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ORIGINAL ARTICLE

Discrimination of Korean *Rehmannia glutinosa* from Chinese *Rehmannia glutinosa* Using Sequence-Characterized Amplified Region Marker

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Abstract *Rehmanniae* Radix, from the roots of *Rehmannia glutinosa* Libosch has been used in traditional herb medicine for the treatment of fever and strengthening liver function, among others. Information on the phylogenetic relationship is very limited in the region of its cultivation. It is very important to know the information of the close relatives of *R. glutinosa* Libosch and *R. glutinosa* Libosch. f. *hueichingensis* Hsiao, *R. glutinosa* produced in Wen County, Meng County, Bo'ai County, Qinyang County in Henan province, China. In this study, we examined the polymorphism analysis of *Rehmanniae* Radix originated from both Korea and China to compare the difference at the genomic DNA level. Results revealed that ITS and rps16 region sequences of *R. glutinosa* in Korea and *R. glutinosa* in China were correspond, while randomly amplified polymorphic DNA analysis showed a difference in UBC 301 primer. The specific primer designed was amplified at 334 bp for *R. glutinosa* originated from China. This primer (HRgF and HRgR) would be used efficiently to distinguish *R. glutinosa* from different sources.

Keywords randomly amplified polymorphic DNA analysis · *Rehmannia glutinosa* · *Rehmanniae* Radix · sequence characterized amplified regions marker

Introduction

Rehmannia glutinosa Libosch is a perennial plant belonging to the family Scrophulariaceae, endemic to China. This species is widely distributed in China, Korea, Japan, Vietnam, among others. (Seong et al., 1996). *R. glutinosa*, one of the important medicinal plants, has been used as a traditional Chinese medicine for thousands of years and is an important herbal medicine ingredient most commonly used in the prescription of samul-tang (四物湯 in Chinese) (Heojun, 2006). Presently, it is used for treatments of nervous breakdown, diabetes, hypertension, hematopoietic, immuno-enhancing, and tonic purposes (Zhang, 2008; Poon, 2011). The original *R. glutinosa* is defined as the root of *R. glutinosa* Libosch libschitz var. purpurea Makino in the Korea Pharmacopoeia (9th edition) (KFDA, 2007), the China Pharmacopoeia (CP, 2010) and the Japan Pharmacopoeia (JP, 2011). However, *R. glutinosa* produced in Onhyeon, Muchuck, Barkae, Shenyang and Maenghyeon of Hanan province in China, is exceptionally named as *R. glutinosa* Libosch f. *hueichingensis*. *R. glutinosa* f. *hueichingensis* has characteristics of rich essential oil composition, thin shell, asymmetric root thickness, and chrysanthemum-shaped lead. It is classified as non-variant, and both *R. glutinosa* f. *hueichingensis* and *R. glutinosa* are classified as *R. glutinosa*. Presently, *R. glutinosa* f. *hueichingensis* is sold in China and exported in large quantity without any distinction from *R. glutinosa*.

The sequence analysis of 18S rRNA gene, and taxonomic studies have been actively conducted (Kim, 2000; Bae, 2006). On the other hand, *R. glutinosa* f. *hueichingensis* have been rarely studied. The relationship between *R. glutinosa* f. *hueichingensis* and *R. glutinosa* in genomic DNA level has not been established yet. In particular, study on the development of DNA markers to distinguish Korean *R. glutinosa* from Chinese *R. glutinosa* (particularly *R. glutinosa* f. *hueichingensis*) has not been conducted, despite the difficulty of distinguishing *R. glutinosa* and *R. glutinosa* f. *hueichingensis* in the market. Therefore, the present study was conducted to analyze whole genomic DNA of both Korean and Chinese *R. glutinosa* by DNA fingerprinting analysis to identify the place-of-origin of *R. glutinosa* distributed in the market.

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Materials and Methods

Plant materials. Thirty-three samples were used, of which eight samples of fresh *R. glutinosa* were collected from habitats and farms in Korea, seven samples of *R. glutinosa* f. hueichingensis from farms in China (Table 1). These samples were identified by Je Hyun Lee, professor of herbal oriental medicine, College of Oriental Medicine, Dongguk University, and deposited in the Korea Institute of Oriental Medicine (KIOM). For monitoring, eighteen samples of Rehmanniae Radix (d-R.g-1–18) were collected from local markets in Korea and China.

DNA extraction. Total DNA from the *R. glutinosa* was extracted and purified using a Nucleospin® plant II kit (MACHEREY-NAGEL, Neumann-Neander, Germany) according to the manufacturer's protocol. The DNA quantification was determined using ND-1000 spectrophotometer (NanoDrop Tech, Wilmington, DE) and was diluted to 20 ng/μL or lower. DNA purity measured as a ratio of A_{260}/A_{280} was 1.5 or higher.

ITS and rps16 sequence analysis. The Internal transcribed spacer (ITS) region of nuclear DNA (nDNA) and the Ribosomal protein S16 gene (rps16) intron of chloroplast DNA (cpDNA) were amplified from total DNA using the polymerase chain reaction (PCR). The ITS region was amplified using primers ITSp 1 (5'-TACCGATTGAATGRTCCG-3') and ITS4 (5'-TCCTCCGC TTATTGATATGC-3') (White et al., 1990). Primers used for amplification of rps16 region, rps16-F (5'-GATACGATCCATTGT GG-3') and rps16-R (5'-GTGCAACAATTCGATAGAC-3'), were made on the basis of *R. glutinosa* (FJ770249) registered in the NCBI. For a 25-μL polymerase chain reaction (PCR) reaction, 1 μL of genomic DNA (~20 ng) was added to 12.5 μL PrimeSTAR HS Premix (Takara, Otsu, Japan) containing 0.63 U PrimeSTAR HS DNA Polymerase, 0.4 mM dNTP Mixture, 2 mM PrimeSTAR Buffer and 1 μL each forward- and reverse- primers (10 pM). PCR was carried out with a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA) following the instruction of the manufacturer: PCR was initiated by 5 min of pre-denaturation at 98°C, 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 final extension at 72°C. The PCR product was purified with a ready to use PCR purification kit (LaboPass™ PCR, Cosmo Genetech, Seoul, Korea) in accordance to the manufacturer's protocol. The cycle sequencing and the sequence reaction were performed by an external company (Solgent, Daejeon, Korea). All sequences were aligned with the BioEdit program version 7.0.9 and DNASIS MAX Version 2.5 (MiraiBio, San Francisco, CA) program. All sequence distances were calculated with DNASIS MAX Version 2.5. Additionally, a dendrogram phylogenetic tree was calculated according to Kimura2-parameter. Each sequence was subjected to comparative analysis using Database of BLAST search network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of National Center for Biotechnology of Information (NCBI).

Randomly amplified polymorphic DNA (RAPD) analysis. RAPD amplification with 48 UBC primers (University of British Columbia, Vancouver, Canada), was repeated two times (Table 2.). The amplification was performed according to the protocol of Williams (Williams, 1990; Shin, 2009). RAPD was carried out with 1 μL of genomic DNA (~20 ng) in a 20-μL reaction vial containing 10 μL 2X EF-Taq Premix (Solgent) and 2 μL of 10 pM primer. The PCR was carried out with a C1000™ Thermal Cycler (Bio-Rad) as follows; PCR was initiated with 5 min at 95°C followed by 35 cycle 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 a constant voltage of 130 V for approximately 40 min, visualized with ultraviolet light, and photographed.

Analysis of sequence-characterized amplified region (SCAR). The diagnostic band of *R. glutinosa* from China with RAPD-

Table 1 List of Rehmannia Radix

No.	Species or Latin name	Collection place	Collection Date
f-R.g-1	*R.glutinosa* Libosch (Fresh Rehmannia root)	Korea	09.06.29
f-R.g-2		Korea	09.06.30
f-R.g-3		Korea	09.06.31
f-R.g-4		Korea	09.06.13
f-R.g-5		Korea	09.07.03
f-R.g-6		Korea	09.07.07
f-R.g-7		Korea	09.07.07
f-R.g-8		Korea	09.07.07
d-R.g-1	Rehmannia Radix (Commercial dried root)	Korea	09.06.24
d-R.g-2		Korea	09.07.24
d-R.g-3		Korea	09.08.14
d-R.g-4		Korea	09.08.14
d-R.g-5		Korea	09.08.14
d-R.g-6		Korea	09.08.14
d-R.g-7		Korea	09.08.14
d-R.g-8		Korea	09.08.14
d-R.g-9		Korea	09.08.14
d-R.g-10		China	08.06.02
d-R.g-11		China	09.06.24
d-R.g-12		China	09.06.24
d-R.g-13		China	09.06.29
d-R.g-14		China	09.06.29
d-R.g-15		China	09.08.14
d-R.g-16		China	09.08.14
d-R.g-17		China	09.08.14
d-R.g-18		China	09.08.14
h-R.g-1	*R. glutinosa* Libosch f.*huechigensis** (Fresh Rehmannia root)	China	09.10.01
h-R.g-2		China	09.10.01
h-R.g-3		China	10.04.09
h-R.g-4		China	10.04.09
h-R.g-5		China	10.04.09
h-R.g-6		China	10.04.09
h-R.g-7		China	10.04.09

*: *R. glutinosa Libosch* f. *hueichingensis* is *R. glutinosa* produced in Wen County, Meng County, Bo'ai County, Qinyang County in Henan province, China (Zhang et al., 2008).

Table 2 UBC primers selected for RAPD analysis

Primer No.	Sequence (5'-3')	Primer No.	Sequence (5'-3')	Primer No.	Sequence (5'-3')
UBC-1	CCTGGGCTTC	UBC-301	CGGTGGCGAA	UBC-531	GCTCACTGTT
UBC-11	CCCCCCTTTA	UBC-305	GCTGGTACCC	UBC-541	GCCCCTTTAC
UBC-21	ACCGGGTTTC	UBC-311	GGTAACCGTA	UBC-551	GGAAGTCCAC
UBC-30	CCGGCCTTAG	UBC-315	GGTCTCCTAG	UBC-561	CATAACGACC
UBC-40	TTACCTGGGC	UBC-321	ATCTAGGGAC	UBC-571	GCGCGGCACT
UBC-51	CTACCCGTGC	UBC-325	TCTAAGCTCG	UBC-581	CCCGTTAAGG
UBC-60	TTGGCCGAGC	UBC-331	GCCTAGTCAC	UBC-591	TCCCTCGTGG
UBC-71	GAGGGCGAGG	UBC-335	TGGACCACCC	UBC-601	CCGCCCACTG
UBC-80	GTGCTCTAGA	UBC-341	CTGGGGCCGT	UBC-611	CCATCGTACC
UBC-100	ATCGGGTCCG	UBC-345	GCGTGACCCG	UBC-621	GTCTGCGCTA
UBC-101	GCGGCTGGAG	UBC-401	TAGGACAGTC	UBC-631	GGCTTAACCG
UBC-111	AGTAGACGGG	UBC-405	CTCTCGTGCG	UBC-641	TGGAACCATG
UBC-121	ATACAGGGAG	UBC-411	GAGGCCCGTT	UBC-651	TCATTTCGCC
UBC-131	GAAACAGCGT	UBC-501	CGGATATACC	UBC-701	CCCACAACCC
UBC-141	ATCCTGTTCG	UBC-511	GAATGGTGAG	UBC-711	CCCTCTCCCT
UBC-151	GTCGTAGTGT	UBC-521	CCGCCCCACT	UBC-721	CCCTTCCCTC

primer UBC 301 (5'-CGGTGGCGAA-3') was extracted from the gel at amplicon length of approximately 450 bp, purified with a LaboPass^{TM} gel extraction kit (Cosmo Genetech). The resulting product was sub cloned into a T-Blunt PCR Cloning Kit (Solgent) according to the manufacturer's protocol. Plasmid DNA was isolated and used as template in a sequence reaction with primers M13-F and M13-R. These sequences were used to construct alignments based on SCAR-markers and primers for HRg-F/HRg-R and were designed with Primer Express (Bioneer, Daejeon, Korea). All primers were synthesized by Bioneer. The following PCR conditions were used for a 20-μL PCR; 1 μL of genomic DNA (~20 ng) was added to 10 μL Smart 2 × PCR pre-mix (Solgent) containing 0.63 U Prime STAR HS DNA Polymerase, 0.4 mM dNTP Mixture, 2 mM Prime STAR Buffer), and 1 μL each of 10 pM: HRg-F (5'-TTAGGCTCTGATGCGAT-3') and HRg-R (5'-CGAATATAGGTACCCGCACTA-3') primer, InRg-F (5'-AGGATCTAGGGTTAATGCCA-3') and InRg-R (5'-CCGGTT TTTTTCTAGCA-3'). The PCR was carried out with a C1000^{TM} Thermal Cycler followed by 5 min pre-denaturation at 95°C, 30 cycle of 20 s at 95°C, 40 s at 55°C with a ramp time of 1°C/s, 1 min at 72°C, and 5 min of final extension at 72°C.

Results and Discussion

For a DNA-based identification of *R. glutinosa* from two different regions, genomic DNAs were used for comparison of ITS and rps16 sequences. Upon comparison of 610 bp of ITS sequence between Korean and Chinese *R. glutinosa* in the analysis of ITS region from nDNA, no variation was observed. Korean and Chinese *R. glutinosa* were confirmed to be 100% identical to *R. glutinosa* (FJ770249) registered in the NCBI, by comparison of the sequence analyses data with the sequences containing the analyzed samples (ITS region sequences) using blast search in the Genbank of NCBI. In addition, *matK* and *rbcL* in the chloroplast genome were analyzed; no significant difference was observed between the two groups.

The sequence of Chinese *R. glutinosa* showed 1 bp (C→T) substitution compared to that of Korean *R. glutinosa*, exhibiting the genetic difference upon comparison of total 521 bp in the analysis of rps16 region from cpDNA (Fig. 1). In addition, when the result of rps16 region was analyzed via NCBI Blast search, Korean *R. glutinosa* was confirmed to have 100% homology with *R. glutinosa* (DQ856487) and Chinese *R. glutinosa* was confirmed to have 100% homology with *R. glutinosa* (FJ172697).

The result of sequence analysis of the two specific regions (ITS and rps16 regions) showed that Korean and Chinese *R. glutinosa* have more than 99.9% homology. SCAR markers based on the unique RAPD-amplified PCR products of *R. glutinosa* and Rehmanniae herbs must be developed prior to the development of a multiplex PCR method that can be applied for the discrimination of Korean *R. glutinosa* of from Chinese *R. glutinosa*. Therefore, in the present study, initial attempted was made to ascertain whether *R. glutinosa* could be discriminated by RAPD analysis using 48 UBC primers, because ITS and rps16 gene analyses were limited to discrimination of Korean *R. glutinosa* from Chinese *R. glutinosa*. Among the non-specific primers that showed polymorphism between the Rehmanniae herbs, primer UBC 301 (5'-CGGTGGCGAA-3') generated strong amplified products of 350–900 bp in size, specifically in Korean and Chinese *R. glutinosa* (Fig. 2). However, primer UBC 301 generated strong amplified products with a size of 450 bp only from Chinese *R. glutinosa*. The polymorphism of the amplified products by RAPD is attributed to genetic characteristics such as genetic changes and interspecific hybridization due to the difference in growth environments.

Based on the sequence analysis of the amplified specific band, SCAR markers distinguished Chinese *R. glutinosa* only. The

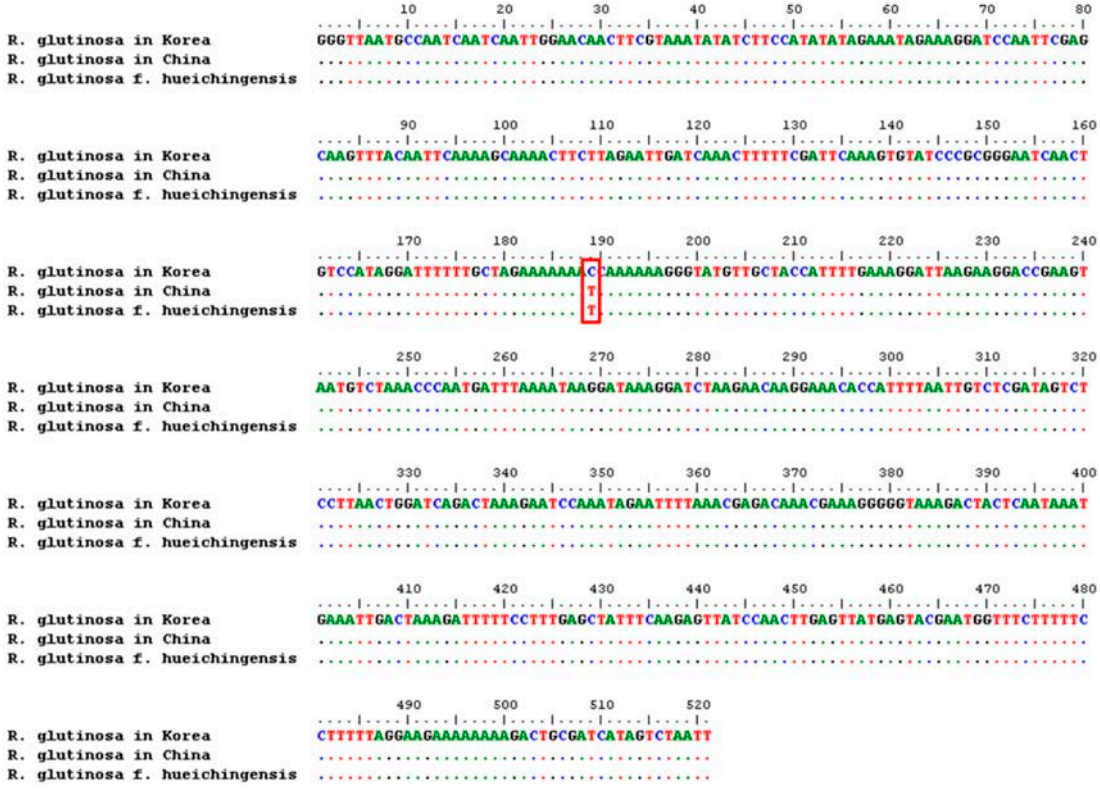

Fig. 1 Comparison of the partial rps16 gene sequence among Korean *R. glutinosa*, Chinese *R. glutinosa*, and *R. glutinosa* f. *hueichingensis*. Dots in the sequences denote same nucleotide state as *R. glutinosa* in Korea.

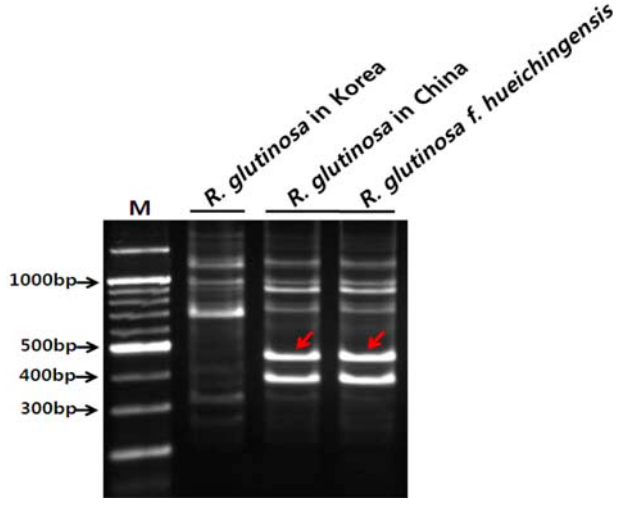

Fig. 2 Randomly amplified polymorphic DNA analysis of Rehmanniae Radix plants (Korean *R. glutinosa*, Chinese *R. glutinosa*, and *R. glutinosa* f. *hueichingensis*) using primer UBC 301. M: 100 bp DNA ladder.

specific primer pair (HRg-F/HRg-R) was designed using UBC 301 primer (Fig. 3), and 334 bp band was detected only in Chinese *R. glutinosa*. HRg-F (5'-TTAGGCTCTGATGCGAT-3') and HRg-R (5'-CGAATATAGGTACCCGCACTA-3') were devised as forward- and reverse primers, respectively. In addition, for a control that tests PCR errors, common primers that can be used in both Korean and Chinese of *R. glutinosa* were designed using rps16 region sequences. The result confirmed that 195 bp band was amplified in both *R. glutinosa*. According to these results, the HRg-F/HRg-R primer set was capable of amplifying the SCAR marker that distinguished Chinese *R. glutinosa* from Rehmanniae herbs.

Multiplex PCR was performed to develop a convenient marker for the discrimination of *R. glutinosa*. The result of PCR amplification using two pairs of primers (HRg-F, HRg-R/InHRg-F, InHRg-R) showed that one band with 195 bp was detected in Korean *R. glutinosa* and two bands with 334 and 195 bp, were detected in Chinese *R. glutinosa*. This result suggests that SCAR markers developed in the present study could be useful for the place-of-origin discrimination of *R. glutinosa* (Fig. 4). Therefore, this multiplex PCR method could be applicable to the discrimination of *R. glutinosa* from other Rehmanniae herbs.

We attempted to devise a multiplex PCR method as a more certain and useful discrimination method on the basis of the nucleotide sequences of some polymorphic PCR products of the herbs after RAPD. These results suggested that PCR product amplified uniquely from *R. glutinosa* could indeed be applicable to the discrimination of Korean *R. glutinosa* from other Rehmanniae Radix constituents. However, the heat-processed samples such as *R. glutinosa* and dried *R. glutinosa* werenot amplified. This result

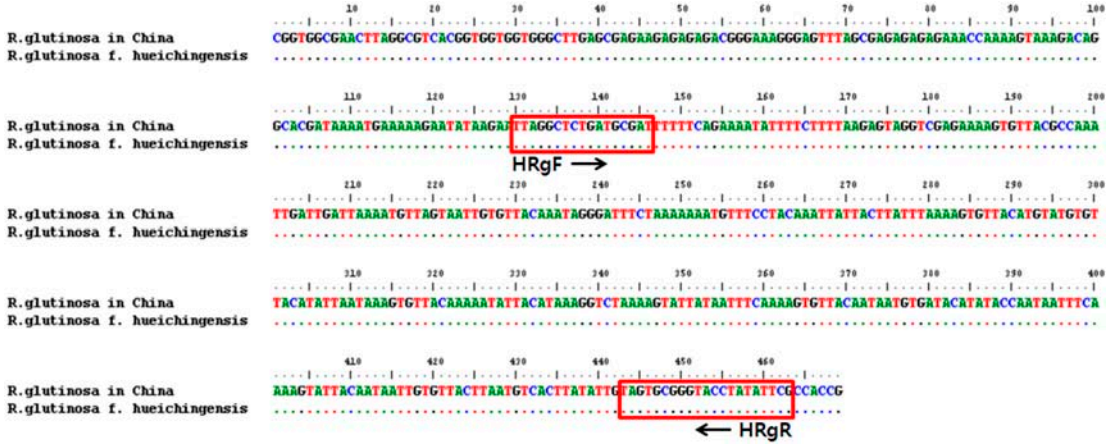

Fig. 3 Characteristics of specific primers designed for discrimination of Chinese *R. glutinosa*. Sequence of Chinese *R. glutinosa* and *R. glutinosa* f. *hueichingensis* amplified by UBC 301 universal primer and Chinese *R. glutinosa*-specific RAPD amplicon (HRg-F/HRg-R).

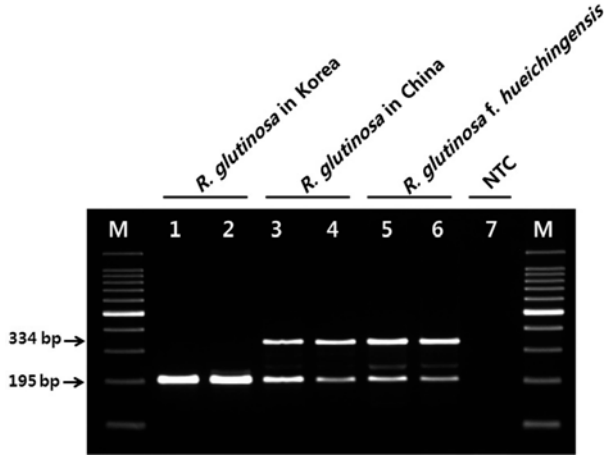

Fig. 4 Profiles of PCR products obtained from specific primer (HRg-F/HRg-R). 334 bp-sized bands were detected in *R. glutinosa* in China and *R. glutinosa* f. *hueichingensis*. Lane M, 100 bp DNA ladder; NTC, No Template Control; Lanes 1-2, *R. glutinosa* in Korea; Lanes 3-4, *R. glutinosa* in China; Lanes 5-6, *R. glutinosa* f. *hueichingensis*.

is likely to be attributable to the unstable preservation of DNA during the heat process. Furthermore, PCR was performed under pre-established conditions using the biological samples. This process confirmed the presence of species-specific amplification products of 334 and 195 bp in Chinese *R. glutinosa*. To validate the SCAR marker, we conducted PCR amongcommercial Rehmanniae Radix (d-R.g-1–18). As a result, the listed samples were classified into two groups. One of the groups (No. d-R.g-1–7 of Table 1) was clustered to be *R. glutinosa* and another (No. d-R.g-8–18 of Table 1) was clustered to be *R. glutinosa* f. *hueichingensis*. Among the samples, numbersd-R.g-8 and 9 showed two bands with 334 bp and 195 bp in *R. glutinosa* f. *hueichingensis* (Fig. 5). With the increasing international trade, the import of *R. glutinosa* from China to Korea has been growing. In China, *R. glutinosa* f. *hueichingensisis* is considered to be the same as *R. glutinosa*, reported by Zhou et al. (2007) These results confirmed that discrimination of Rehmanniae Radix is possible.

In conclusion, Chinese *R. glutinosa* and *R. glutinosa* f. *hueichingensis* are genetically identical, and most *R. glutinosa*

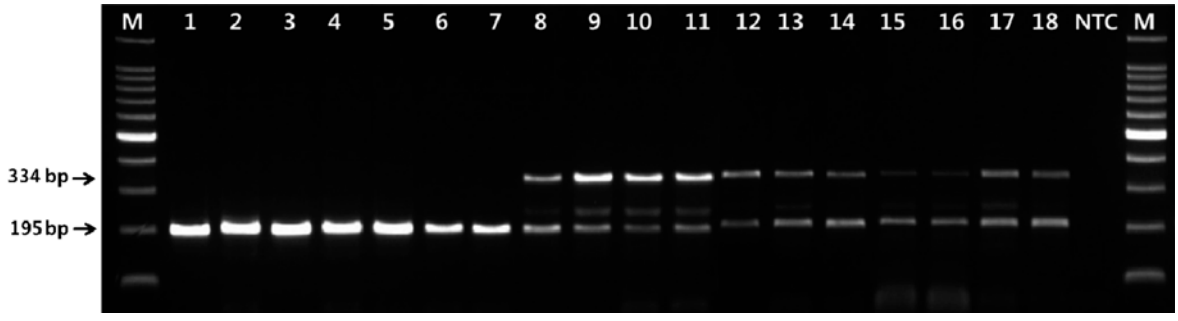

Fig. 5 PCR Profiles of commercial dried Rehmannia Radix obtained from specific primer (HRg-F/HRg-R). M, 100 bp DNA ladder; NTC, No Template Control; Lanes 1-9 (d-R.g-1–9 in Table 1), Rehmanniae Radix collected in Korea; Lanes 10-18 (d-R.g-10–18 in Table 1), Rehmanniae Radix collected in China.

distributed in China are considered as *R. glutinosa* f. *hueichingensis*. Further studies are required to verify the results of the present study by securing more samples from more diverse regions.

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