BIODIVERSITAS
Volume 18, Number 3, July 2017
Pages: 1243-1249

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d180349

Short Communication: Rapid and accurate genetic authentication of *Penthorum chinense* by improved RAPD-derived species-specific SCAR markers

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Manuscript received: 28 June 2017. Revision accepted: 29 July 2017.

Abstract. *Mei Z, Khan MA, Zhang X, Fu J. 2017. Short Communication: Rapid and accurate genetic authentication of* Penthorum chinense *by improved RAPD-derived species-specific SCAR markers. Biodiversitas 18: 1243-1249.* For the genetic identification and authentication of living organisms, development of Sequence-Characterized Amplified Region (SCAR) markers from Random Amplified Polymorphic DNA (RAPD) fragments is a valuable molecular approach. By using SCAR markers, molecular analysis is simplified to a Polymerase Chain Reaction (PCR) analysis using PCR primers designed from specific sequences of the RAPD amplicons. In this study, RAPD fragments from improved RAPD amplification of a perennial herb *Penthorum chinense* Pursh from China were cloned into a T-vector, and positive clones were identified by PCR amplification, and sequenced with the Sanger sequencing method for the SCAR marker development. Five SCAR markers were developed that were very specific to *P. chinense*, and deposited in GenBank (accession numbers: KX671029, KX671030, KX671031, KX671032 and KX671033). BLAST searches of these five nucleotide sequences in the GenBank database showed no identity with markers from other species. This study was developing five specific SCAR markers, has enabled reliable genetic identification of the herbal plant species *Penthorum chinense* Pursh, useful for authenticating future samples of this important medicinal herb.

Keywords: Genetic identification, molecular markers, *Penthorum chinense*, RAPD, sequence characterized amplified region

INTRODUCTION

Penthorum chinense Pursh is a perennial herb found predominantly in East Asia, particularly in China, Korea, Thailand, Vietnam, Laos, Mongolia and some parts of Russia (Flora of China 2017). Hence the plant is often called Oriental penthorum, having long been used in traditional Chinese medicine. Nevertheless, in modern biological science, there have been only a few reports concerning this useful plant. Some studies have reported on antioxidant and anti-cancer cancer properties of *P. chinensis,* as well as on potential hepatoprotective, antiviral and anti-diabetic uses (Cao et al. 2015; Hu et al. 2015; Huang et al. 2015; Lu et al. 2012; Wang et al. 2015; Zhang et al. 2013). Certain bioactive compounds including quercitrin, quercetin-3-O-rhamnoside, gallic acid, β-sitosterol, thonningianins A, 5-hydroxy-flavanone-7-O-beta-D-glucoside, 2,4,6-trihydroxybenzoic acid, pinocembrin-7-O-beta-glucoside, 5-methoxy-pinocembrin-7-O-β-D-glucoside and pinocembrin-7-O-[3"-O-galloyl-4",6"-hexahydroxydiphenoyl]-beta-glucose have been isolated from this plant species (Feng et al. 2001; Lu et al. 2012; Wang et al. 2006, 2014). These compounds might play important roles in its medicinal properties. The recent interest of medicinal chemists in the plant have focused attention on the need for a reliable system of identification for the species, and on its genetic authentication and conservation.

In recent developments of biotechnology, a number of molecular techniques have been used for the genetic identification and authentication of plant, animal and bacterial species, such as Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter-Simple Sequence Repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP) analyses (Williams et al. 1990, Fu et al. 2013; Khan et al. 2016). One particularly useful approach, based on initial RAPD sequencing, is Sequence Characterized Amplified Region (SCAR) analysis, which has the advantage of generating markers that are usually very stable and which therefore provides a high level of authenticity in genetic identification (Fu et al. 2015; Khan et al. 2016; Fu et al. 2017). By using the SCAR markers, molecular analysis has been simplified to a PCR reaction using PCR primers designed from the sequences of RAPD amplicons (Kumla, et al. 2012; Fu et al. 2015).

In the study reported here, we have sought to apply such molecular techniques, to reliably identify specimens of the plant *P. chinense* sampled from different geographical locations in China. For the molecular identification, we have developed SCAR markers, originated from RAPD analysis.

MATERIALS AND METHODS

Samples and DNA extraction

A total of nine samples of *Penthorum chinense* Pursh and one sample of the species *Eclipta prostrata* (L.) L. were gathered from different regions of China (Table 1). Genomic DNA was extracted from fresh leaves using a previously described modified cetyl Trimethyl Ammonium Bromide (CTAB) method (Fu et al. 2013). Briefly, the plant materials were first fixed in fixing solutions containing chloroform, PVP, 2-Hydroxy-1-ethanethiol (without liquid nitrogen), and ground into tiny pieces by silica (SiO_2) for DNA extraction. The DNA quality was checked by electrophoresis on 0.8% agarose gel and spectrophotometry (Fu 2012). For PCR, the final concentration of all DNA samples was adjusted to 10 ng/μL, and stored at -20^{o}C till use.

Improved RAPD Amplification

Different DNA samples were amplified with RAPD primers A12, I19, I19, Q19 and Q19 using Tiangen reagents (Beijing, China) according to the manufacturer's protocol. The sequences of these primers are listed in Table 2. The PCR reaction system of 10 μL contents consisted of 5 μL 2×Taq PCR MasterMix, 1 μL 2.5 μM primer, 1.5 μL genomic DNA, and 2.5 μL ddH_2O. Amplification reactions were performed in a PCR machine "Applied Biosystems Veriti® 96-Well Thermal Cycler" (Life Technology, USA) using the following steps: initial denaturation at 95°C for 90 s, 40 cycles of denaturation at 94°C for 40 s, annealing at 36°C with the RAMP rate from annealing to extension adjusted to 0.125ºC/s (5% ramp rate) for 60 s, extension at 72°C for 90 s, and a final extension step at 72°C for 5 min (Mei et al. 2015). PCR products were loaded onto a 1.5% agarose gel for electrophoresis.

Molecular cloning and identification of positive RAPD DNA fragments

Eight different bright bands were excised from the agarose gel, and purified by using TIANgel Mini DNA Purification Kit (DP209, Tiangen reagents, Beijing, China) according to the manufacturer's protocol. The purified DNA fragments were ligated into pGM-T vector (No. VT202) (Tiangen reagents, Beijing, China) by AT cloning, and then transformed into DH5α *E. coli* competent cells. The recombinant clones were spread on LB agar plates, containing ampicillin (100 μg/μL), X-gal (40 mg) and IPTG (160 μg), and incubated at 37°C overnight. The blue white screening method was used and white colonies were screened out. The presence of right insert was verified by PCR from white colonies by using T7/SP6 primer pairs (T7 primer: 5'-TAATACGACTCACTATAGGG-3', SP6 primer: 5'-ATTTAGGTGACACTATAGAA-3'), then run on a 1% agarose gel electrophoresis (Mei et al. 2015; Khan et al. 2016; Fu et al. 2017).

DNA sequencing and bioinformatics

The sequencing of the positive clones was performed by the Sanger method using SP6 of T-vector sequencing primers. To remove the vector sequences and verify whether the sequences of cloned RAPD fragments were novel, the online program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was used for the homology search of our sequenced DNA clones with different species in the GenBank database.

SCAR primer design

By using online program Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), the nucleotide sequence of each of the cloned RAPD fragments was used to design pairs of SCAR primers. The quality of primer pairs was tested. The sequences of each primer, optimized PCR condition, and amplification length are shown in Table 3.

Table 1. Sources of RAPD samples

Name	Sources	Sample
Penthorum chinense Pursh	Yingtan, Jiangxi	YT
Penthorum chinense Pursh	Anqing, Anhui	AQ
Penthorum chinense Pursh	Ankang, Shanxi	AK
Penthorum chinense Pursh	Chenzhou, Hunan	CZ
Penthorum chinense Pursh	Luzhou, Sichuan	LZ
Penthorum chinense Pursh	Xichang, Sichuan	XC
Penthorum chinense Pursh	Zunyi, Guizhou	ZY
Penthorum chinense Pursh	Yichang, Hubei	YC
Penthorum chinense Pursh	Xinzhou, Shanxi	XZ
Eclipta prostrate	Luzhou, Sichuan	EP

Table 2. Sequences of RAPD primers

Primer	Sequence (5'-3)'	Primer	Sequence (5'-3')
SBS-A12	TCGGCGATAG	SBS-I19	AATGCGGGAG
SBS-N19	GTCCGTACTG	SBS-Q19	CCCCCTATCA

Table 3. Sequences of SCAR primers, PCR product size and PCR condition

SCAR	5'-primer	Sequence (5'-3')	3'-primer	Sequence (3'-5')	Size (bp)	Tm (°C)
A12-12	A12-12L	GTTTGGAGGCAAGAAAACCA	A12-12R	TTGTCCTTCTGGTCTATGTGGA	225	60
I19-13	I19-13L	TCGACTTGAAGGGCTCGTTA	I19-13R	AAAAATCCATGGCAAAATGC	213	60
I19-22	I19-22L	GTTAGGGCTTGGTTCACGAG	I19-22R	AACCCGGTAGATGATGATGC	212	60
Q19-11	Q19-11L	TCGTCGAACACTTGGTGTGT	Q19-11R	TCTCCCCATCATTCTTACGG	257	60
Q19-22	Q19-22L	TGGTAGGATATTTCTGCGACTG	Q19-22R	GACTCGAGATGGAGGCACAG	230	60

SCAR markers development and analysis

To develop stable SCAR markers, the PCR amplification was performed by using 22 DNA samples as templates. They are the different samples of *Penthorum chinense* listed in Table 1, and one sample of each of *Eclipta prostrata* (L.) L., *Canarium album* (Lour.) DC., *Dimocarpus longan* Lour., *Litchi chinensis* Sonn., *Mentha haplocalyx* Briq., *Lycium barbarum* L., *Angelica sinensis* (Oliv.) Diels, *Ginkgo biloba* L., Russian Knapweed *Rhaponticum repens* (L.) Hidalgo, *Ganoderma lucidum* (Curtis) Karst., *Ganoderma japonicum* (Fr.) Sawada, *Gardenia jasminoides* J. Ellis, and *Artemisia argyi* H. Lév. & Vaniot. The content of the 10 μL PCR reaction system was as follows: 5 μL 2×Taq PCR MasterMix, 1 μL of 2.5 μM each pair of SCAR primers, and 1μL genomic DNA (10 ng), with the remaining volumes filled by ddH_2O. PCR amplification was performed in the above-mentioned "Applied Biosystems Veriti® 96-Well Thermal Cycler" with an initial pre-denaturation for 90 s at 95°C followed by 30~34 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s. The final extension step was performed at 72°C for 5 min. The amplified PCR products were separated on 1.8% agarose gel in 1 × TAE buffer. Gels were then visualized by 0.5 μg/mL ethidium bromide staining and the images were documented using the ChemiDoc XRS (Bio-Rad, USA) (Fu, 2012).

RESULTS AND DISCUSSION

Cloning of RAPD amplification fragments

Five RAPD primers (A12, I19, I19, Q19, and Q19) were used for the improved RAPD amplification of DNA samples (Figure 1). The improved RAPD amplification results are shown in Figure 2, where the white arrows indicate the bands labeled with primers. The arrow-indicated bands were cut from the agarose gel and purified. Then the purified PCR products were ligated into the pGEM T-vector. The blue and white screening method in LB agar plate was used to screen the positive clones (data not shown). The white clones were then identified by PCR amplification using the SP6/T7 primer pair. The results of positive clones are shown in Figure 3. In Figure 3.A, the positive clone I19-13 shown in lane 1-2, and the clone I19-22 in lane 3-4, have the inserted DNA-fragment with size ~2100bp, whereas the positive clones A12-12, Q19-11 and Q19-22 are shown in Figure 3.B as three inserted DNA-fragments with right length in sizes 2000~2400 bp.

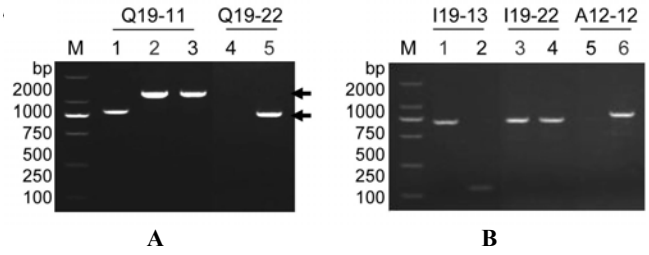

Figure 2. Recovery of RAPD fragments from *Penthorum chinense* using different RAPD primers

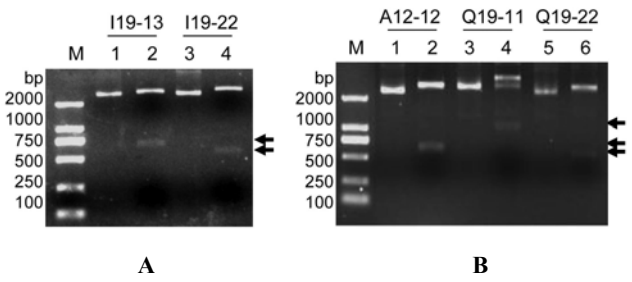

Figure 3. Identification of positive clones after DNA ligation. A. Clone identification of RAPD fragments I19-13 and I19-22. B. Clone identification of RAPD fragments A12-12, Q19-11, Q19-22. The black arrows indicate expected PCR bands in size of different clones. *Lane M* indicates the DNA molecular weight marker DL2000 with the fragment size (bp).

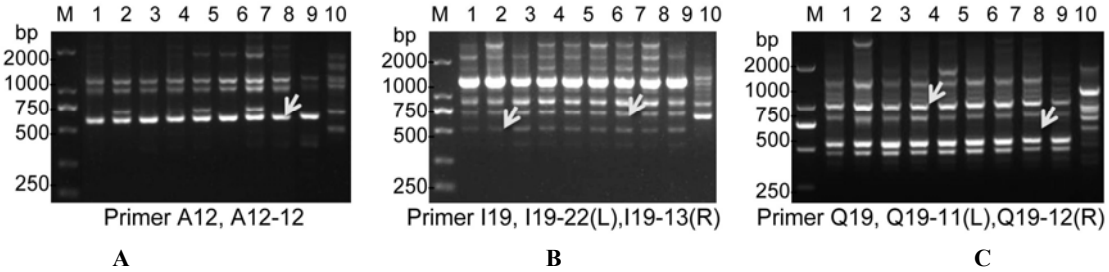

Figure 1. Improved RAPD amplification from DNA samples of *Penthorum chinense* (listed in Table 1) using different RAPD primers.

Sequencing and characterization of specific RAPD fragments

The sequencing of the above-mentioned five RAPD fragments of clones A12-12, I19-13, I19-22, Q19-11 and Q19-22 were performed. This was followed by BLAST searches of the nucleotide sequences in the GenBank database, which indicated that the five clones had no significant identity with that of any other species in the database. The sequenced results revealed that clone A12-12 consisted of 598 nucleotides. It was deposited into GenBank with accession number KX671029 (Figure 4.A). Clone I19-13 consisted of 718 nucleotides, and was deposited into GenBank with accession number KX671030 (Figure 4.B); clone I19-22 consisted of 502 nucleotides, and was deposited into GenBank with accession number KX671031 (Figure 4.C); clone Q19-11 consisted of 572 nucleotides, and was deposited into GenBank with accession number KX671032 (Figure 4.D); and clone Q19-22 consisted of 524 nucleotides, and was deposited into GenBank with accession number KX671033 (Figure 4.E). Here the actual lengths of the high-quality nucleotide sequences obtained using SP6 vector primer are shorter than the RAPD fragments.

A

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  1 AGGTGGAAGGATCAGCAAGCAAATCTCCTTCAAAAGAACTTAATCTAGTCTTGGGATCCATAGGAATGGTAACTGGTTTG
 81 GAGGCAAGAAAACCAGTGTCTTCAAGGAGCAGTAATGCATACTTGCGTTGTGACATAAAAATCCCTTTACTAGATTGTGC
161 TATTTCAAGTCCAAGAAAATATTTTAAAATTCCCAAATCTCTTAGCTTGAACTGAGATTAGAGAAATTGCTTCAGAGCAG
241 AAATAATAGTTTTATTAAGCCCAGTTATAAAAATATCATCCACATAGACCAGAAGGACAATAAAATCAGAACCAGTTCCT
321 TTAGTGAACAAAGAATAATCTGACTTAGATTGTTGAAAACTGTAAGCAAGCAAAGAGTAAGAGAATTTTTCAAACCATTA
401 CTGGGAGGTCTACTTCAAACCATAAATGGATTTATGTAACTTGCACACCAACTTCTCCCATTGAGTGATAGCTTGAGAAG
481 GAACTTTGTAGCCTAAAGGCAAATCCATATGCACTTCTTCAAATAGTTCGTCATCAAGAAAAGCATTATTCACATCTAGT
561 TGGACAATAAACTATTATTTAGAAGCTGCAATAGAA
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B

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  1 ATGAAATTTTAGTAATGAAAGATGCTTGTACAATAATGAATTCGGGATAAATTATAAGTTAAATTTTTAACAAGTGGTGT
 81 TTGTACCGGTATAATATGTGAATTTAATCTCTAATTCAATTTACATAAATTCAATTTATACGATATAAAAAATATGTATT
161 CTATATAAATTTGTTCTTATATTTAAATATGAGAAATTTGAGATTATCTTTTATTTTTACTTTATGTAATTGAAATTTTA
241 TTCGAATAGAATAAAGGACTCAATATCATAGCATTTACATTATATTATTAGATTAAAATAAAAAGTTCATATGTAGAATT
321 AGGGGTATACTCGAGCCGAGCTCAAAACGAACTGTTCGGGCTCGGTTCGAGCTCGTTTAAGACGAGCTCGTTAAGGGCTC
401 GATTAAGTTTTTTTTTTCCTTCATAATATTCGGCTCGACTTGAAGGGCTCGTTAACGAGCTCGAGCGTTAATGGGGAAGA
481 ATTTCGAATATTGATACCAAAAACGTGGTGTTTTGGTGTTTTTTTTTCAAAACGTAGCGTTTTGTTAAGTGTTCGAATAT
561 TAATACCCAAAATGTGGCGTTTTGCGTATCAATGTAGACAAAATGTGGCTTTTTAGACCAAAACGCCGCATTTTGCCATG
641 GATTTTTTCCCAAATCCGAAATTGCAAAACCTTAGAAATTGATCCTACCACCCGTCCGTTCCCTCCTCCCGCATTAAT
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C

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  1 AGCGAATTGCCGGGATCTGGCGGAGAAAGGTACTAAGTAATAGTTGTTAACAAAATCGTTGCCACCCAGGGTGATCAGCG
 81 TTAGGGCTTGGTTCACGAGCTGTGCTGCCTGATCAAACCCAACGAGTGCAGCCAGTCTTTGCTGGTACTGTTGGAAGTAT
161 TCCAATTGCCTGCCGATCCTTATTATATTTATCTGCACAAGTGTAAAATATGTTACAAAAGTAGCTTTCTTGCGTGTCTG
241 ACCCCTCATACTCGTGATAGGGGGAGCGGGAGCATCATCATCTACCGGGTTTGCCCAATCAGACATGCTGAGTTATGACT
321 ATTTTTCTTCTTCTTTCCAACATCATTTATTTTGGTTATGAATGAGAGATTGCCATGCCCCTCTCAATTAATGCATCTTT
401 GGGTCAAAATAGCCATCCTTTTCTTTTCCTGATGCTAAGTGAGCAAAAGAATACGGCACTCTCGTTCAACTAAATGATTG
481 CTTTTTTGGTCAATTTTAACCT
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D

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  1 TAACCAGCCATCCAAGAACATATTGCACATGACGCTCATATAATTCCTCATTCTTAAAAATAGTCGGGTCAAATACCAAT
 81 GATGATGAAGCAATTTTATGCTTTTAGGAGGAGCTCGCTTGTGAGTGTTTCGGAGCCATGTGTTTAACAACGCAAAAGTT
161 TCCGAAAGTGGATCAAAATTAGACAAGCACACGAATTGCACATCAGAACAACTATCCTCTCTAATTGCAATAAAAATGGG
241 ACACTAAAACCCACCAAAAACTCACTTAGGCAATTCGTCGAACACTTGGTGTGTAGTGTGATTTATGGTGAAGGAATGAG
321 AAGTACAATCCAATTAAAACTCCCAATCGGTTAAATATGCACCACAATACACAAATATACAAGAAAACACACATTTTATG
401 GTAGAAAAACTTACGGACCAAGTGTAGCACTTAAACCCAATATTGGAGAGAGAAGATTTGTGAGAAATACCTCCAATTCC
481 AATCAAATTCCGATAGGACTCCGGCCAAATTCCGTAAGAATGATGGGGAGAGTGATGTTAATGTGATGTACGTGTTTGGA
561 TTGAGGACAAGT
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E

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  1 CAAACATGTCAGAATTTAAACATTTGTGTTCTCATTAAAAAGCCTTAATATAAATCTCATATAGAATACAACTTGAGTCA
 81 AAACTGATTGGTAGGATATTTCTGCGACTGATTCTCTACAAATTCGTACTAGTTTTATCTGGGCCACTTCTTTTAATGCA
161 ACGACTTCTATGCAATATTGCATTGTAGTCAAAGGAATAATATTTTCTCATTTAAAGCAATTCTATGTACGCAAACTAAA
241 CTATAAAATATACAATTTTTCCTGTATAATCTTTATCTCAACTTGCTCCTATTATTGTCTGTGCCTCCATCTCGAGTCCA
321 CTTTATAAGTTCAATATTTCACCACTAATCCTAAGATATGATTTTCTTGGAATAAATTTCCATTTACGAATCAATGATAA
401 TTTCGACAACTAGAAAACTTAATACGTTGTATTCTTATTATCAAATCAATATGTGATCAAGTTGTGGTACTATTCGCATT
481 TTAATACAGAATTGTTGCATTTTACGATATTCTTAAACTAATAT
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Figure 4. The cloned nucleotide information by Sanger-sequencing. A. The sequences of clone A12-12 with 598bp [The GenBank accession number: KX671029]; B. The sequences of clone I19-13 with 718bp [The GenBank accession number: KX671030]; C. The sequences of clone I19-22 with 502bp [The GenBank accession number: KX671031]; D. The sequences of clone Q19-11 with 572bp [The GenBank accession number: KX671032]; E. The sequences of clone Q19-22 with 524bp [The GenBank accession number: KX671033]

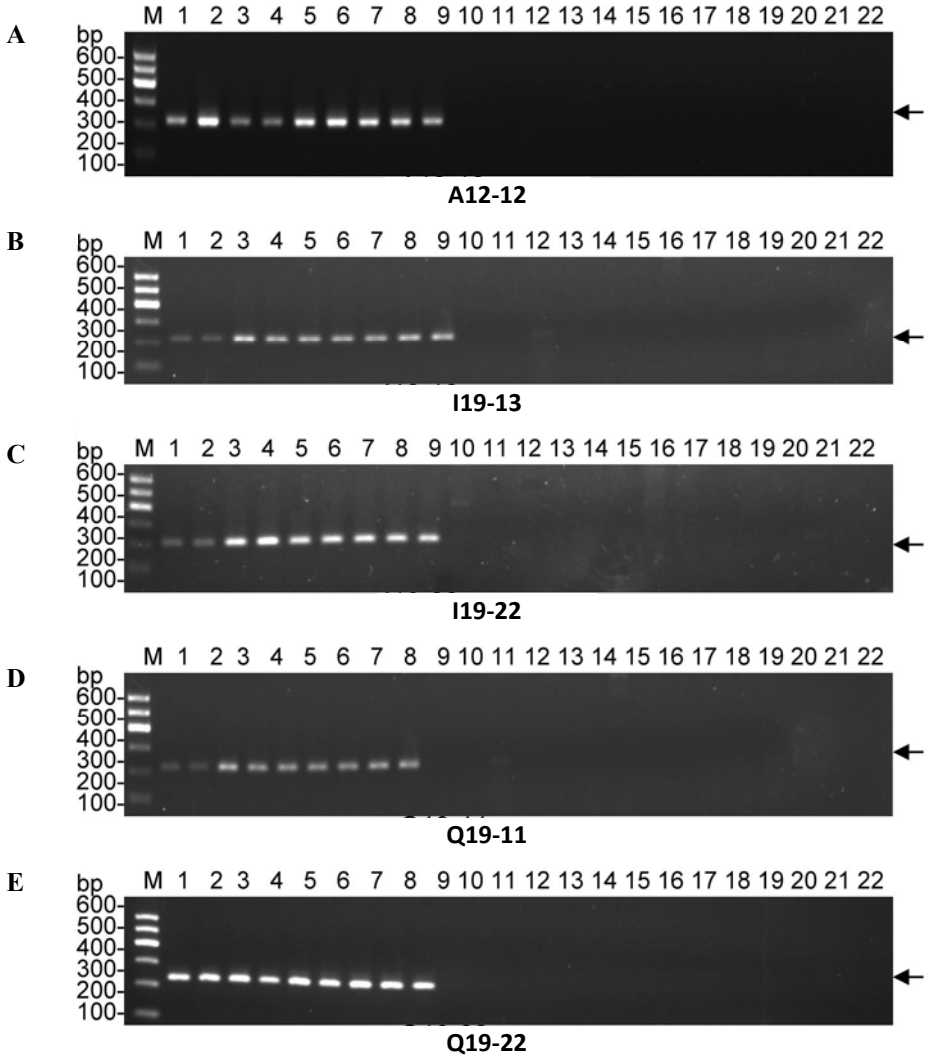

Figure 5. Development of stable RAPD-SCAR markers for A12-12, I19-13, I19-22, Q19-11 and Q19-22. Lane 1-9 is *Penthorum chinense* Pursh, 10-22 is *Eclipta prostrata* (L.) L., *Canarium album* (Lour.) DC., *Dimocarpus longan* Lour., *Litchi chinensis* Sonn., *Mentha haplocalyx* Briq., *Lycium barbarum* L., *Angelica sinensis* (Oliv.) Diels, *Ginkgo biloba* L., Russian Knapweed *Rhaponticum repens* (L.) Hidalgo, *Ganoderma lucidum* (Curtis) Karst., *Ganoderma japonicum* (Fr.) Sawada, *Gardenia jasminoides* J. Ellis, and *Artemisia argyi* H. Lév. & Vaniot. Lane M indicates the DNA molecular weight marker DL600

Authentication of *Penthorum chinense* with specific SCAR markers

To generate more stable specific diagnostic SCAR markers for *Penthorum chinense*, five pairs of primers were designed and synthesized based on our cloned sequences (Table 3). The designed SCAR primer pairs were then used to amplify the genomic DNA collected from 22 samples of different species or cultivars to test amplification species-specificity. The PCR amplification results are shown in Figure 5. The PCR results for the SCAR markers A12-12, I19-13, I19-22, Q19-11 and Q19-22 indicate that amplification products with expected size were observed in samples of *Penthorum chinense* (lane 1-9)(Figure 5.A-E). But there was no amplification for the other species. Therefore, we confirm that the specific SCAR markers were successfully developed, and can in future be used for authentication of cultivars of *Penthorum chinense*, and for distinguishing this species from other species currently listed in GenBase.

Discussion

Genetic characterization and identification of living organisms have been revolutionized by the development of molecular marker technologies. These have become valuable tools for systematists, botanists, zoologists, and even for medicinal chemists. Most of these techniques are simple, cheap and easy to carry out, and do not require whole-DNA sequencing of target species. Yet, they can reveal high degree of polymorphism (Williams et al. 1990, Fu et al. 2013, 2015). RAPD, developed in the 90s of last

century, is still a popular technique. When RAPD is combined with SCAR, it can improve the stability and specificity, making genetic identification and authentication more efficient in studying different alleles. In our lab, we have successfully characterized a number of plant or fungal species by developing SCAR markers based on RAPD amplicons; namely *Dimocarpus longan* (Yang et al. 2013), *Acorus* species (Ryuk et al. 2014), *Lonicera japonica* (Yang et al. 2014), *Trichoderma cf. harzianum* (Pérez et al. 2014), *Cordyceps sinensis* (Lam et al. 2015), *Litchi chinensis* (Cheng et al. 2015), *Angelica sinensis* (Zhang et al. 2015), and *Ganoderma lucidum* (Khan et al. 2016).

In this study, we collected samples of the species *Penthorum chinense* Pursh from nine different geographical locations of China. We have generated RAPD fragments by using DNA materials extracted from these plants and cloned in T-vectors. Finally, we have developed five SCAR markers (GenBank accession numbers: KX671029, KX671030, KX671031, KX671032, KX671033), which are strictly specific to the nine samples of *Penthorum chinense* Pursh. These markers can be used for the genetic identification and authentication of *P. chinense*. The BLAST searches of these five nucleotide sequences (KX671029, KX671030, KX671031, KX671032, KX671033) in GenBank database did not show any identity with that of other species currently represented in the database, indicating that these five SCAR markers are novel molecular markers for the *Penthorum chinense* Pursh species.

In a previous study, two different species of the genus *Penthorum* (i.e. *Penthorum sedoides* L. from Virginia, USA and *P. chinense* Push from Sichuan, China) were genetically distinguished and SCAR markers were developed for their identification (Duan et al. 2011). However, there was no report on genetic identification and marker development for *P. chinense* from different geographical locations.

For the first time, this study has developed novel SCAR biomarkers for *P. chinense*, which can be used for the genetic identification and authentication of this plant. Establishment of these SCAR markers will provide a more reliable way to authentically identify *P. chinense* species than by traditional morphological methods, and can contribute to the biological conservation of this plant. We suggest future works to conserve this plant and save it from extinction. More scientific studies should be performed to identify and isolate the active ingredients from this plant and to study their medicinal properties.

ACKNOWLEDGEMENTS

The work was supported by the Joint Program of the Science & Technology of Luzhou and the Science & Technology Department of Sichuan (14JC0154), The Sichuan Provincial Health Department (130279) and Scientific Research Fund of Sichuan Provincial Education Department (14ZA0152, 17ZA0427).

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