Genetics and Molecular Research

Development of novel SCAR markers for genetic characterization of *Lonicera japonica* from high GC-RAMP-PCR and DNA cloning

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ABSTRACT. Sequence-characterized amplified region (SCAR) markers were further developed from high-GC primer RAMP-PCR-amplified fragments from *Lonicera japonica* DNA by molecular cloning. The four DNA fragments from three high-GC primers (FY-27, FY-28, and FY-29) were successfully cloned into a pGM-T vector. The positive clones were sequenced; their names, sizes, and GenBank numbers were JYHGC1-1, 345 bp, KJ620024; YJHGC2-1, 388 bp, KJ620025; JYHGC7-2, 1036 bp, KJ620026; and JYHGC6-2, 715 bp, KJ620027, respectively. Four novel SCAR markers were developed by designing specific primers, optimizing conditions, and PCR validation. The developed SCAR markers were used for the genetic authentication

of *L. japonica* from its substitutes. This technique provides another means of developing DNA markers for the characterization and authentication of various organisms including medicinal plants and their substitutes.

Key words: High GC-content primers; RAMP-PCR; Sequence-characterized amplified region (SCAR); Genetic authentication; *Lonicera japonica*; Substitutes

INTRODUCTION

Since the 1990s, a number of genetic markers and DNA marker techniques have been developed. They include random amplified polymorphic DNA (RAPD), internal transcribed spacer (ITS), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), inter-retrotransposon amplified polymorphism (IRAP), amplified fragment length polymorphism (AFLP), and restriction fragment length polymorphism (RFLP). The techniques can be used singly or in combination for the assessment of genetic diversity and the characterization of germplasms, fingerprinting of genotypes, and molecular-assisted breeding (Williams et al., 1990; Agarwal et al., 2008).

RAPD is based on the amplification of genomic DNA where whole-genome information is unavailable, and uses single primers comprising 10-base arbitrary nucleotide sequences. These primers detect polymorphisms in the absence of specific nucleotide sequence information; the polymorphisms function as genetic markers and can be used to construct genetic maps and carry out DNA fingerprinting, etc., (Agarwal et al., 2008). RAPD has several advantages: it requires fewer DNA templates, does not involve hazardous contamination, and is simple and inexpensive. However, it does have some disadvantages such as poor reproducibility and low productivity (Williams et al., 1990; Fu et al., 2013; Fu et al., 2015). To improve the effectiveness of the RAPD method, a modified RAPD technique, known as improved RAPD or RAMP-PCR, has been developed (Fu et al., 2000). The resolution and production of RAPD are greatly increased by prolonging the RAMP time from the stage of annealing to extension in PCR. This improved RAMP-PCR, whether used singly or in combination with other maker techniques, has been successfully applied to the characterization and authentication of some plants.

The sequence-characterized amplified region (SCAR) marker is a stable marker and is generally derived from random amplified polymorphic DNA (RAPD) or improved RAPD fragments (Yang et al., 2013; Fu et al., 2015). The basic principle is to convert the dominant markers into co-dominant markers to reduce the tedious procedures of RAPD. These markers generally reveal higher levels of polymorphism owing to higher annealing temperatures and longer primer sequence specificity. With a SCAR marker, analysis is reduced to a simple PCR analysis using PCR primers designed from the sequence of the amplicon of RAPD fragments (Kiran et al., 2010; Fu et al., 2013).

We developed a more effective DNA marker technique called "high GC-RAMP-PCR", in which we attained a GC content of 8-10 nucleotides over a length of 10 oligonucleotides (80-100%) by using RAMP-PCR (Unpublised Data). In this study, four SCAR makers produced by molecular cloning were developed from high GC-RAMP-PCR products of *Lonicera japonica* (*L. japonica*). This technique may provide a valuable approach for the genetic characterization of *L. japonica* and a means of distinguishing it from its substitutes.

MATERIAL AND METHODS

Primers for RAPD

Three standard 10-bp oligonucleotides (oligos) (90% G+C) for RAPD have been used. The sequences of the high GC-content primers are presented in Table 1.

DNA preparation

The five samples of *L. japonica* were collected from different regions of southern China: Shenzhen City in Guangdong Province (SZ), Yichang City in Hubei Province (YC), Leshan City and Emei City in Sichuan Province (LS and EM, respectively), and Loudi City in Hunan Province (LD), as described previously (Fu et al., 2013). Another *L. japonica* sample was taken from Houston in the USA. Samples of dried flowers from *Lonicera macranthoides* and *Lonicera confusa* were collected from Xiushan County in Sichuan Province. The DNA material from the leaf samples of *L. japonica* and from the leaves or dried flowers of other plant species was extracted using a slight modification to the CTAB method (Fu et al., 2013). We used the phenol/chloroform method for the extraction of DNA from other animal samples (Fu, 2012). DNA quality was determined by 1% agarose gel electrophoresis and by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE 19810, USA). The final concentration of all the DNA samples was adjusted to 10 ng/μL, and they were stored at -20°C until required (Fu et al., 2013).

Amplification of DNA by RAMP RAPD PCR

The PCR system (10 μL in total) was prepared as follows: 1 μL 2.5 μM high GC-content primer, 1 μL DNA template (10 ng) from *L. japonica* samples or from other species, 5 μL 2X PCR Taq MasterMix (TianGen Biotech Co. Ltd., Beijing, China), and 3 μL deionized water. The sequences of the high GC-content primers are presented in Table 1.

For RAMP PCR, a RAMP rate of 5% (0.125°C/s) was used to amplify the different species in an Applied Biosystems Veriti® 96-Well Thermal Cycler (Life Technology, CA, USA).

Agarose gel electrophoresis

The amplified high GC-RAMP-PCR products, screened PCR products, enzyme-digested plasmids, or SCAR analysis products were resolved by electrophoresis on a 1.5% agarose gel in 1X TAE (Tris-acetate-ethylenediamine tetra acetic acid [EDTA]) buffer. The gels were visualized by 0.5 mg/mL ethidium bromide staining, and the images were documented using a ChemiDoc XR imaging system (Bio-Rad, CA, USA). A DL2000 DNA Marker was used in the electrophoresis (TaKaRa Biotechnology Co., Ltd., Dalian, China).

Molecular cloning of improved RAPD fragments amplified by high GC-content primers

The cloning of improved RAPD fragments or high-GC-RAMP-PCR products has been described previously (Fu et al., 2015). Different bright bands were excised from agarose gels and purified using a DP209 TIANgel Mini Purification Kit (TIANGEN Biotech Co., Ltd.,

Beijing, China) according to the manufacturer instructions. Purified DNA fragments were ligated into a VT202 pGM-T vector (TIANGEN Biotech Co., Ltd., Beijing, China). Positive clones identified by PCR and DNA digestion were selected for Sanger sequencing.

Sequence homology searches and bio-informatic analysis

Homology searches of different species were performed in GenBank by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) using the cloned nucleotide sequences described above.

Development of SCAR markers and SCAR analysis

The sequences of each of the cloned RAPD fragments or high-GC-RAPD-PCR products were used to design pairs of SCAR primers using Primer 3 software from the http://frodo.wi.mit.edu/primer3 website. The primers for SCAR analysis were synthesized. The sequences of the SCAR primers, their amplification lengths, and the optimized PCR conditions are given in Table 2. Twenty-two DNA samples were used as templates for PCR amplification for validation of SCAR markers. They comprised five from *L. japonica*, and 17 from other species/varieties, including *Viola philippica*, *Penthorum sedoides*, *P. chinense*, *Ganoderma lucidum*, *Gardenia jasminoides*, *Litchi chinensis*, *Dimocarpus confinis*, *Canarium album*, *Gastrodia elata*, and *Rattus norvegicus*. The PCR reaction solution comprised 5 μL Taq 2X PCR MasterMix, 1 μL 2.5 μM primers, and 1 μL genomic DNA (10 ng), making a total volume of 10 μL. The amplification reactions comprised an initial pre-denaturation for 90 s at 95°C followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. The final extension step was performed at 72°C for 5 min. The amplified PCR products were resolved by 1.5% agarose electrophoresis (Fu, 2012).

To distinguish between *L. japonica* and *L. confusa*, PCR amplifications were performed using the four pairs of SCAR primers and the amplification conditions described above (Table 2).

RESULTS

Amplification of *L. japonica* DNA using the improved RAPD technique with high GC-content primers

RAMP-PCR with the high GC-content primers FY-27, FY-28, and FY-29 (Table 1) was used to complete amplification of the *L. japonica* DNA samples (Figure 1). We then cut specific bands, as shown in Figure 1, which we called JYHGC1 and JYHGC2 (Figure 1A), and JYHGC6 and JYHGC7 (Figure 1B). The PCR products were purified. Quality assessment was performed on an agarose gel and determined by a single band of the correct size (Figure 2A and data not shown).

Table 1. Sequences of high GC-content primers.

Name	Sequences (5'-3')
FY-27	GCAGGCGGCG
FY-28	CGAGCCCGCC
FY-29	GCCGACCGGC

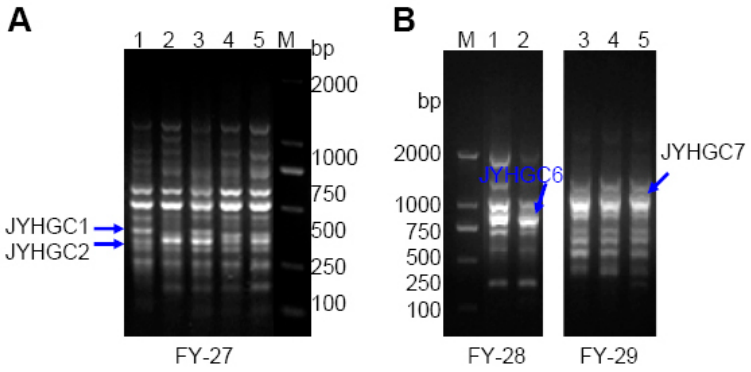

Figure 1. Bands amplified by RAMP-polymerase chain reaction (PCR) (5% RAMP rate) with three different high GC-content primers from *Lonicera japonica* samples. **A.** The fragments JYHGC1 and JYHGC2 from the indicated bands using high GC-content primer FY-27. *Lanes 1-5* = different *L. japonica* samples taken from Shenzhen City in Guangdong Province (SZ), Yichang City in Hubei Province (YC), Leshan City and Emei City in Sichuan Province (LS and EM, respectively), and Loudi City in Hunan province (LD), respectively. **B.** The fragment JYHGC6 from the indicated band using high GC-content primer FY-28 and the fragment JYHGC7 from the indicated band using high GC-content primer FY-29. *Lanes 1-5* = different *L. Japonica* samples taken from Leshan City and Emei City in Sichuan (LS and EM, respectively), Leshan City and Emei City in Sichuan (LS and EM, respectively), and Loudi City in Hunan (LD), respectively. The blue arrows indicate bands cut from the agarose gels. *Lane* "*M*" = the DL2000 DNA marker with the indicated sizes (bp).

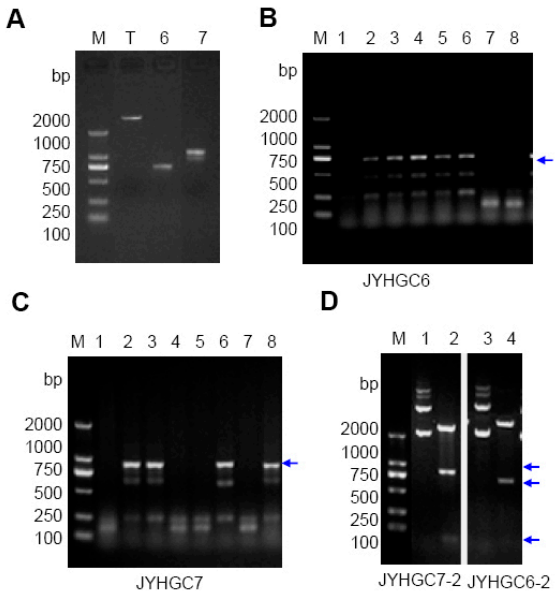

Figure 2. Cloning of RAPD fragments. **A.** Agarose gel electrophoresis from purified bands. *Lane* "*T*" = T-vector; *lane* "*6*" = fragment JYHGC6 using high GC-content primer FY-28; *lane* "*7*" = fragment JYHGC7 using high GC-content primer FY-29. **B.** Colonial polymerase chain reaction (PCR) screening for different clones of JYHGC6. **C.** Colonial PCR screening for different clones of JYHGC7. d. *Eco*RI cut for positive clone verification with extracted plasmids. *Lanes 1* and *2* = cloned JYHGC7-2 plasmids without and with *Eco*RI digestion. Two inserts were identified by *Eco*RI digestion indicating there was one *Eco*RI site in the fragment JYHGC7, which was verified by DNA sequencing. *Lanes 3* and *4* = cloned JYHGC6-2 plasmid without and with *Eco*RI digestion. *Lane* "*M*" = the DL2000 DNA marker.

DNA cloning, sequencing, and characterization of high-GC-RAPD PCR products

The four RAPD fragments or high-GC-RAPD PCR fragments were successfully cloned into the pGM-T vector. Then PCR and enzymatic verifications were performed, and all showed the predicted sizes (Figure 2B, C and data not shown). The positive clones were then sequenced, and four DNA sequences were obtained; their names, sizes, and GenBank Nos. were: JYHGC1-1, 345 bp, KJ620024 (Figure 3A); YJHGC2-1, 388 bp, KJ620025 (Figure 3B); JYHGC7-2, 1036 bp, KJ620026 (Figure 3C); and JYHGC6-2, 715 bp, KJ620027 (Figure 3D), respectively.

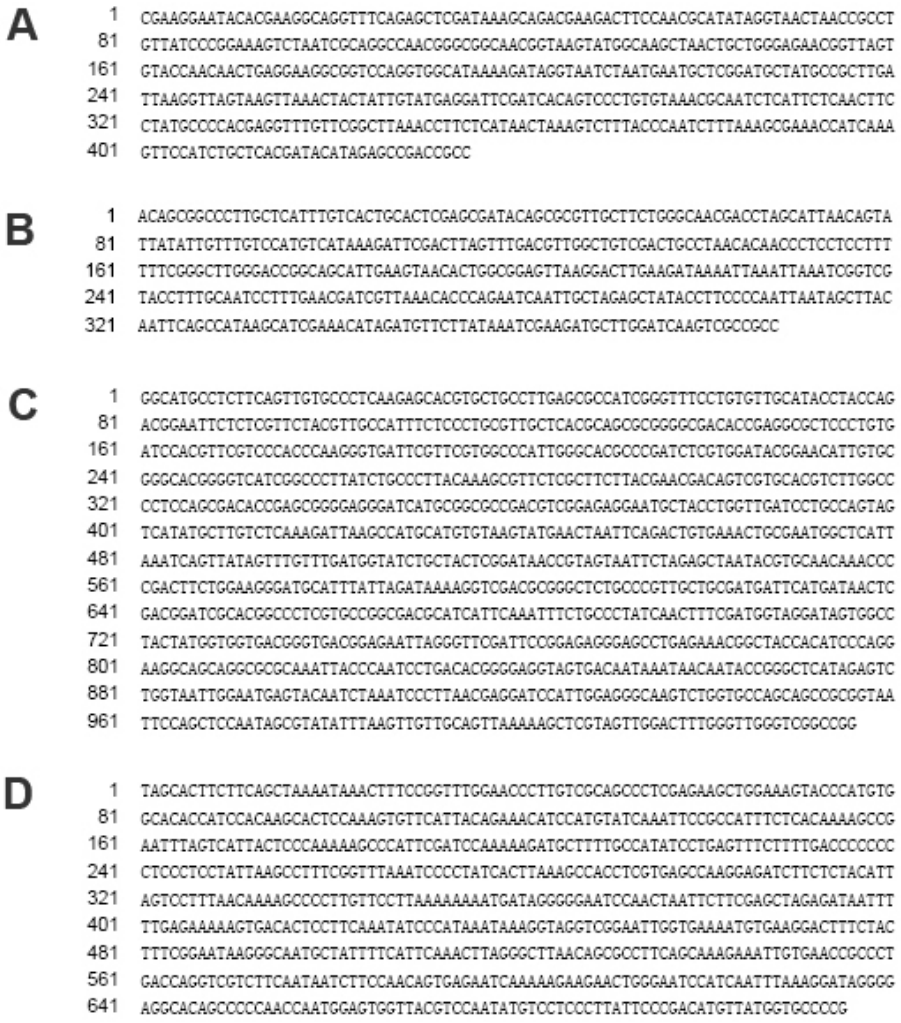

Figure 3. Cloned nucleotide sequence information from Sanger sequencing. **A.** The sequence of clone JYHGC1-1. **B.** The sequence of clone JYHGC2-1. **C.** The sequence of clone JYHGC7-2. **D.** The sequence of clone JYHGC6-2.

BLAST searches of the nucleotide sequences in GenBank showed that 290 nucleotides of clone JYHGC1-1 shared 95% identity with *Phoenix dactylifera* mitochondrion, complete genome (Sequence ID: JN375330.1); 179 nucleotides of clone JYHGC2-1 shared 88% identity with *Glycine max*, strain Williams 82, clone GM_WBb0158O04, complete sequence (Sequence ID: AC235444.1); 820 nucleotides of clone JYHGC7-2 shared 92% identity with *Solanum lycopersicum* chromosome 3, clone EcoRI0042E07, complete sequence (Sequence ID: AC246968.1); and 670 nucleotides of clone JYHGC6-2 shared 89% identity with *P. dactylifera* mitochondrion, complete genome (Sequence ID: JN375330.1) (Figure 4A, B, C and D).

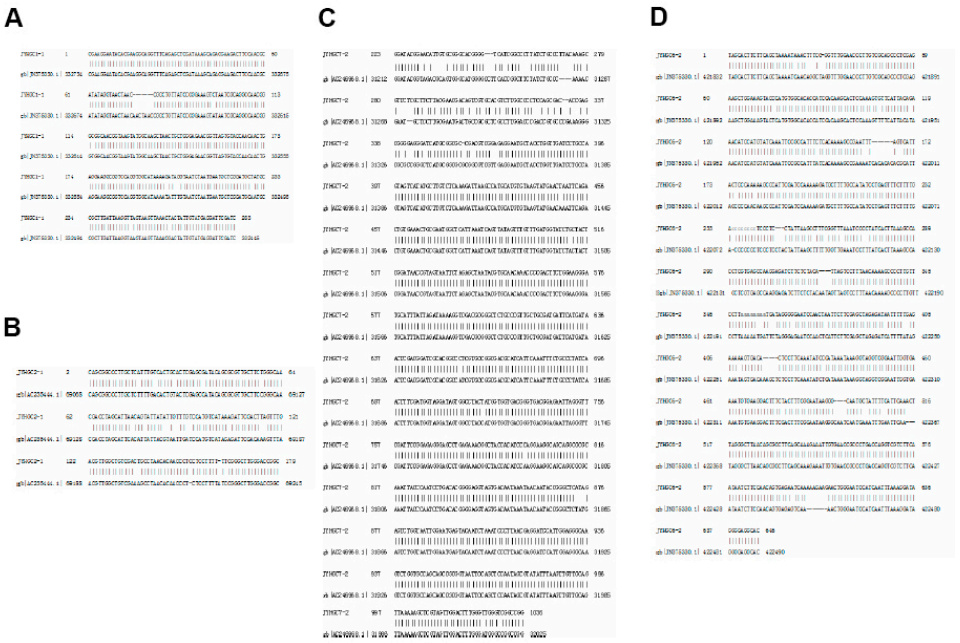

Figure 4. BLAST search results for the four cloned nucleotide sequences. **A.** BLAST of JYHGC1-1. **B.** BLAST of JYHGC2-1. **C.** BLAST of JYHGC7-2. **D.** BLAST of JYHGC6-2.

Development of *L. japonica*-specific SCAR markers

To generate stable *L. japonica*-specific diagnostic SCAR markers from the cloned high-GC-RAMP-PCR products mentioned above, four pairs of primers (Table 2) were designed and synthesized based on cloned sequences.

The designed SCAR primer pairs were then used to amplify the genomic DNA from 18 of the collected DNA samples to test species-specific amplification. The PCR amplification results indicated that the products of the expected size were only observed in five *L. japonica* samples using the SCAR markers JYHGC2-1 and JYHGC7-2; there was no amplification in other species (Figure 5, panel 2 & 3), which indicates that SCAR markers JYHGC2-1 and JYHGC7-2 are *L. japonica* species-specific.

Table 2. Sequences of sequence-characterized amplified region (SCAR) primers, polymerase chain reaction (PCR) conditions, and amplified product sizes.

SCAR	5'-primer	Sequence (5'-3')	3'-primer	Sequence (5'-3')	Size (bp)	Tm (°C)
JYHGC1-1	JYHGC1-1L	AAGTCTAATCGCAGGCCAACG	JYHGC1-1R	TCGTGGGGCATAGGAAGTTGA	242	61
JYHGC2-1	JYHGC2-1L	CATTTGTCACTGCACTCGAGC	JYHGC2-1R	GTGTTACTTCAATGCTGCCGG	180	61
JYHGC7-2	JYHGC7-2L	TACGTTGCCATTTCTCCCTGC	JYHGC7-2R	AGGTAGCATTCCTCTCCGACG	285	61
JYHGC6-2	JYHGC6-1L	CCTCGTGAGCCAAGGAGATCT	JYHGC6-2R	GGTCAGGGCGGTTCACAATTT	275	61

The PCR amplification results using SCAR marker JYHGC1-1 showed a specific band of the expected size in *L. japonica* samples from Shenzhen in Guangdong and Leshan in Sichuan (Figure 5, panel 1), without any amplification in the other 16 samples, which indicates that the marker JYHGC1-1 is *L. japonica* strain-specific or *L. japonica* cultivar-specific.

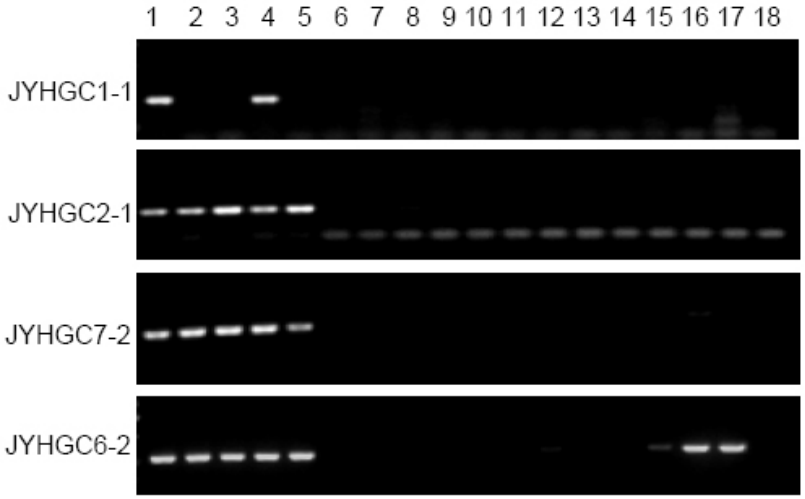

Figure 5. Analysis of sequence-characterized amplified region (SCAR) markers JYHGC1-1, JYHGC2-1, JYHGC7-2, and JYHGC6-2 in the various species. *Lanes 1-5* = samples of *Lonicera japonica* from Shenzhen City in Guangdong, Yichang City in Hubei, Emei City and Leshan City in Sichuan, and Loudi City in Hunan, respectively; *lanes 6-7 = Dimocarpus longan* from Fujian and Hainan; *lanes 8-9 = Ganoderma gibbosum* (Blumii et Nees) Patouillard and *Ganoderma lucidum*; *lane 10 = Gardenia jasminoides*; *lane 11 = Litchi chinensis* from Guangdong, *lane 12 = Dimocarpus confinis* from Guangxi; *lane 13 = Canavium album* from Luzhou City in Sichuan; *lane 14 = Gastrodia elata* from Liangshan City in Sichuan; *lane 15 = Viola philippica*; *lane 16 = Penthorum chinense* from Gulin County in Sichuan; *lane 17 = Penthorum sedoides*; *lane 18* = from *Rattus norvegicus* (rat) liver.

The PCR amplification results using SCAR marker JYHGC6-2 showed a specific band of the expected size in all five *L. japonica* samples and the other three species (*V. philippica*, *P. chinense* and *P. sedoides*), which indicates that the SCAR marker JYHGC6-2 is not *L. japonica* species-specific (Figure 5, panel 4).

Authentication of *L. japonica* from *L. confusa*

To distinguish between *L. japonica* and *L. confusa* or its substitutes, PCR amplification was performed using the four pairs of developed SCAR primers (Table 1) with

samples mentioned in the Material and Methods section and one representative sample of *L. macranthoides* for *L. confusa*. The result showed that the PCR products with expected size were observed in only six *L. japonica* samples using the SCAR markers JYHGC2-1 and JYHGC6-2, without any amplification in the sample of *L. confusa*, which indicated that these two makers are *L. japonica*-specific, and are useful for distinguishing between *L. japonica* and *L. confusa* (Figure 6, panels 2 and 4). However, we also noticed the PCR product from *L. confusa* had the same expected size as *L. japonica* using SCAR marker JYHGC7-2, which indicated that this marker is not *L. japonica*-specific, and cannot be used to distinguish between *L. japonica* and *L. confusa* (Figure 6, panel 3).

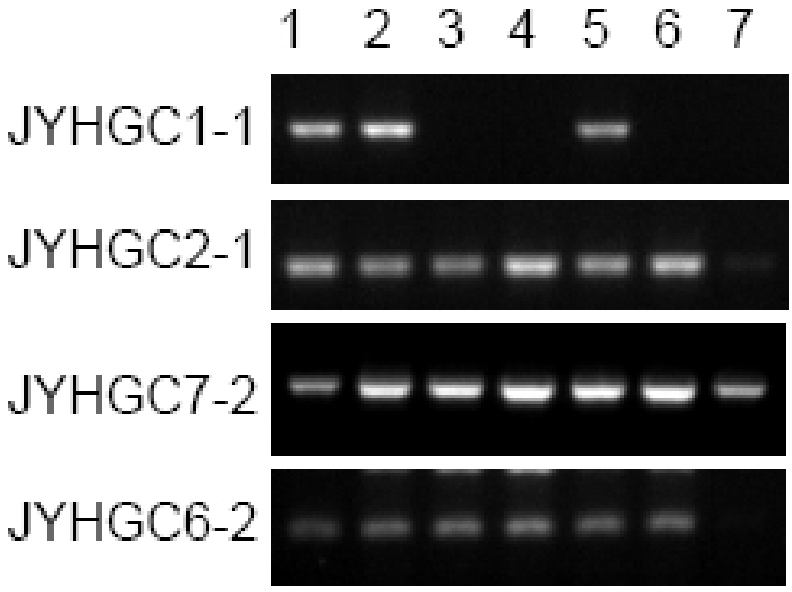

Figure 6. Authentication of *Lonicera japonica* from *Lonicera confuse* by sequence-characterized amplified region (SCAR) markers JYHGC1-1, JYHGC2-1, JYHGC7-2, and JYHGC6-2. *Lanes 1-6* = samples of *L. japonica* from Houston in the USA, Shenzhen City in Guangdong, Yichang City in Hubei, Emei City and Leshan City in Sichuan, and Loudi City in Hunan, respectively; *lane 7* = *Lonicera macranthoides* of *L. confuse* sample from Xiushan County in Sichuan.

The SCAR marker JYHGC1-1 amplified a specific band of the expected size only in the samples of *L. japonica* from Houston in the USA, Shenzheng in Guandong Province, and Leshan in Sichuan Province (Figure 6, panel 1), which indicated that this marker is not *L. japonica*-specific. However, it is specific to *L. japonica* cultivars, and can be used for the authentication of specific *L. japonica* samples from different localities.

DISCUSSION

Analysis of genetic variations can help us understand the molecular basis of various biological phenomena. It can also aid gene mapping, DNA fingerprinting, and genetic characterization and authentication of different organisms, including medicinal plants, from

their substitutes. Advanced marker techniques tend to amalgamate the advantageous features of several basic techniques (Kiran et al., 2010; Fu et al., 2015). RAPD has been widely used as a technique for the genetic characterization and authentication of various organisms since 1990 (Williams et al., 1990). One of the limitations of this technique is the lower level of repeatability of banding patterns if the amplification reactions are not optimized (Williams et al., 1990; Agarwal et al., 2008). In our previous studies we used an improved RAPD technique with a prolonged RAMP time, which increased the band numbers, repeatability, and DNA production (Fu et al., 2000; Mei et al., 2014; Fu et al., 2015), as confirmed by ISSR (Fu et al., 2013). We successfully used high GC-content primers with a GC content of 8-10 nucleotides over a length of 10 oligonucleotides (80-100% GC content of nucleotides) and improved RAPD (high GC-RAMP-PCR), to further increase the RAPD specific bands and the amplifying efficiency. A previous study reported that regular RAPD analysis with high GC-content primers only amplified well in certain plants owing to the absence of RAMP-PCR (Kubelik and Szabo, 1995).

SCAR markers are stable diagnostic markers, generally derived from RAPD fragments linked to a trait of interest, and convert the dominant markers into co-dominant markers to reduce the tedious procedures of RAPD. These markers generally reveal higher levels of polymorphism and specificity owing to higher annealing temperatures and longer primers (Yang et al., 2013). With SCAR markers, analysis is reduced to a simple PCR analysis using specific PCR primers designed from the cloned RAPD sequence (Kiran et al., 2010; Yang et al., 2013; Fu et al., 2015; Mei et al., 2015). Therefore, four fragments from improved RAPD PCR using high GC content have been cloned, and SCAR markers have been developed. PCR products of the expected size were observed in five *L. japonica* samples by SCAR markers JYHGC2-1 and JYHGC7-2, which are *L. japonica* species-specific. The lack of non-specific amplicons in the other species indicates the efficacy of these markers in distinguishing the *L. japonica* species from the others. The SCAR marker JYHGC1-1 was found to be specific for *L. japonica* samples only from Shenzhen City in Guangdong Province and Leshan City in Sichuan Province, so this marker may be used to distinguish these specific strains of *L. japonica*. SCAR marker JYHGC6-2 showed a specific band in all the *L. japonica* samples and the other three species (*V. philippica*, *P. chinense* and *P. sedoides*), which indicates that JYHGC6-2 might not be a good marker for distinguishing *L. japonica* from other species. Thus, stable diagnostic SCAR markers for the authentication of *L. japonica* were developed here.

Jin Yin Hua comprises the dried flower-buds or flower-buds of early-opening *L. japonica* (Thunb.). *L. japonica* and other *Lonicera* species, as traditional Chinese medicine, are well known for their anti-inflammatory, anti-cancer, anti-virus, anti-angiogenic, wound healing, hepato-protective, and anti-oxidant activities. A number of compounds that have been identified and isolated from *Lonicera* species, including biflavonoids, quercetin, phenolic acids, and dicaffeoylquinic acid, are supposed to be responsible for these various pharmacological and biological activities (Shang et al., 2011; Fu et al., 2013). *L. confusa* is known as *Shan Yin Hua* in markets, but its pharmacological components and biological activities might be different, and it is considered a substitute for *L. japonica* (Shang et al., 2011; Yang et al., 2014). In addition, *L. japonica* is more expensive than *L. confusa*. Therefore, to distinguish between *L. japonica* and *L. confusa*, an *L. macranthoides* dried flower sample (*L. macranthoides* sample as a representative sample of *L. confusa*) was collected and PCR amplification was performed using the four pairs of SCAR primers (Table 1). The results showed that the PCR products of expected size were observed in only six *L. japonica* samples using the SCAR

markers JYHGC2-1 and JYHGC6-2; there was no amplification in the sample of *L. confusa*, indicating that those two makers are *L. japonica*-specific, and are useful for distinguishing *L. japonica* from *L. confusa* (Figure 6, panel 2 & 4). However, we noticed that the PCR product from *L. confusa* had the same expected size as *L. japonica* using SCAR marker JYHGC7-2, which indicated that this marker is not *L. japonica*-specific, and cannot be used to distinguish between *L. japonica* and *L. confusa*

(Figure 6, panel 3). However, we should point out that SCAR marker JYHGC6-2 showed a specific band in three other species (*V. philippica*, *P. chinense*, and *P. sedoides*), which indicates that JYHGC6-2 might not be a good marker for distinguishing *L. japonica* from other species except *L. confusa* (Figure 5, panel 4). The fact that SCAR marker JYHGC1-1 amplified specific bands only in the samples of *L. japonica* from Houston in the USA, Shenzheng in Guandong Province, and Leshan in Sichuan Province (Figure 6, panel 1), indicates that this marker is not *L. japonica*-specific, but is specific to *L. japonica* cultivars, and can be used in the authentication of *L. japonica* from specific samples of different localities.

In conclusion, we have developed highly effective SCAR markers from high GC-RAMP-PCR products by molecular cloning. This is the first time that specific SCAR markers have been developed from high GC-RAMP-PCR products. We hope that our results may make a valuable contribution to the development of DNA markers for the genetic characterization and authentication of various organisms, including medicinal plants and their substitutes.

Conflicts of interest

The authors declare no conflict of interest.

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