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Development of SCAR Markers Based on Improved RAPD Amplification Fragments and Molecular Cloning for Authentication of Herbal Medicines *Angelica sinensis, Angelica acutiloba* and *Levisticum officinale*

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Molecular cloning from DNA fragments of improved RAPD amplification of *Angelica sinensis, Angelica acutiloba* and *Levisticum officinale*, provided novel sequence-characterized amplified region (SCAR) markers A13, A23, A1-34 and A1-0 whose sequences were deposited in the GenBank database with the accession numbers KP641315, KP641316, KP641317 and KP641318, respectively. By optional PCR amplification, the SCAR markers A13 and A23 are *Levisticum officinale*-specific, whereas the SCAR marker A1-34 is *Angelica acutiloba*-specific, and the SCAR marker A1-0 is *Angelica sinensis*-specific. These diagnostic SCAR markers may be useful for genetic authentications, for ecological conservation of all three medicinal plants and as a helpful tool for the genetic authentication of adulterant samples.

Keywords: *Angelica sinensis*, *Angelica acutiloba*, *Levisticum officinale*, Sequence-characterized amplified region, Random amplified polymorphic DNA, Molecular Cloning, Substitute, Genetic authentication.

Umbelliferous drugs are important in traditional medicine. The dried root of *Angelica sinensis* (Oliv.) Diels ("Danggui"), is commonly used in traditional Chinese medicine (TCM). It was mentioned as a women's medicine 2000 years ago in the famous *Shen-nung Pents'ao ching* for treatment of menstrual disorders, amenorroehea and rheumatism [1-3]. Compounds isolated from the herb show anticancer activities [4,5], and good effects on cardiovascular complications, hepatic diseases, gastric ulcer healing, etc [6-8]. The demand for *Danggui* is increasing and the export to western countries becomes more important.

For cost concerns *Angelica acutiloba* (Sieb. & Zucc.) Kitagawa (*A. acutiloba)* and *Levisticum officinale* Koch (*L. officinale*) are often used as adulterants or substitutes for *A. sinensis* in China. *A. acutiloba* is one of the most intensively cultivated medicinal plants in Korea and Japan. The roots of this plant have been used for the treatment of various female disorders and cardiovascular disease in Korea and other Asian countries [9]. It was introduced to China and cultivated in Sichuan and northeast of China [10]. *L. officinale* ("European Danggui") is a well-known drug in Europe and also listed in the German Pharmacopoeia. It was officially introduced from Europe in 1957 as a substitute to mitigate the large demand of *A. sinensis* and then was widely cultivated in provinces of Mongolica, Hebei, Shandong, Henan, Shanxi and Shaanxi [9, 10]. However, the chemical constituents of *A. acutiloba*, *L. officinale* and *A. sinensis* are very different and pharmacological experiments indicated that the medicinal efficacy of *A. acutiloba* and *L. officinale* is lower than that of *A. sinensis*, and cannot substitute for *A. sinensis*. Thus, *A. acutiloba* and *L. officinale* was prohibited to be used as TCM instead of *A. sinensis* in China [1, 11].

Traditional discrimination among *A. sinensis*, *A. acutiloba* and *L. officinale* is very difficult because of the similar morphology of the root and subterranean structures. It is also hard to differentiate by physical and chemical traits due to the small amounts of samples, the complexity and instability of chemical constituents and the source variability. Molecular markers provide an efficient tool for identification of species or cultivars, assessment of genetic variability in plants, and are not restricted by the form of the samples, such as different tissues or methods of preparation. A number of studies were performed using various molecular markers, such as randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs) and DNA sequencing, in order to authenticate medicinal plant species [12-14]. Among these methods, RAPD is one of the most widely applied for genetic diversity assessment techniques since it is easy, rapid, and requires no prior sequence information [15, 16]. However, it is sensitive to the change of polymerase chain reaction (PCR) conditions and thus has poor reproducibility. A sequence-characterized amplified region (SCAR) analysis requires knowledge of a unique region in the genome that is not conserved in other species, it is conveniently conducted and has good specificity and reproducibility [17-21]. An improved method of RAPD analysis named RAMP-PCR instead of traditional RAPD-PCR has been established by our earlier study on the identification of *A. sinensis* and its substitutes [22]. However, the complex operation was not practical for the present application. Therefore, clear discrimination markers for *A. sinensis* and its adulterants were needed. In this study, SCAR is developed and applied based on the improved RAPD amplification of *A. sinensis* (cultivated from different places), *A. acutiloba* and *L. officinale*, in order to establish a stable

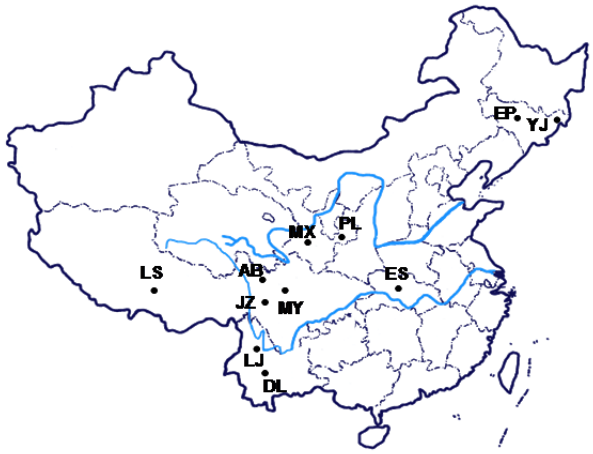

Figure 1: The localities of sample sources. The localities of samples of different cultivars of *A. sinensis*, *A. acutiloba*, and *Angelica apaensis* Shan & Yuan and *L. officinale* from different regions in China [22].

Table 1.The localities of samples of Angelica sinensis and its substitutes in China

No.	*Species*	Source	Sample
1	*Angelica sinensis* (Oliv.) Diels	Minxian,Ganshu	MX
2	*Angelica acutiloba* (Sieb. & Zucc.) Kitagawa	Yanji,Jilin	JP
3	*Angelica apaensis* Shan & Yuan	Aba,Sichuan	AB
4	*Angelica sinensis* (Oliv.) Diels	Pingliang,Gansu	PL
5	*Angelica sinensis* (Oliv.) Diels	Enshi,Hubei	HB
6	*Angelica sinensis* (Oliv.) Diels	Jiuzhaigou,Sichuan	JZ
7	*Levisticum officinale* Koch	Changchun, Jilin	EP
8	*Angelica sinensis* (Oliv.) Diels	Lijiang,Yunnan	LJ
9	*Angelica sinensis* (Oliv.) Diels	Mianyang,Sichuan	MY
10	*Angelica sinensis* (Oliv.) Diels	Lasa, Tibet	TB
11	*Angelica sinensis* (Oliv.) Diels	Dali, Yunan	DL

molecule identification method as a foundation for further application.

Cloning of RAPD amplification fragments: Two RAPD primers SBS-A1 and SBS-A2 were used for the improved RAPD amplification by different samples (Table 1) (Figure 1). The improved RAPD amplification results are shown in Figure 2, where the red arrows indicate the bands labeled by primer SBS-A1 (Figure 2A) and SBS-A2 (Figure 2B). The red arrow-indicated bands were cut from the agarose gel and purified. Then the purified PCR product was ligated into pGEM T-vector. The blue and white screening method in LB agar plate was adopted firstly to screen the positive clones (data not shown). The white clones were then identified by PCR amplification using SP6/T7 primer pair. The results of positive clones are shown in Figure 3. In Figure 3A, the positive clone A13 in lane 3 has an expected inserted DNA-fragment with ~850bp in size, whereas the positive clones A23, A1-34 and A1-0 are shown in Figure 3B, 3C, 3D, as three inserted DNA-fragments with right length in sizes 1.4 kb, 2 kb and 1.4 kb, respectively.

Sequencing and characterization of specific RAPD fragments: The sequencing of the above mentioned four cloned RAPD fragments of clones A13, A23, A1-34 and A1-0 were performed, then BLAST searches of the nucleotide sequences in GenBank database

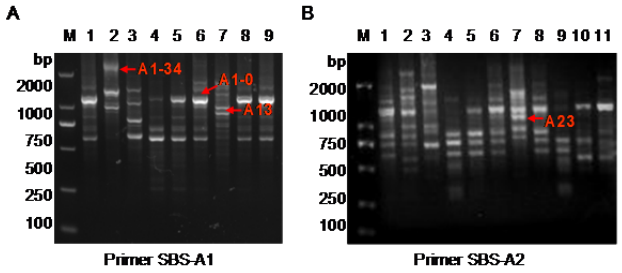

Figure 2: Improved RAPD amplification and recovery of fragments from *Angelica sinensis* and adulterants. Improve RAPD amplification from DNA samples of *A. sinensis*, *A. acutiloba*, *A. apaensis* and *L. officinale* listed in Table 1. A. The primer SBS-A1. B. The primers SBS-A2. The red arrows indicate bands before cut. *Lane M* indicates the DNA molecular weight marker DL2000 with indicated fragment size (bp).

indicated that they have no significant identity to that of any species (data not shown). The sequenced results revealed that clone A13, consisted of 722 nucleotides, and was deposited into GenBank with accession number KP641315 (Figure 4A); clone A23, consisted of 903 nucleotides, and was deposited into GenBank with accession number KP641316 (Figure 4B); clone A1-34, consisted of 310 nucleotides, and was deposited into GenBank with accession number KP641317 (Figure 4C); and clone A1-0, consisted of 782 nucleotides, and was deposited into GenBank with accession number KP641318. Note that the actual lengths with high quality nucleotide obtained using SP6 vector primer are shorter than the RAPD fragments.

Development of specific SCAR markers for authentication A. sinensis: To generate more stable specific diagnostic SCAR markers for species for *A. sinensis*, or *A. acutiloba*, or *L. officinale*, four pairs of primers were designed and synthesized based on our cloned sequences (Table 2) (Figure 4). The designed SCAR primer pairs were then used to amplify the genomic DNA collected from 18 samples of different species or cultivars to test amplification species-specificity. Figure 5 shows the PCR amplification results. The PCR results by SCAR markers A13 and A23 indicated that the amplification products with expected size were observed only in samples of *L. officinale* (Figure 5, A&B, lane 7), and not in other species including different cultivars of *A. sinensis*, or *A. acutiloba* tested. Negative controls without DNA template did not show any PCR product (data not shown). This indicated that these two SCAR markers A13 and A23 are *L. officinale*-specific. The lack of these specific amplicons in the samples from other species or cultivars indicates the efficacy of this marker in distinguishing the samples of *L. officinale* from other species. The PCR results by SCAR marker A1-34 indicated that the PCR products with expected size were observed only in sample of *A. acutiloba* (Figure 5, C, lane 2), and no amplification in other species or cultivars, which indicates that the SCAR marker A1-34 is specific for *A. acutiloba* of the sample of Yanji in Jilin province (JP). The PCR results by SCAR marker A1-0 indicated that the PCR products with expected size were observed only in all samples of *A. sinensis* (Figure 5, D, lanes 1, 4, 5, 6, 8, 9), and no amplification in other species including *A. acutiloba, A. apaensis,* or *L. officinale*. This indicates that SCAR marker A1-0 is useful for distinguishing *A. sinensis* from *A. acutiloba*, *A. apaensis*, or *L. officinale*.

Specificity for SCAR markers A13 and A23, and A1-34: To further demonstrate the specificity for these SCAR markers, PCR on more samples of *L. officinale*, and *A. acutiloba* were used. We tested the SCAR specificity for markers A13 and A23 on two samples of *L. officinale* from Ganzi of Sichuan and Changchun of Jilin, and the

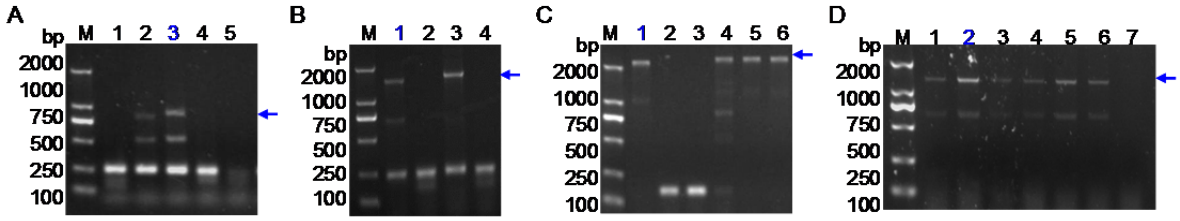

Figure 3: Identification of positive clones after DNA ligation. A. Clone identification of RAPD fragment A13. *Lanes 1~5* indicate different clones. B. Clone identification of RAPD fragment A23. *Lanes 1~4* indicate different clones. C. Clones identification of RAPD fragment A1-34. *Lanes 1~6* indicate different clones. D. Clone identification of RAPD fragment A1-0. *Lanes 1~7* indicate different clones.

Table 2. Sequences of SCAR primers, PCR product size

SCAR	5'-primer	Sequence (5'-3')	3'-primer	Sequence (5'-3')	Size (bp)	Tm (° C)
A13	A13-L	AAAAGCCGATTTCCTGTCCT	A13-R	AGCGAAGAGGACAGGTTTGA	294	60
A23	A23-L	CCACACTCAACTGGGTCCTT	A23-R	GTGCTTTTCCACCCAAGAAA	274	60
A1-34	A1-34-L	CCCTTTATCGGCTACGGACT	A1-34-R	TCTGCACGAATAGGGTCTCC	177	60
A1-0	A1-0-L	AAGGCAAAACTTCAGGCTCA	A1-0-R	CTTCCTTCTCCCCCTTTTTG	253	65

A

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1   CTTACTTACCTAAAGTGAACTTTTGGCTTACCTAAAAAGTGGACTGAAGGAACTGACCTAAGCATAGCTCTCTCAAATAA
81  AAATGCCTTTCATTTCTCTTTTAAGACCTTTGTCCTACTTCTAGTCTCAGTCCTCCAGCCTATCGAAAGTCATCTTTTTA
161 TTAACATTAACCAACAACACATGCACTTTGTTGGCACTAAAAAAAAGGTTATTCAATCCACTAGTGCAACTGAAGGAATT
241 ACCTCTTCTGAACCGGAATAAGAAAGAGCTCATAACCACTACTTGTGAACAGCTCTCCTCAACTGAAGGCGCACCTACTG
321 TTACTAGAAGTCTAGTCTCTCTCAGGTCCAGTTTCGATCTCGAACTACAACATAGGCGGGAAAAGAGATATTCAGAAAAG
401 CCGATTTCCTGTCCTGTCTTAAGAACCTGACTCAAAAGAGAAAAGAAGACCGGACTAAAAGAGATCTCACTTTCGACGAT
481 TGAACTCCACTTTCATATCAGGCTTGCACTGGAATTCACTTTCAGGAATCCTTGCTCCTGGCTCATATTCACATAGGCCA
561 CTCATAAGAATAAGACGTTCAACTCCCTCAAACACGTGTAATTGAGAGAGTCAGAATATCAGTGCCTTGGGTAAATGTCT
641 ACCTTCGGGAGAATCTTACCGAACGACTTTCAAACCTGTCCTCTTCGCTTCCCAAGAGATTGGATTTAGGTAAAAGGGCC
721 TG
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B

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1   GCCGAGCTGTATGAACAACTTGTCGAGTTCATCTTCAACTGATCAAAAGGTCCTGTCCTTGACTCGCCGTGTTGTATGAA
81  CAACTCATAGAGTTCATCTTCATCCATAAGAAGATTGCCTTAGACTCGCCGAGTCTGCTCATGCACTCGCTGAGTCCCAT
161 GAATACCCAGAACAATGGCTTAGTCCAGAATGTTGGGTCACTTCCGGGACTCCCTAAAACCTTTCCACACTCAACTGGGT
241 CCTTTTGACCCTCAACGAATTTGGGACTCAAGCTTGGACTCGCCGAGTCCAAGGACGGACTCGCCGAGTCTATCTCGTGT
321 TCTCACTAGATCTTCAATTTCTGATGTACAACTCTTGATCAAAGGATAGATCTATGCTCCAAGGACCGTTCTAGCACGCA
401 AATTTACAAACTTTACGTGTTTGCATGGGACTTTAAGCTCAAAAGGTCCTAAAATAAGCCCTTGAATTGCATGGGGCCTT
481 TCTTGGGTGGAAAAGCACCCTCAACTTGTAAAAACTTTTATTCAATCATCAAATGTCAACTACATTTTACAAGTTTCAGA
561 AATCTAATTATTGAAACTAATATCATCCTTCAGCCCGATGCGCCTAGTATGCTGCAAATGTTTAACCCTACTCAGTCCCT
641 TCATGAGGGGATCTACTAGGTTCTCATCTGATGATACCCTCTTTGCCACAAGGAGTCCTTCTATTCGATGTCTGATGAAA
721 TGGTATTTTCTGTCAATGTGCCTAGATCTCCCGTGATCTCTTGGTTTCCTTAGCCAAAGCAACCGCACTTTTTCTGTCAC
801 AGAAAAGTTTCATTGGCTCCTTTATAGCTGGTACAACTCCAGGTCTCCGATGAAGTTCTTTAGCCATATTGCTCTTTGCT
881 ACCTCGCTGCTGCTATATACTCT
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C

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1   CAGGCCCTTTATCGGCTACGGACTAATAAACAATGCCAACACAAGAAAATATCAACACCTCACTATAATAAAAGGGTCAA
81  TAGAAATTAGGGTTTTGATTTTAAAAACCCCCAAATTAAAGCTTACCAATTTCAGTAGAGAAGGCCCTAGAAATAGTAT
161 AGGAGACCCTATTCGTGCAGAGAACACCCAAATTAAAGCTTACCAATTTGGACACGAGCAAAGGAGAGACGGGAGCAGAG
241 GTGTTTGGTAATGGTGGTGAGTAGAGAAGGACGGAGGTGAGTAGAACAGAGGTGAGTGGTGGTGGTGGTG
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D

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1   TTATGTACTGGAGATCTTCCTGAGTGGCTATTTGATGCATATCAATCTACTAGAGACCCAGATGTTGAGGTTCTAGTCCA
81  GTACAAGCGTGCCACTCAGGAACCAATCAATGATGCAAACCTCAAGGAGCATATCCTCCAAAACTTCATAGGTACCCTGA
161 AGCTGCAGAAACATCTAATCATCCAGCAAAGGACTGATACAATGGAGATCAAAGACAACATTCAGGATGCCAAGGCAAAA
241 CTTCAGGCTCAAGTGAATGAACTTCTACCACCCACCATCATGGAAAATATACGGACAAAACTGAGGAAGGATGATGAGGT
321 GTCTAGGAAAGTTGAAGCCCTCACAACCAGGGTTGCTACCATTGAAAGCACCCTAGGTCAATTGCTTGCCAATCAAACTG
401 CACAGACAACCCTCCTCCAACAACTACTCCAAGCCACTACAACCAGTCAAACCCTTGATGATAACAAAAGGGGGAGAAG
481 GAAGTAGGAGCTTCATCTCAGGGGGAGCCTGTTGTGCAAGTGCCAACAACTGAGGGGGAGCATGTGGATCAAGAGGACTC
561 CATTACCAATCGACTGGCAAACTCAGTTGGAGTCTCCACAGCTCTAATTCCAGTTCACACAACCATCCTAAGAGTTCTCA
641 CTCCTGAGGTTGTGTGTCCTGTGACAAGAAAGACATGAGAGCCATCCTCAATCTGTGAGTTCAAACCCATCTTGGGTAAA
721 GATCTCTCTCCTGTTAAATCAAAAGGCAAAAAACTCTCTAAGGACTGAATATGCAATGTCAA
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Figure 4: The cloned nucleotide information by Sanger-sequencing. A. The sequences of clone A13 (722 bp). B. The sequences of clone A23 (903 bp). C. The sequences of clone A1-34 (310 bp). D. The sequences of A1-0 (782 bp).

SCAR markers A1-34 on three samples of *A. acutiloba* from Nanchuan of Chongqin, Emei of Sichuan and Yanji of Jilin, respectively. The PCR results by SCAR markers A13 and A23 indicated that the amplification products with expected size were observed only in two samples of *L. officinale* (Figure 6, A & B), SCAR marker A1-34 was amplified only in three samples of *A. acutiloba* (Figure 6, C). Whereas SCAR marker A1-0 is only amplified in the sample of *A. sinensis* (Figure 6, D).These results provide stronger support for markers A13, A23, A1-34 and A1-0 being useful in *L. officinale* and *A. acutiloba* identification. This therefore confirms that the specific SCAR markers were successfully developed, and provide authentication of *A. sinensis* and other species or cultivars, which can be used for the genetic authentication of these medicinal plants.

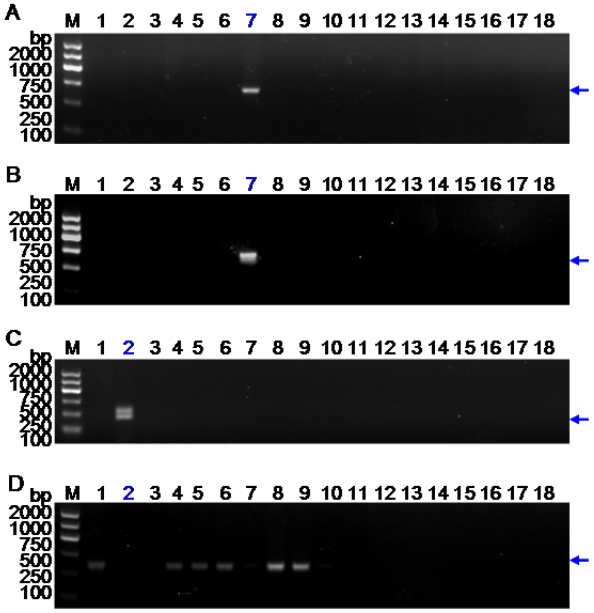

Figure 5: Development of stable RAPD-SCAR markers for A13, A23, A1-34 and A1-0. A. A SCAR marker A13. B. A SCAR marker A23. C. A SCAR marker A1-34. D. A SCAR marker A1-0. *Lanes 1, 4, 5, 6, 8 and 9* are samples of *A. sinensis* as specified in table 1; *Lane 2* is one sample of *A. acutiloba*; *Lane 3* is one sample of *A. apaensis*; Lane 7 is one sample of *L. officinale*. *Lane 10* is one sample of *G. biloba* from Hunan; *Lane 11* is one sample of *L. chinesis* from Sichuan; *Lane 12* is one sample of *D. Longan* from Sichuan; *Lane 13* is one sample of *C. album* from Sichuan; *Lane 14* is one sample of *L. japonica* from Hunan; *Lane 15* is one sample of *G. jasminoides*; *Lane 16* is one sample of *G. lucidum*; *Lane 17* is one sample of *D. confinis* from Guangxi; *Lane 18* is one sample of *P. chinense* from Luzhou of Sichuan. The blue arrows indicate expected PCR products in size. Lane M indicates the DNA molecular weight marker DL2000.

Twenty varieties of *Angelica* are endemic to the Hengduan Mountains and its adjacent regions in China, which appear to be both a refuge and a major diversification center for *Angelica* [9, 22, 23, 24]. The wild resources of *A. sinensis* are distributed in the provinces of Shaanxi, Sichuan, Gansu and Tibet, which belongs to the Hengduan Mountains. There are great genetic differences of *A. sinensis* from the cultivation area in Gansu province such as Pinliang, Minxian, Zhangxian and Weiyuan because of the natural environment and local ecological conditions [25-27]. *L. officinale* and *A. acutiloba* are used as adulterants and substitutes for *A. sinensis* in many areas of China [22, 28]. It is difficult to identify

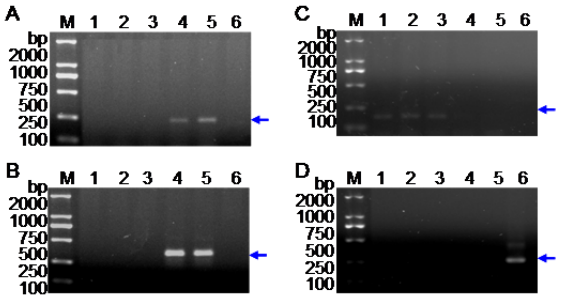

Figure 6: The representative results of specific SCAR amplification for A13, A23, A1-34 and A1-0. A. A SCAR marker A13. B. A SCAR marker A23. C. A SCAR marker A1-34. D. A SCAR marker A1-0. Lanes 1~3 indicate the different samples of *A. acutiloba* from Nanchuan of Chongqin, Emei of Sichuan and Yanji of Jilin. Lanes 4~5 indicate the different samples of *L. officinale* from Ganzi of Sichuan and Changchun of Jilin. Lane 6 indicates *A. sinensis.* Lane M indicates the DNA molecular weight marker DL2000.

them by only using the methods of morphology, physical and chemical characteristics. *A. sinensis* could be accurately distinguished from *L. officinale* and *A. acutiloba* by the specific molecular markers. The genetic distance between different species or cultivars might have grown during the course of evolutionary speciation, and the distance between intra-species samples might have grown due to the geographical isolation which has been indicated in previous studies [22], so the medicinal efficacy might also have changed. We have employed improved RAPD amplification or RAMP-PCR [12, 22] with more DNA bands, to produce highly informative bands [12, 22, 28-30]. Accordingly, in this study developed SCAR markers based on improved RAPD fragments of *A. sinensis* cultivated from different places, *A. acutiloba* and *L. officinale*, and established stable molecule identification method as a foundation for their application.

Totally, the SCAR markers A13 and A 23 are *L. officinale*-specific, whereas the SCAR marker A1-34 is *A. acutiloba*-specific, and the SCAR marker A1-0 is *A. sinensis*-specific. The results highlighted that simple rapid PCR-based molecular method could be used as a helpful assistant tool for the genetic authentication of adulterant samples.

Experimental

Samples and DNA extraction: A total of eleven *Angelica* cultivars or species, including eight samples of *A. sinensis* from Minxian and Pingliang of Gansu, Mianyang and Jiuzhaigou of Sichuan, Lijiang of Yunnan, Lasa of Tibet, Dali of Yunnan and Enshi of Hubei province of China, and one sample of *A. acutiloba* from Yanji of Jilin Province, one sample of *A. apaensis* Shan & Yuan from Aba of Sichuan province, one sample of *L. officinale* Koch from Changchun of Jilin, were collected from different geographic locations of China in this study (Figure 1) (Table 1). The genomic DNA was extracted from dried roots or fresh leaves, and commercially crude materials using a modified CTAB method described previously [12]. The plant materials were first fixed in fixing solutions containing chloroform, PVP, 2-Hydroxy-1-ethanethiol, and then ground into tiny pieces by silica (SiO_2) for the DNA extraction. The DNA quality was checked by 0.8% agarose gel electrophoresis and spectrophotometry (nanodrop 2000). The final concentration of all DNA samples was adjusted to10 ng/μL for PCR, and stored at -20^{o}C till use.

Improved RAPD amplification: The different DNA samples were amplified with RAPD primers SBS-A1 (5'-CAGGCCCTTC-3') or SBS-A2 (5'-TGCCGAGCTG-3') using Tiangen reagents (Beijing, China) according to the manipulation protocol. A total 10 μL PCR reaction system was 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 an PCR machine "Applied Biosystems Veriti® 96-Well Thermal Cycler" (Life Technology, USA) using the following program: 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 [22]. PCR products were loaded into a 1.5% agarose gel for electrophoresis.

Cloning and identification of positive RAPD DNA fragments: Four different bright bands were excised from the 1.5% agarose gel, and purified by using TIANgel Mini DNA Purification Kit (DP209, Tiangen reagents, Beijing, China) according to the manipulation 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 for 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 [21, 22].

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

Design of SCAR primers: By using online program Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), the nucleotide sequence of each of the cloned RAPD fragment was used to design pairs of SCAR primers. The quality of primers pair was tested. The sequences of each primer, optimized PCR condition and amplification length are shown in Table 2.

Development SCAR markers and SCAR analysis: To develop stable SCAR markers, the PCR amplification was performed by using eighteen of DNA samples as templates. They are the different samples of *A. sinensis*, *A. acutiloba* and *L. officinale* listed in Table 1, one sample of *Ginkgo biloba* L from Hunan, one sample of *Litchi chinensis* Sonn. from Sichuan, one sample of *Dimocarpus longan* Lour. from Sichuan, one sample of *Canarium album* (Lour.) Raeusch. from Sichuan, one sample of *Lonicera* japonica *Thunb* from Hunan, one sample of *Gardenia jasminoides* Ellis, one sample of *Ganoderma lucidum* (Leyss. Ex Fr.), one sample of *Dimocarpus* confinis (How & Ho) H. S. from Guangxi, one sample of *Penthorum chinense* Pursh from Luzhou of Sichuan. The content of 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 an 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 a

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) [12].

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