland is the original species in Korea (KP), whereas *A. tatarinowii* Schott, which is similar to *A. gramineus.* Soland is the original species in China (CP). The use of *A. calamus* and *A. gramineus* is ambiguous [4]. The aerial and subterranean parts of *A. gramineus*, *A. tatarinowii*, and *A. calamus,* both are medicinal herbs, show similar morphologies. Patients should be sure to use the correct plant. However, more accurate and effective

Table 1. List of *Acorus* specimens used in this study

No.	Locality	Collection Date	Identification results
1	Jeju, Korea	2010.2	*A. gramineus*
2	Jeonbuk, Korea	2010.2	*A. gramineus*
3	Jeju, Korea	2010.2	*A. gramineus*
4	Seoul, Korea	2010.2	*A. gramineus*
5	Banan, China	2010.2	*A. gramineus*
6	Guangxi, China	2002.7	*A. gramineus*
7	Szechuan, China	2010.2	*A. calamus*
8	Gyeongju, Korea	2010.2	*A. calamus*
9	Seoul, Korea	2002.9	*A. calamus*
10	Guangxi, China	2002.9	*A. calamus*
11	Bonghwa, Korea	2010.2	*A. calamus*
12	Uiseong, Korea	2002.7	*A. calamus*
13	Zhejiang, China	2010.2	*A. tatarinowii*
14	Nanjing, China	2010.7	*A. tatarinowii*
15	Nanjing, China	2010.7	*A. tatarinowii*
16	Changsha, China	2010.7	*A. tatarinowii*

Table 2. Amplification and sequencing primers used in this study

Primer name	Direction	Sequence (5' to 3')
ITSp 1	Forward	TACCGATTGAATGRTCCG
ITS 4	Reverse	TCCTCCGCTTATTGATATGC
rbcL-1F	Forward	ATGTCACCACAAACAGAAAC
rbcL-1352R	Reverse	CAGCAACTAGTTCAGGRCTCC

screening methods need to develop before this is feasible.

With recent advances in analytical methods in molecular biology such as PCR and sequence analysis, more accurate and objective methods for classifying medicinal herbs have been developed [5, 6]. Of these, randomly amplified polymorphic DNA (RAPD) has been widely used for accurately identifying plant species [7, 8]. In particular, with the introduction of more accurate and stable sequence-characterized, amplified region (SCAR) markers developed using species-specific amplified products obtained from RAPD analysis [9], studies involving screening of medicinal herbs have been actively conducted both domestically and internationally.

The objective of this study was to analyze the genetic diversity of Korean *Acorus* using RAPD markers and to examine the efficacy of SCAR markers for distinguishing members of the genus *Acorus* [10, 11]. Monitoring of AGR purchased in Korean markets using genomic DNA markers has not been conducted previously.

Accordingly, our goals were to establish a screening method and monitoring system at the genomic DNA level by developing markers using RAPD analysis to prevent the misuse and mix use of A. gramineus, and to identify its inappropriate use.

Materials and methods

Plant materials

A total 16 *Acorus* samples were used in this study. Six of which were *A. gramineus*, six were *A. calamus*, and four were *A. tatarinowii* (**Table 1**). Materials came from dried roots or purchased from commercial suppliers in Korea and China. These samples were preserved at the Korea Institute of Oriental Medicine.

DNA extraction

Total DNA was extracted from the *Acorus* samples and purified using a Nucleospin® plant II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. DNA was quantified using an ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE, USA).

ITS and rbcL sequence analysis

ITS regions were amplified from total DNA by PCR. The ITS region was amplified using the primers ITSp 1 and ITS4 (**Table 2**) (White et al., 1990). For a 25 μL PCR reaction, 1 μL of genomic DNA (~ 20 ng) was added to 12.5 μL PrimeSTAR HS Premix (Takara, Shiga, Japan) containing 0.63 U PrimeSTAR HS DNA Polymerase, 0.4 mM dNTP mixture, 2 mM PrimeSTAR Buffer, and 1 μL forward and reverse primers (10 pM). PCR was performed using a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the following program: denaturation was followed by 30 cycles of 10 s at 98°C, 15 s at 50°C with a ramp time of 1°C/s, 1 min at 72°C, and 5 min of final extension at 72°C. The PCR product was purified

Table 3. List of the sequences of the UBC 10-mer oligonucleotide primers used for RAPD analysis

Primer sequences					
No.	Sequences (5' to 3')	No.	Sequences (5' to 3')	No.	Sequences (5' to 3')
UBC-001	CCT GGG CTT C	UBC-315	GGT CTC CTA G	UBC-491	TCC TGT CAA G
UBC-011	CCC CCC TTT A	UBC-321	ATC TAG GGA C	UBC-495	CTT TCC TTC C
UBC-021	ACC GGG TTT C	UBC-325	TCT AAG CTC G	UBC-501	CGG ATA TAC C
UBC-030	CCG GCC TTA G	UBC-331	GCC TAG TCA C	UBC-511	GAA TGG TGA G
UBC-040	TTA CCT GGG C	UBC-335	TGG ACC ACC C	UBC-521	CCG CCC CAC T
UBC-051	CTA CCC GTG C	UBC-341	CTG GGG CCG T	UBC-531	GCT CAC TGT T
UBC-060	TTG GCC GAG C	UBC-345	GCG TGA CCC G	UBC-541	GCC CCT TTA C
UBC-071	GAG GGC GAG G	UBC-351	CTC CCG GTG G	UBC-551	GGA AGT CCA C
UBC-080	GTG CTC TAG A	UBC-355	GTA TGG GGC T	UBC-561	CAT AAC GAC C
UBC-100	ATC GGG TCC G	UBC-361	GCG AGG TGC T	UBC-571	GCG CGG CAC T
UBC-101	GCG GCT GGA G	UBC-365	TAG ACA GAG G	UBC-581	CCC GTT AAG G
UBC-111	AGT AGA CGG G	UBC-371	TCT CGA TTG C	UBC-591	TCC CTC GTG G
UBC-121	ATA CAG GGA G	UBC-376	CAG GAC ATC G	UBC-601	CCG CCC ACT G
UBC-131	GAA ACA GCG T	UBC-381	ATG AGT CCT G	UBC-611	CCA TCG TAC C
UBC-141	ATC CTG TTC G	UBC-386	TGT AAG CTC G	UBC-621	GTC TGC GCT A
UBC-151	GTC GTA GTG T	UBC-391	GCG AAC CTC G	UBC-631	GGC TTA ACC G
UBC-161	CGT TAT CTC G	UBC-395	TCA CTT GAG G	UBC-641	TGG AAC CAT G
UBC-171	TGA CCC CTC C	UBC-401	TAG GAC AGT C	UBC-651	TCA TTT CGC C
UBC-181	ATG ACG ACG G	UBC-405	CTC TCG TGC G	UBC-661	CCT GCT TAC G
UBC-191	CGA TGG CTT T	UBC-411	GAG GCC CGT T	UBC-671	CAT TAA GGC G
UBC-201	CTG GGG ATT T	UBC-415	GTT CCA GCA G	UBC-681	CCC CCG GAC T
UBC-211	GAA GCG CGA T	UBC-421	ACG GCC CAC C	UBC-691	AAA CCA GGC G
UBC-221	CCC GTC AAT A	UBC-425	CGT CGG GCC T	UBC-701	CCC ACA ACC C
UBC-231	AGG GAG TTC C	UBC-431	CTG CGG GTC A	UBC-711	CCC TCT CCC T
UBC-241	GCC CGA CGC G	UBC-435	CTA GTA GGG G	UBC-721	CCC TTC CCT C
UBC-251	CTT GAC GGG G	UBC-441	CTG CGT TCT T	UBC-731	CCC ACA CCA C
UBC-261	CTG GCG TGA C	UBC-445	TAG CAG CTT G	UBC-741	CCT CCC TCT C
UBC-271	GCC ATC AAG A	UBC-451	CTA ATC TCG C	UBC-751	CCC ACC ACA C
UBC-281	GAG AGT GGA A	UBC-455	AGC AAG CCG G	UBC-761	GAG AGG AGG G
UBC-291	AGC TGA AGA G	UBC-461	CCC GTA TGT C	UBC-771	CCC TCC TCC C
UBC-301	CGG TGG CGA A	UBC-471	CCG ACC GGA A	UBC-781	GGG AAG AAG G
UBC-305	GCT GGT ACC C	UBC-481	GTA ATT GCG C	UBC-791	GTG GGT TGT G
UBC-311	GGT AAC CGT A	UBC-485	AGA ATA GGG C		

using a PCR purification kit (LaboPass™ PCR; Cosmo Genetech, Seoul, Korea), according to the manufacturer's instructions. Cycle sequencing and the sequencing reactions were performed by an external company (Solgent, Daejeon, Korea). All sequences were edited using Chromas (Technelysium, South Brisbane, Australia) and aligned using the ClustalW algorithm of the software MEGA3. All sequence distances were calculated using MEGA3. Additionally, a dendrogram phylogenetic tree was constructed according to Kimura2-parameters.

RAPD analysis

RAPD amplification using 95 primers (**Table 3**) was repeated twice. The amplification was performed according to the protocol previously described by Williams. Briefly, amplification was performed with 1 μL of genomic DNA (~ 20 ng) in a 20 μL reaction volume containing 10 μL 2 × EF-Taq Premix (Solgent) and 2 μL 10 pM primer (UBC, Canada). PCR was performed using a C1000™ Thermal Cycler (Bio-Rad) under the following conditions: initiation with 5 min of denaturation at 95°C, followed by 35

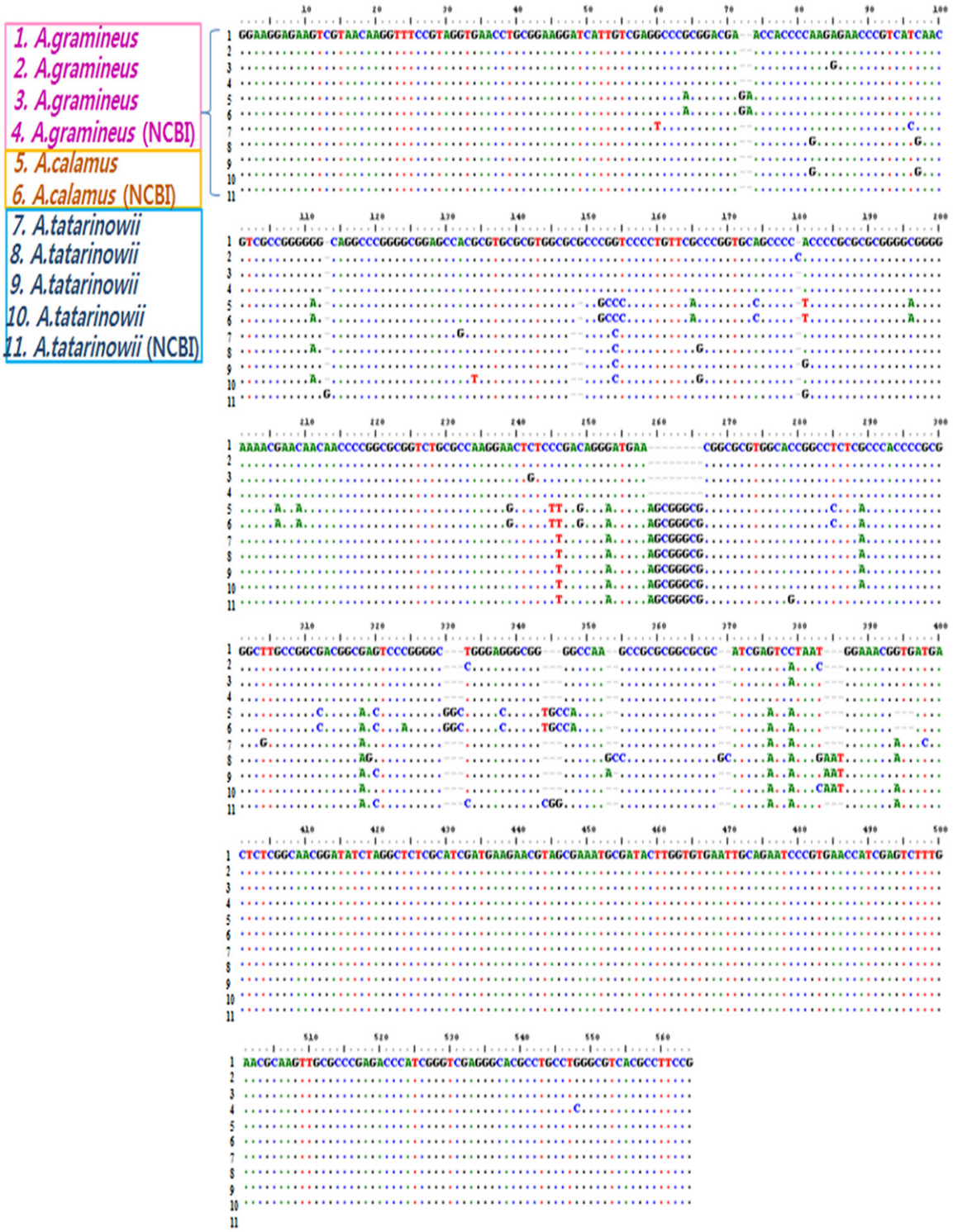

Figure 1. Partial ITS gene sequence alignment for *Acorus gramineus*, *A. calamus,* and *A. tatarinowii* compared with sequences from the NCBI database.

cycles of 40 s at 95°C, 30 s at 38°C with a ramp time of 1°C/s, 1 min at 72°C, and 5 min of final extension at 72°C. PCR products were analyzed on 1.5% agarose gels at 130 V constant voltage for approximately 40 min, visualized under ultraviolet light and photographed.

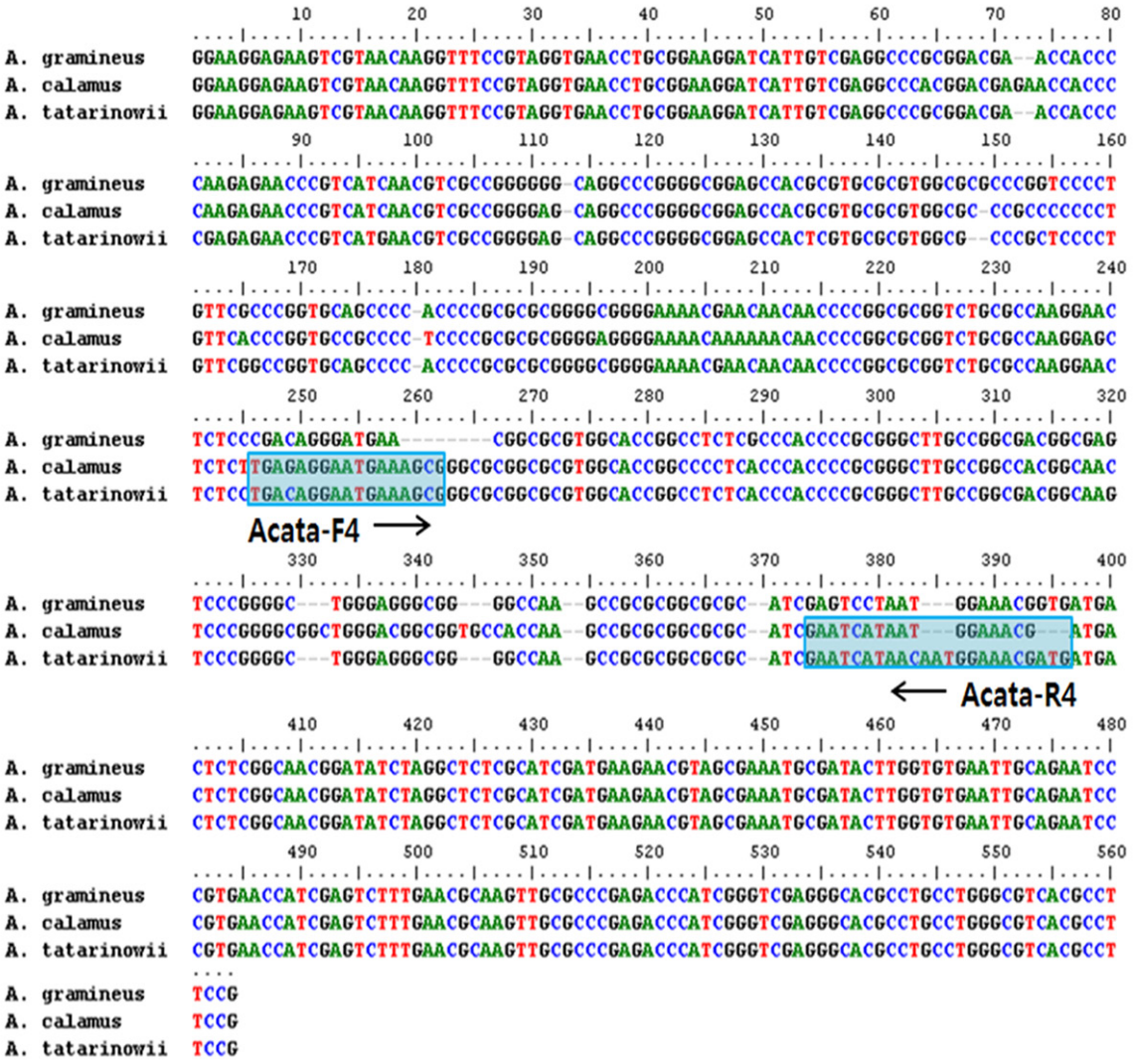

Figure 2. Alignment of ITS sequences from *Acorus gramineus*, *A. calamus,* and *A. tatarinowii,* showing the sequence used to design the primer pair.

SCAR analysis

The diagnostic band for *A. calamus* amplified using RAPD primer UBC-681 (5'-CCCCCG-GACT-3'), which was approximately 246 bp in length, was purified using a LaboPass™ gel extraction kit (Cosmo Genetech). Cycle sequencing and the sequencing reaction itself were performed by an external company (Solgent). These sequences were used to construct alignments based on SCAR markers, and primers for Aca681-F/R and were designed using Primer Express (Bioneer, Daejeon, Korea). The PCR conditions for each 20 μL reaction were as follows: 1 μL of genomic DNA (~ 20 ng) was added to 10 μL 2 × EF-Taq Premix (Solgent) and 1 μL each of 10-pM primers. PCR was performed using a C1000™ Thermal Cycler (Bio-Rad), with 5 min of denaturation at 95°C, 30 cycles of 40 s at 95°C, 30 s at 55°C with a ramp time of 1°C/s, 1 min at 72°C, and 5 min of a final extension at 72.

Results

ITS sequence analysis and the design of specific primers for distinguishing A. calamus and A. tatarinowii from A. gramineus

Amplified ITS sequences, 564 bp long, from the 3 species of *Acori* were compared. Compared to *A. gramineus*, the sequence of *A. calamus* showed 27 bp of substitutions, 16 bp of insertions, and 4 bp of deletions, which correlates to

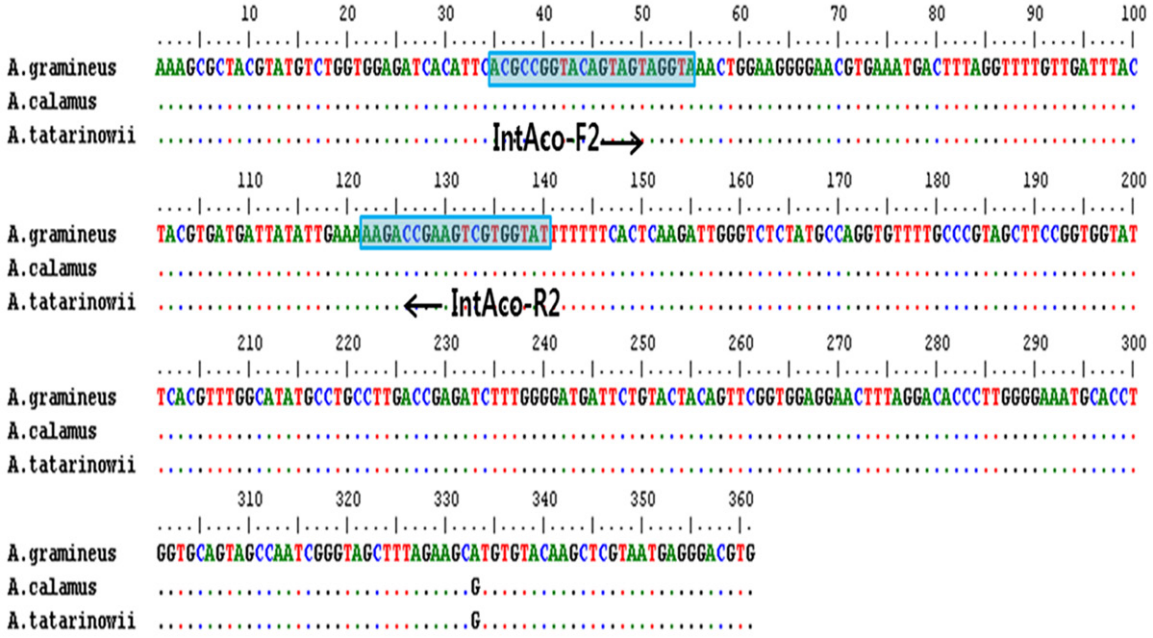

Figure 3. Comparison of rbcL sequences from *Acorus gramineus*, *Acorus calamus,* and *Acorus tatarinowii* showing the sequences used to design the internal primer pair (IntAco-F2/R2).

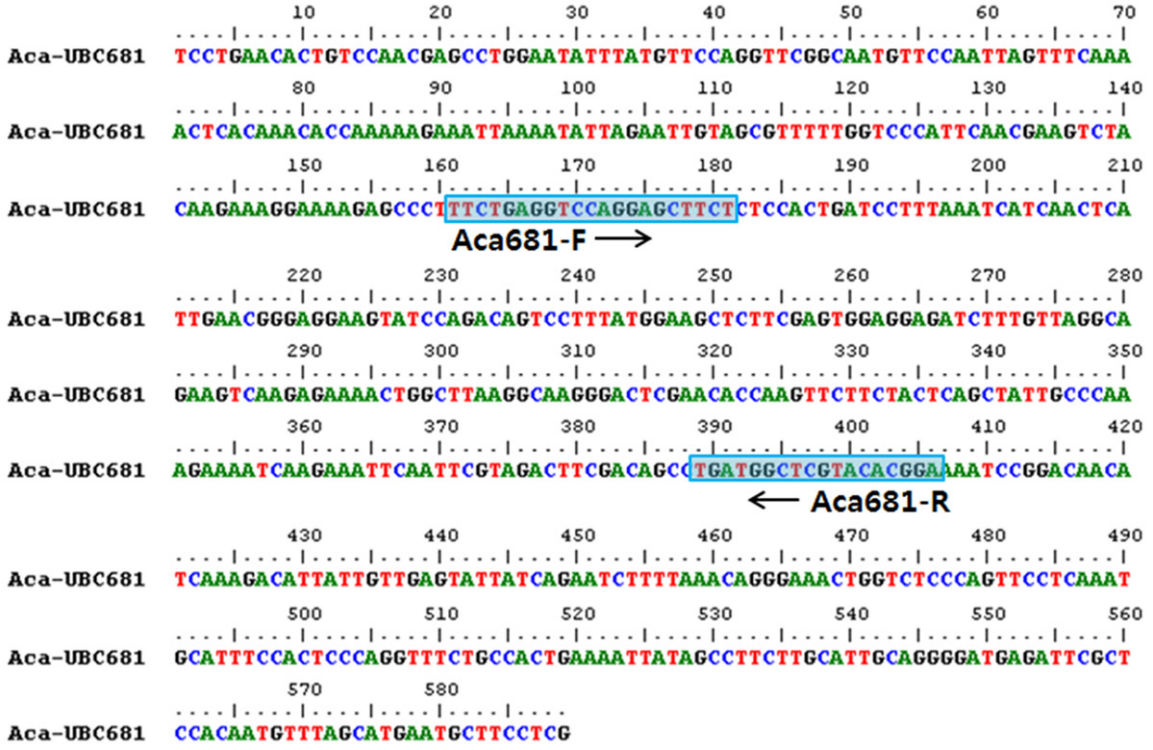

Figure 4. Characteristics of specific primers designed for discrimination of *A. calamus*. Sequence of *A. calamus* amplified by the UBC-681 RAPD primer and the *A. calamus*-specific RAPD amplicon (Aca681-F/R).

92% (517 bp/564 bp) homology. Additionally, 15 bp of substitutions, 15 bp of insertions, and 2 bp of deletions were found in *A. tatarinowii*, correlating with 94% (532 bp/564 bp) homolo-

Table 4. The sequence of the primers designed in this study

Primer name	Direction	Sequence (5' to 3')
Aca681-F	Forward	TTC TGA GGT CCA GGA GCT TCT
Aca681-R	Reverse	TCC GTG TAC GAG CCA TCA
Acata-F4	Forward	TGA CAG GAA TGA AAG CGG
Acata-R4	Reverse	CAT CGT TTC CAT TCT TAT GAT TC
IntAco-F2	Forward	ACG CCG GTA CAG TAG TAG GTA
IntAco-R2	Reverse	ATA CCA CGA CTT CGG TCT T

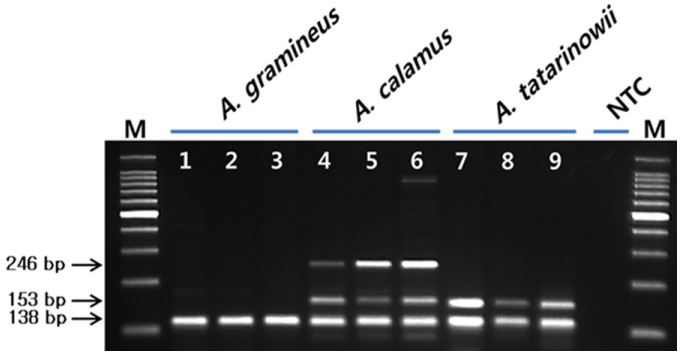

Figure 5. PCR profile using Acata F4/R4 primer designed from the partial ITS gene sequence and Aca681-F/R primer designed form RAPD analysis using the random primer UBC 681 (M: size marker, 1-3: *Acorus gramineus*, 4-6: *A. calamus*, 7-9: *A. tatarinowii*, NTC: No Template Control).

gy (**Figure 1**). Analysis of the phylogenetic tree constructed using ITS sequences showed that *Acori* can be classified into 3 groups: *A. gramineus*, *A. calamus*, and *A. tatarinowii*. *A. gramineus* was shown to be more closely related to *A. tatarinowii* than to *A. calamus* (data not shown).

To distinguish between the 3 species of *Acori*, ITS-specific primers were designed and tested. However, the specific primers proved to be inappropriate for distinguishing between the 3 species because of low reproducibility. Accordingly, a primer recognizing the same locus in both *A. calamus* and *A. tatarinowii* was designed (**Figure 2**). This specific primer was confirmed to be suitable for distinguishing *A. calamus* and *A. tatarinowii* from *A. gramineus*.

Analysis of the rbcL sequence from chloroplast DNA and design of internal primers

Analysis of nucleotide sequences amplified using primers for the rbcL region of chloroplast DNA (cpDNA) showed that the size of the amplified fragment was 523 bp in *A. gramineus*, 437 bp in *A. calamus*, and 380 bp in *A. tatarinowii*. The rbcL region showed no intraspecific nucleotide variation, at the interspecific level, the sequences shared 99% homology, with a 1 bp variation. Thus, the rbcL region of cpDNA was observed to be highly homologous between species, and we designed a pair of primers common to the 3 species of *Acori* based on this region (**Figure 3**).

Development of a pair of specific primers for identifying A. calamus by RAPD analysis

To identify *A. calamus*, a pair of specific primers (Aca681-F/R) was designed based on analysis of the sequencing results from the UBC 681 primer. This primer pair produced a 246 bp amplicon and only recognized *A. calamus* (**Figure 4**). Additionally, a pair of specific primers (Aca681-F/R) was confirmed for all samples.

Multiplex PCR analysis

We developed primers that could be used to simultaneously distinguish between *A. gramineus*, *A. calamus*, and *A. tatarinowii*. Six primers were designed, including A. cata-F4 and A. cata-R4, which amplifies sequences in both *A. calamus* and *A. tatarinowii*, Aca681-F and Aca681-R, which amplifies sequence in *A. calamus*, and IntAco-F2 and IntAco-R2, which simultaneously amplifies sequences in all 3 species (**Table 4**). When multiplex PCR was conducted using all 6 primers, 246 bp and 153 bp bands were observed in *A. calamus*, a 153 bp band was observed in *A. tatarinowii*, and an identical 138 bp band was observed in all 3 species (**Figure 5**). These results confirmed that the specific primers developed in this study can be used to distinguish between the 3 species of *Acori* using multiplex PCR. Additionally, optimal PCR conditions were determined.

Monitoring

For monitoring, an allelic discrimination assay was used. This method was also applied to commercially distributed AGR (monitoring no. 1-69) in Korea. A total 52 of monitoring sam-

Table 5. Monitoring of Acori Graminei Rhizoma purchased from Korean markets

Monitoring No.	Identification Results	Monitoring No.	Identification Results	Monitoring No.	Identification Results
1	*A. gramineus*	24	*A. tatarinowii*	47	*A. gramineus*
2	*A. calamus*	25	*A. gramineus*	48	*A. gramineus*
3	*A. tatarinowii*	26	*A. gramineus*	49	*A. gramineus*
4	*A. gramineus*	27	*A. gramineus*	50	*A. gramineus*
5	*A. gramineus*	28	*A. gramineus*	51	*A. tatarinowii*
6	*A. gramineus*	29	*A. gramineus*	52	*A. gramineus*
7	*A. gramineus*	30	*A. gramineus*	53	*A. gramineus*
8	*A. gramineus*	31	*A. gramineus*	54	*A. gramineus*
9	*A. gramineus*	32	*A. gramineus*	55	*A. gramineus*
10	*A. gramineus*	33	*A. gramineus*	56	*A. tatarinowii*
11	*A. tatarinowii*	34	*A. gramineus*	57	*A. gramineus*
12	*A. gramineus*	35	*A. gramineus*	58	*A. calamus*
13	*A. gramineus*	36	*A. gramineus*	59	*A. calamus*
14	*A. gramineus*	37	*A. tatarinowii*	60	*A. calamus*
15	*A. tatarinowii*	38	*A. gramineus*	61	*A. calamus*
16	*A. gramineus*	39	*A. gramineus*	62	*A. gramineus*
17	*A. gramineus*	40	*A. gramineus*	63	*A. calamus*
18	*A. calamus*	41	*A. gramineus*	64	*A. tatarinowii*
19	*A. gramineus*	42	*A. gramineus*	65	*A. gramineus*
20	*A. gramineus*	43	*A. gramineus*	66	*A. gramineus*
21	*A. gramineus*	44	*A. gramineus*	67	*A. tatarinowii*
22	*A. gramineus*	45	*A. gramineus*	68	*A. gramineus*
23	*A. gramineus*	46	*A. tatarinowii*	69	*A. gramineus*

ples clustered with the sequence of *A. gramineus*, 7 monitoring samples clustered with the sequence of *A. calamus*, and 10 monitoring samples clustered with the sequence of *A. tatarinowii* (**Table 5**). All samples had been sold as *A. gramineus* in the commercial market. These results suggest that our method can be used to identify AGR in the Korean market. The SCAR marker was used to confirm that identification of AGR in the market is possible.

Discussion

Analysis of nuclear ribosomal DNA sequence data showed that the ITS regions of *A. gramineus*, *A. calamus*, and *A. tatarinowii* are 601 bp, 660 bp, and 624 bp long, respectively. When these ITS regions were compared with nucleotide sequences from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) using a BLAST search, *A. gramineus* was confirmed to share 99% homology (600 bp/601 bp) with NCBI *A. gramineus* DQ008849, with only a single one-bp substitution. No nucleotide sequence variation was observed among the 6 samples of *A. gramineus*. *A. calamus* share 99% homology (550 bp/551 bp) with NCBI *A. calamus* DQ008848, with only a single one-bp substitution. No nucleotide sequence variation was observed among the six samples of *A. calamus*. Samples of *A. tatarinowii* were confirmed to share 95-97% homology (529-536 bp/552 bp) with NCBI *A. tatarinowii* DQ008845. Because each sample showed substitutions and insertions of 16-23 bp in diverse locations, *A. tatarinowii* appears likely to contain a large number of intraspecific nucleotide polymorphisms.

Identifying the origin of medicinal herbs is very important because their origin is relevant to the effects of the herbs. However, nearly all imported medicinal herbs are dried or processed, making it difficult to distinguish between similar species and production regions based on appearance. In this study, we developed specific primers that can be used to distinguish between three species of herbs in the genus *Acorus* using multiplex PCR, along with optimal PCR conditions for their use. These specific

primers can be used to identify both dried and fresh plants. Additionally, a survey of samples purchased at markets in Korea using these primers confirmed the incorrect labeling of *A. gramineus* bands and showed that most plants from the Acorus are *A. gramineus* in the Korean market.

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Disclosure of conflict of interest

None.

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