Molecular authentication of *Gynostemma pentaphyllum* through development and application of random amplification polymorphic DNA sequence-characterized amplified region marker

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ABSTRACT. Due to the morphological similarities of aerial parts, it is difficult to distinguish *Gynostemma pentaphyllum* from *Cayratia japonica*, which is usually an adulterant of the former. To develop a reliable method for the identification and authentication of *G. pentaphyllum*, a combination of random amplification polymorphic DNA (RAPD) technique with sequence-characterized amplified region (SCAR) markers was studied. Twenty-five samples of *G. pentaphyllum* and two samples of *C. japonica* were collected from different regions in Guangxi or bought from different provinces in China. Through the RAPD analysis, significant genetic polymorphism was observed among the intraspecies samples of *G. pentaphyllum.* Furthermore, a specific marker, J-750, was obtained for authentication.

Therefore, the SCAR marker for *G. pentaphyllum* (359 bp) was developed from the RAPD amplicon. With PCR amplification using the SCAR primers, a specific band of 359 bp was distinctly visible for all tested samples of *G. pentaphyllum*, but was absent in the samples of *C. japonica*. Furthermore, the results revealed that the SCAR marker was useful for the identification and authentication of *G. pentaphyllum* irrespective of whether samples were fresh, dry, or of commercial origin. The SCAR marker obtained in this study successfully authenticated *G. pentaphyllum* through an integrated PCR system containing SCAR and control primer combinations of two pairs. In addition, it was also used for simultaneous discrimination of *G. pentaphyllum* from *C. japonica*.

Key words: Sequence-characterized amplified region; *Gynostemma pentaphyllum*; Random amplification polymorphic DNA; Molecular authentication

INTRODUCTION

Gynostemma pentaphyllum (Thunb.) Makino (Jiaogulan) belongs to *Gynostemma* BL. of the family Cucurbitaceae. It is a widely distributed and abundant species in China and Southeast Asia (Liao et al., 2011) and has a complex genetic background (Jiang et al., 2009b; Liao et al., 2011). Many studies have shown that *G. pentaphyllum* extracts possess various biological properties, such as immunomodulatory (Im et al., 2012), anticancer (Liu et al., 2014; Deng and Yang, 2014; Tsui et al., 2014), antifatigue (Shan and Shi, 2014), and neuroprotective effects (Schild et al., 2009; Choi et al., 2010), and they are also involved in the regulation of lipid metabolism (Wang et al., 2013) and hypoglycemia (Gao et al., 2014; Park et al., 2014). Therefore, *G. pentaphyllum* has been extensively used in traditional Chinese medicine and in diverse industries such as healthcare, food, and cosmetics. However, the quality and safety of medications containing Jiaogulan are influenced by the presence of chaotic adulterants in the medicinal herb markets. In China, some medicinal plants have several synonymous names in different regions, or one name may represent different plants in disparate areas. For example, *Cayratia japonica* of the family Vitaceae is known as “Jiaogulan” in Shandong Province and in the north of Jiangsu Province (Ding 1998). Moreover, due to the morphological similarity of aerial parts, it is difficult to identify *G. pentaphyllum* and discriminate it from *C. japonica*, particularly in the case of dry medicinal materials. The morphologic characteristics of both *G. pentaphyllum* and *C. japonica* are similar, and they both possess leaves with pedate foliolates (usually 5-7), herbaceous, climbing, or prostrate stems, and are often found along brooks, in valleys, forests, thickets, and on roadsides on mountain slopes (Chen, 2007; Lu, 2011).

Authentication of genuine species has become a key issue for the standardization of herbal preparations in traditional medicine, because most of these medications are produced from herbs that are collected from wild habitats or are cultivated on farms. Although some methods have been developed to distinguish *G. pentaphyllum* from *C. japonica* based on morphotypes, microcharacters, or physical and chemical reactions, these are dependent on taxonomy experts. Currently, there are few reports about the authentication of *G. pentaphyllum* at the DNA molecular level. Therefore, the correct genotypic identification of plant material is essential in order to protect public health and for industrial production.

Limitations of chemical and morphological approaches regarding the authentication of medicinal plants have resulted in the development of newer quality control techniques. Genetic polymorphisms in medicinal plants have been widely studied, and have helped to differentiate herbal medicines at the genomic level. Various molecular markers have been explored for use in the authentication of botanical materials such as ginseng (Wang et al., 2011b), atractylodes (Mizukami et al., 2000), and such techniques can provide more objective and reliable tools for the categorization and authentication of plant materials. Conventionally, randomly amplified polymorphic DNA (RAPD), based on polymerase chain reaction (PCR) amplification, has been used to estimate genetic diversity in wild plant populations or cultivars. This method can also be used to effectively authenticate various herbal medicines (Yang et al., 2013; Xin et al., 2014; Mei et al., 2014). However, the repeatability of results obtained by RAPD is dependent on the quality of DNA extracted from the botanical samples. Unfortunately, it is always difficult to obtain high-quality DNA from dry and processed herb materials. Therefore, a technique known as sequence-characterized amplified region (SCAR) has been developed based on RAPD (Bashir et al., 2014), which is specific, sensitive, and reproducible. SCAR markers have been explored and used in many medicinal plants, including *Bulbus fritillariae* (Xin et al., 2014), *Pistacia chinensis* (Sun et al., 2014), *Acorus gramineus* (Ryuk et al., 2014), *Rosa indica* (Bashir et al., 2014), *Miscanthus sacchariflorus* (Kim et al., 2012), and *Eucommia ulmoides* (Wang et al., 2011a). However, there are no suitable SCARs for *G. pentaphyllum*.

The objective of this study was to develop a method to distinguish *G. pentaphyllum* and *C. japonica* using DNA molecular markers. RAPD analysis of DNA from these two plants was completed by screening 20 decamer oligonucleotide primers. In light of the RAPD analysis results, a reliable SCAR marker was developed to enable the identification of these two species. Additionally, a PCR integrated system was developed to allow the simultaneous detection of these two herbal species through the conversion of RAPD to SCAR markers.

MATERIAL AND METHODS

Plant material

Twenty-five samples of *G. pentaphyllum* and two samples of *C. japonica* were used in this study. All plant materials were collected from different regions in Guangxi or bought from different provinces in China (Table 1). The botanical species were identified by Professor Kaijia He, Guangxi Institute of Chinese Medicine and Zhengzhu Ling in Guangxi Medicine Botanical Garden.

Isolation of DNA

Genomic DNA was extracted from fresh and dried samples using the cetyltriethylammonium bromide (CTAB) protocol with minor modifications, as previously described (Jiang et al., 2009a). DNA concentration and purity were determined by spectrophotometry (Smartspec™ plus, Bio-Rad, USA), and electrophoresis on a 1.5% agarose gel with known standards. Gels were stained using ethidium bromide and visualized under ultraviolet (UV) light. The final DNA concentration of each sample was diluted to approximately 40 ng/μL for PCR amplification.

PCR amplification

Twenty random decamer primers were used for RAPD amplification (Table 2), which were

of high homology and aligned with the genome of *Arabidopsis thaliana*. The PCR reaction mixture contained 40 ng template DNA in a 25-µL reaction volume with 2.5 µL 10X reaction buffer, 2 mmol/L $MgCl_2$, 100 µmol/L dNTPs, l U ExTaq DNA polymerase (Dalian TaKaRa Co. Ltd.), and 10 pmol primer. Amplification was performed in a DNA thermal cycler (TGradient PCR, Biometra, Germany) using the following parameters: 94°C for 3 min; 40 cycles at 94°C for 50 s, 37°C for 50 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were subjected to agarose gel (1.5% [w/v]) electrophoresis in 1X Tris acetate buffer, along with DL2000 DNA ladders (Beijing Sunbiotech Co. Ltd.) as size markers. Gels were stained using ethidium bromide and visualized under UV light.

Table 1. Information of the 27 samples (25 *Gynostemma pontaphyllum*, 2 *Cayratia japonica*) used in this study.

No.	Species of herbal plant	Source (county, province, country)	Notes
JI	*G. pentaphyllum*	Nanning, Guangxi, China	Fresh material
J2	*G. pentaphyllum*	Lingyun, Guangxi, China	Fresh material
J3	*G. pentaphyllum*	Nanning, Guangxi, China	Fresh material
J4	*G. pentaphyllum*	Jingxiu, Guangxi, China	Fresh material
J5	*G. pentaphyllum*	Jingxiu, Guangxi, China	Fresh material
J6	*G. pentaphyllum*	Nanning, Guangxi, China	Fresh material
J7	*G. pentaphyllum*	Damingshan, Guangxi, China	Fresh material
J8	*G. pentaphyllum*	Jingxiu, Guangxi, China	Fresh material
JG1	*G. pentaphyllum*	Nanning, Guangxi, China	Dry material
JG2	*G. pentaphyllum*	Jinxiu, Guangxi, China	Commercial material
JG3	*G. pentaphyllum*	Nanning, Guangxi, China	Dry material
JG4	*G. pentaphyllum*	Jinxiu, Guangxi, China	Dry material
JG5	*G. pentaphyllum*	Jinxiu, Guangxi, China	Dry material
JG6	*G. pentaphyllum*	Nanning, Guangxi, China	Dry material
JG7	*G. pentaphyllum*	Damingshan, Guangxi, China	Dry material
JG8	*G. pentaphyllum*	Jinxiu, Guangxi, China	Dry material
JG9	*G. pentaphyllum*	Shanxi, China	Jiaogulan tea
JG10	*G. pentaphyllum*	Guizhou, China	Commercial material
JG11	*G. pentaphyllum*	Hunan, China	Commercial material
JG12	*G. pentaphyllum*	Changde, Hunan, China	Jiaogulan tea
JG13	*G. pentaphyllum*	Nanning, Guangxi, China	Dry material
JG14	*G. pentaphyllum*	Guilin, Guangxi, China	Commercial material
JG15	*G. pentaphyllum*	Lipu, Guangxi, China	Commercial material
JG16	*G. pentaphyllum*	Fusui, Guangxi, China	Commercial material
JG17	*G. pentaphyllum*	Jingxiu, Guangxi, China	Commercial material
W1	*C. japonica*	Lingyun, Guangxi, China	Fresh material
W2	*C. japonica*	Lingyun, Guangxi, China	Dry material

Table 2. Sequences of random decamer primers used in this study.

No.	Primer sequence	No.	Primer sequence
1	CTGCTGGGAC	11	GTTGCCAGCC
2	GTAGACCCGT	12	ACTTCGCCAC
3	CCTTGACGCA	13	TTCCGCCACC
4	TCCGCTCTGG	14	TGGCGCAGTG
5	AGGGAACGAG	15	ACAGGTGCTG
6	GGACCCTTAC	16	TGGCAAGGCA
7	TTCCGAACCC	17	CCAAGAGGCT
8	TGAGCGGACA	18	GAGCACTGCT
9	CATCCGTGCT	19	TGGGCTCTGG
10	CTCACCGTCC*	20	GTGTGCAGTG

*Amplified J-750 for *Gynostemma pentaphyllum*, SCAR marker.

Screening strategy and identification of specific RAPD amplicon

A series of reproducible fragments, which migrated at the same rate, were amplified by primer 10 from 25 samples of *G. pentaphyllum,* including eight fresh and 17 dried materials. Bands of 750 bp were generated from all *G. pentaphyllum* samples but were absent in samples from *C. japonica* species. Specific fragments of the RAPD amplicon from eight fresh *G. pentaphyllum* samples were selected and named J-750 for further cloning and sequencing.

Cloning of RAPD amplicon

The J-750 fragment amplified by primer 10, which is a putative marker, was excised from a 1.5% agarose gel, purified using a Gel Extraction Kit (Biospin, Hangzhou Bioer Technology Co. Ltd.), inserted into the *pMD18*-T vector (Dalian TaKaRa Co. Ltd.), and the recombinant vector was transformed into *Escherichia coli* JM-109 (MBI). Plasmid DNA was extracted using a Plasmid DNA Extraction Kit (Biospin, Hangzhou Bioer Technology Co. Ltd.). Positive clones were confirmed through profile analysis of double restriction digestion with *Bam*HI and *Hin*dIII, and then sequenced by Beijing Sunbio Biotech Co. Ltd.

Development of the SCAR marker integrated amplification system

Based on the sequenced RAPD amplicon, a pair of SCAR oligonucleotide primers (G1 and G2) was designed along the hyperconservative region, which could amplify approximately 359 bp of the genomic DNA of *G. pentaphyllum*. Two pairs of control primers were obtained according to the 18S rDNA sequences of Cucurbitaceae or Vitaceae, respectively, and were named J1-J2 and W1-W2. J1-J2 could amplify a 293-bp fragment within the 18S rDNA region of *G. pentaphyllum*; however, W1-W2 amplified only a 177-bp fragment in *C. japonica.* All primers (Table 3) were synthesized by Beijing Sunbio Tech Co. Ltd. The three pairs of primers were used for PCR amplifications of genomic DNA from all materials. Species-specific amplification was performed using a PCR integrated system with a SCAR primer pair and a control primer pair. Reactions were performed in a 25-μL mixture containing two pairs of primers (10 pmol of each primer), 80 ng DNA template, and the same buffer as previously described. Thermal cycling conditions for amplification were 94°C for 3 min; 10 cycles at 94°C for 30 s, 69°C for 30 s, and 72°C for 30 s. The annealing temperature was reduced by 1°C at every cycle until the “touchdown” annealing temperature reached 59°C; then 20 cycles were performed at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min.

Table 3. Primer sequences for PCR integrated systems 1 and 2.

Primer	Primer sequence	Amplification region
G1	ATAAGATGAACAGGCCATAAG	SCAR of *G. pentaphyllum*
G2	GTAAACTGACCCGAACACTC	
J1	AGACTGTGAAACTGCGAATGG	18S rDNA of Cucurbitaceae
J2	CTCCGTCACCCGTCACC	
W1	CGCGTGGGAATCTGCCGAACA	18S rDNA of Vitaceae
W2	GGAGTCCGGGCCGTGTCT	

Analysis of sequence data

Alignment analysis of sequenced J-750 was performed using Vector NTI and Clustal W. Homology screening was performed using the BLAST algorithm within the GenBank database at http://www.ncbi.nlm.nih.gov/BLAST/ of National Center for Biotechnology Information, using the program BLASTn.

RESULTS

Analysis and identification of RAPD marker for *G. pentaphyllum*

High-molecular weight genomic DNA was extracted from various sources of fresh and dry materials of *G. pentaphyllum* and *C. japonica* (Table 1). Some degraded DNA fragments were observed in the DNA extracted from dry samples. Species-specific genomic sequences were screened using the RAPD method. Of the 20 random decamer primers selected, PCR with 19 primers separately produced a distinct and reproducible amplification profile for all samples of *G. pentaphyllum*. Among the 19 primers, primer 10 consistently amplified an intense band of approximately 750 bp only in *G. pentaphyllum* samples, but this band was absent in the *C. japonica* samples (Figure 1A-C). This band, named J-750, was selected as a putative-specific RAPD marker of *G. pentaphyllum*.

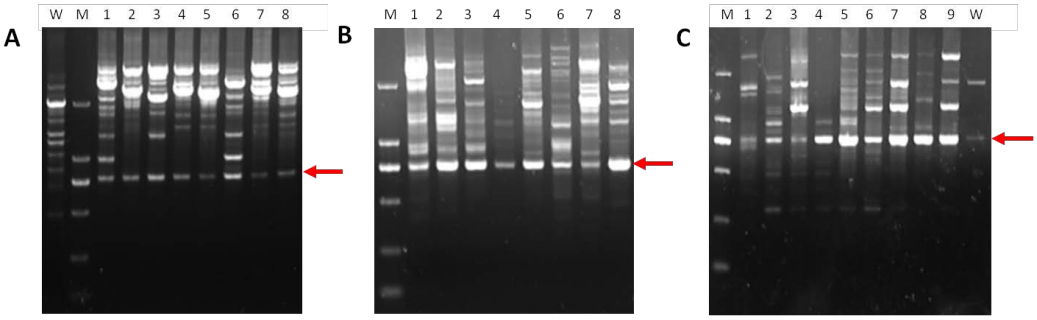

Figure 1. Random amplified polymorphic DNA (RAPD) amplification profile using primer. **A.** RAPD amplification of fresh samples. *Lane M* = DL 2000 marker; *lanes 1-8* = J1-J8 of *Gynostemma pentaphyllum*; W: *Cayratia japonica*. Arrow: consistently amplified bands in *G. pentaphyllum*, J-750. **B.** RAPD amplification of dry samples. *Lane M* = DL 2000 marker; *lanes 1-8* = JG1-JG8 of *G. pentaphyllum.* Arrow: consistently amplified bands from *G. pentaphyllum*, J-750. **C.** RAPD amplification of dry samples. *Lane M* = DL 2000 marker; *lanes 1-9* = JG9-JG17 of *G. pentaphyllum*; W: *C. japonica*. Arrow: consistently amplified bands from *G. pentaphyllum*, J-750.

Cloning and sequencing of RAPD marker

To convert the specific RAPD marker to a SCAR marker for *G. pentaphyllum*, specific bands of DNA (about 750 bp) were amplified using primer 10 from eight fresh samples of *G. pentaphyllum* and were named J1-750-J8-750, respectively. Later, all fragments of J1-750-J8-750 were cloned in pMD18-T and sequenced. Restriction endonuclease (RE) digestion analysis revealed that a *Hin*dIII site existed in all J-750 samples, which could be used to cut the latter into two fragments of about 500 and 270 bp on a 1.5% w/v agarose gel (Figure 2). The size of these

amplified fragments was consistent with those of the corresponding RAPD. This means that the RAPD marker was successfully cloned. Subsequently, all sequences obtained of the RAPD marker were submitted to GenBank (accession No. HQ900672-HQ900679).

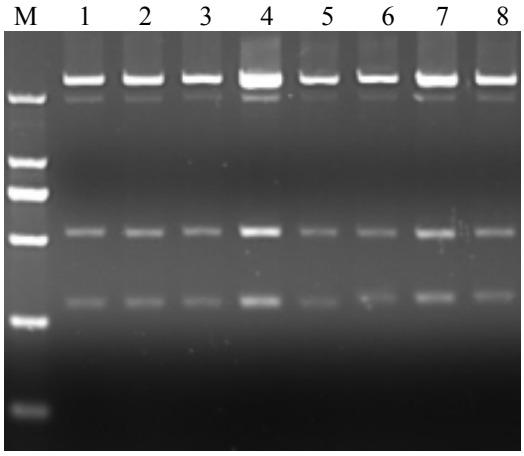

Figure 2. Double-restriction digestion profile from *Bam*HI and *Hin*dIII for eight recombinant plasmids of the J-750 random amplified polymorphic DNA (RAPD) amplicon. *Lane M* = DL 2000 marker; *lanes 1-8* = positive recombinant plasmids of J-750 from J1-J8 samples of *Gynostemma pentaphyllum.*

Sequence analysis of RAPD amplicon

The length and consistency of the eight RAPD amplicon sequences were analyzed (Table 4). These were highly conserved sequences with >97% similarity. The length of the eight RAPD marker sequences ranged from 766 to 770 bp, comprising approximately 68% A-T. The results from BLAST analysis revealed that the homology of these sequences with known plant nucleotide sequences was at a low sequence-similarity level, <40-90 bp. Two possible coding regions were found with 48 or 45 encoded amino acids. The function and possible protein-coding of two open reading frames requires further study. These eight sequences were found to contain several RE sites. The number of RE *Ssp*I sites on J-750 were polymorphic, such that one *Ssp*I site was found on J1-750 and J6-750, two *Ssp*I sites on J3-750, J4-750, J5-750, J7-750, and J8-750, and three *Ssp*I sites were found on J2-750. Most other RE digestion sites were single (**Table S1**).

Table 4. Comparison of the length and consistency of eight *Gynostemma pentaphyllum* sequences.

Length (bp)	Specimen No.	J1-750 (%)	J2 -750 (%)	J3-750 (%)	J4-750 (%)	J5-750 (%)	J6-750 (%)	J7-750 (%)	J8-750 (%)
769	J1-750	100.0							
766	J2-750	97.7	100.0						
768	J3-750	99.3	98.0	100.0					
767	J4-750	98.6	98.2	99.0	100.0				
770	J5-750	98.3	97.4	98.4	98.8	100.0			
769	J6-750	99.9	97.8	99.5	98.7	98.4	100.0		
766	J7-750	98.3	97.8	98.7	99.2	98.6	98.4	100.0	
769	J8-750	98.1	97.5	98.4	99.0	98.3	98.2	98.8	100.0

Amplification using SCAR primers

Based on the eight J-750 RAPD marker sequences, SCAR primers were designed to amplify genomic DNA from 27 specimens (including two samples of *C. japonica*). Two pairs of control primers were used to amplify 18S rDNA, and the length of the 18S rDNA fragment from *G. pentaphyllum* was 293 bp, and that from *C. japonica* was 177 bp. As shown in Figure 3A-C, a SCAR primer pair, G1 and G2, generated a single, distinct, and brightly resolved 359-bp band in all *G. pentaphyllum* cultivars and dry materials, but no specific amplicons were observed from *C. japonica* species. Even though some degraded fragments were observed in the genomic DNA extracted from dry material, including commercial samples, SCAR bands were still strong and distinct for the specimens inspected. An internal control of approximately 290 bp for all *G. pentaphyllum* species and another of approximately 180 bp for *C. japonica* species were observed on agarose gel (Figure 3A-C). Therefore, these data show that it is possible to discriminate *G. pentaphyllum* from *C. japonica* using PCR amplification of SCAR and control primers (**Table S2**).

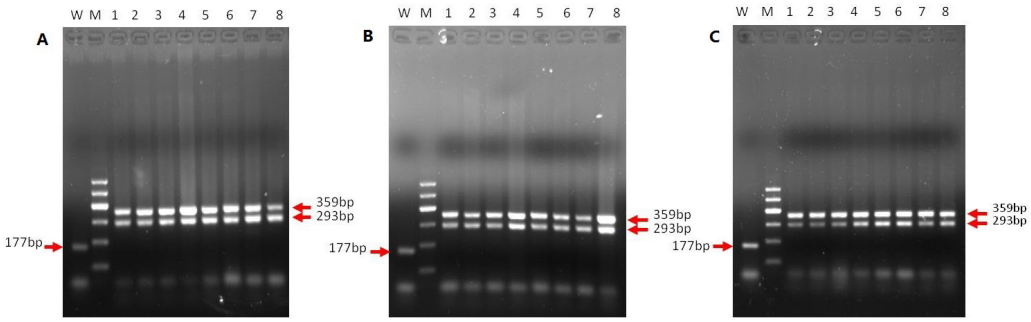

Figure 3. Amplification profile of sequence-characterized amplified region (SCAR) marker and control. **A.** Amplification of fresh samples. W: *Cayratia japonica*. *Lane M* = marker 1; *lanes 1-8* = J1-J8 samples from *Gynostemma pentaphyllum*. **B.** Amplification of dry samples. W: *C. japonica*; *lane M* = marker 1; *lanes 1-8* = JG1-JG8 dry samples of *G. pentaphyllum*. **C.** Amplification of dry samples. W: *C. japonica*; *lane M* = marker 1; 1-8: JG10-JG18 dry samples of *G. pentaphyllum*.

DISCUSSION

Traditionally, common methods used to authenticate herbal medicines have depended on histological and morphological evaluations, but the accuracy of these methods is often influenced by environmental and subjective factors (Wang et al., 2012). Chemical analyses such as high-performance liquid chromatography and capillary electrophoresis are useful for identification; however, quantitative variations in characteristic compounds within herbal materials impede the confirmation of their botanical identities (Hon et al., 2003; Xie et al., 2011). It is clear that compounds within herbal medicines present chemical complexity, which are usually affected by growth, drying, and storage conditions, as well as the time of collection. Therefore, techniques involving molecular markers are more dependable as they are dependent on genetic composition and are not influenced by other factors (Wang et al., 2011b; Wu et al., 2011).

RAPD analysis could be used to explore the high degree of genetic polymorphisms in various herbal medicines; not only is there no requirement for prior DNA sequence information of these species, but this technique is also easy to perform. In the present RAPD analysis, significant genetic polymorphism was observed among different specimens of *G. pentaphyllum*. The 19 random decamer primers screened could be used in research on germplasm resources. The genetic polymorphism in tested cultivars was distinguishable and could be utilized to develop markers for subsequent species identification. In this study, one consistent band of J-750 was amplified by random decamer primer 10 from *G. pentaphyllum* specimens but not from *C. japonica*. Therefore, this band could be selected as a genetic marker of *G. pentaphyllum.*

Based on sequence analysis of the cloned J-750 fragment, one pair of intraspecies-specific primers (G1 and G2) specific for genomic DNA of *G. pentaphyllum* was designed and a SCAR marker amplification system was developed for the simultaneous authentication of *G. pentaphyllum* and *C. japonica*. The annealing site of G1 and G2 with the RAPD amplicon is shown in Figure 4. The size of the RAPD amplicon was sufficient for the layout of SCAR primers; meanwhile, the variable region could be evaded. Previously, we identified another species-specific RAPD amplicon (about 500 bp), but the length was not adaptive to conversion to a SCAR marker (data not shown). Here, J-750 was a more appropriate RAPD amplicon to be converted to a SCAR marker. In comparison with other assay methods of authentication, the detection of SCAR marker needs only a gel-based assay and requires no sequence analysis of the PCR products (Yang et al., 2013). In addition to SCAR marker primers, two pairs of control primers were used to amplify fragments of 18S rDNA from *G. pentaphyllum* (J1 and J2) or *C. japonica* (W1 and W2), which were used as internal controls, respectively. The length of the SCAR marker is 359 bp, while the length of the control fragment from *G. pentaphyllum* and *C. japonica* was 293 and or 177 bp, respectively, and easily distinguished. In this assay, primer pairs G1-G2 and J1-J2 amplified the predicted 359- and 293-bp DNA fragments in all samples of *G. pentaphyllum* but not *C. japonica*. However, W1-W2 generated only a 177-bp fragment in *C. japonica* samples. Therefore, the SCAR marker amplification system is simple, reliable, repeatable, and cost-effective. The results substantiate the applicability of the SCAR amplification system as a qualitative diagnostic tool for the authentication and identification of *G. pentaphyllum.* Therefore, this system could be employed to discriminate crude and processed commercial materials of *G. pentaphyllum* from their adulterants.

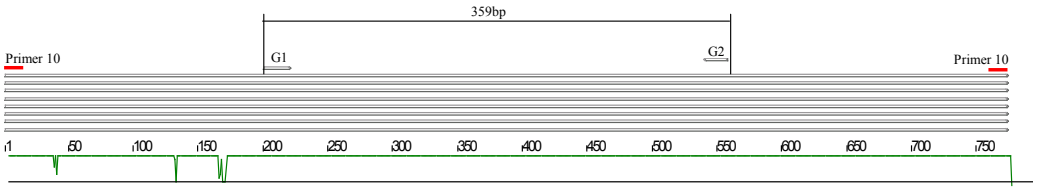

Figure 4. Alignment of species-specific primers G1 and G2 with random amplification polymorphic DNA (RAPD) marker amplicon.

Sequence analysis of the J-750 RAPD amplicon has revealed the presence of several restriction endonuclease sites. One of the RE sites, *Ssp*I, is polymorphic in the inspected specimens of *G. pentaphyllum*, which could be developed as cleaved amplified polymorphic sequence and may be appropriate for the detection of single-nucleotide variations (Kim et al., 2006). In conclusion, the SCAR marker obtained in this study could be used for the identification of *G. pentaphyllum*, as

well as for the simultaneous discrimination of *G. pentaphyllum* from *C. japonica* through the primer combination of two pairs of SCAR markers (G1-G2) and a control (J1-J2). Furthermore, the SCAR primers and the marker amplification system have been awarded the title of national patent by the Chinese State Bureau of Intellectual Property Office (ZL201110139269.4).

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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