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Identification of Medicinal *Viola philippica* from *V. mandshurica* using SCAR Markers

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Abstract: *Viola philippica* is a widely used herb in Chinese traditional medicine. However, *V. mandshurica* is found as one of common substitutes or adulterants of *V. philippica* in the commercial market. In order to authenticate the two herbal species, an improved randomly amplified polymorphic DNA (RAPD) and Sequence-Characterized Amplified Region (SCAR) were performed to obtain species-specific DNA fragments. Three 10-mer random primers SBS-A8, SBS-I10 and SBS-Q6 were used for screening of specific RAPD markers and the former two primers demonstrated different amplification band patterns. Two candidate specific bands showed in two taxa by SBS-A8 primer were successfully cloned and sequenced respectively. Based on the RAPD marker sequences, four pairs of SCAR primers from *V. philippica* (VPF1/VPR1 and VPF2/VPR2) and *V. mandshurica* (VMF1/VMR1 and VMF2/VMR2) were designed. VPF1/VPR1 and VPF2/VPR2 primers yielded expected 694 bp and 403 bp amplicons with the *V. philippica* DNA only. DNA amplification using VMF1/VMR1 primers generated a single 574 bp band only in *V. mandshurica*, while VMF2/VMR2 primers produced 261 bp bands in two species. The results suggested that RAPD and SCAR analysis are effective in distinguishing genuine *V. philippica*. The methodology is a valuable tool to authenticate other morphologically similar herbal medicinal materials.

Key words: Molecular authentication, *Viola philippica*, RAPD, genetic characterization, non-coding regions

INTRODUCTION

Viola philippica Cav. in genus *Viola* L. (Violaceae) is a perennial herb distributed widely in China (Zhang, 2008; Su *et al*., 2009). As a traditional medicine, the dried whole herb is effective in eliminating toxicant, reducing inflammation and releasing pains (Zhang *et al*., 2010). With a common name Diding, *V. philippica* is generally recognized as genuine drug possessing better potent clinical effects (Zhu and Chen, 2010). However, *Viola mandshurica* W. Beck. is used commercially as one of common substitutes or adulterants of *V. philippica* and it is difficult to discriminate them due to their similar morphological characteristics and close phylogenetic relationships (Dong and Hu, 2006; Liang and Xing, 2010).

Since the favorable therapeutic effects, an increasing quantity of Diding is needed in recent years. In the modernization of traditional Chinese medicine, permanent quality controls are necessary to ensure a continuous safety and efficacy of the drug and in which an important order is the regular verification of the species being used as source material (Ruzicka *et al*., 2009). To obtain the better therapeutic effects, it is essential to discriminate *V. philippica* from *V. mandshurica*.

Commonly, strategies to herbal authentication are relied upon morphological, histological or chemical inspections. Recently, mass spectra analysis showed an alternative approach for the ingredients identification of Diding. However, the morphological and histological characteristics of closely related herbs are usual similar in their features and the chemical ingredients are variable during the plant harvest (Yu *et al*., 2011). Therefore, it is difficult to distinguish the closely related herbs with visible inspection as well as through analytical tools (Na *et al*., 2004). By contrast, DNA markers are clearly desirable in unlimited numbers, irrespective of environment and the herb development stage and can be accomplished in a relativelyshorter period (Manimekalai and Nagarajan, 2006; Zhou *et al*., 2008; Diao *et al*., 2009). The Randomly Amplified Polymorphic DNA (RAPD) technology has been shown to be powerful in analysis of species on molecular level (Hoque *et al*.,

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2005; Salem *et al*., 2005; Kumar *et al*., 2006; Haddadi *et al*., 2008). Subsequently, the Sequence Characterized Applied Region (SCAR) which is commonly converted from RAPD by specific oligonucleotide primers, is a locus-specific technique with more reliability and more reproducibility for molecular identification. Currently, reliable SCAR markers have been already derived from RAPD fragments in different herbs (Devaiah and Venkatasubramanian, 2008; Liao *et al*., 2009). The aim of this study was to develop new reliable and reproducible molecular markers for *V. philippica* authentication based on the RAPD and SCAR analysis.

MATERIALS AND METHODS

The study was conducted from March 2010 to December 2010 at Luzhou City, Research Center for Preclinical Medicine of Luzhou Medical College.

Plant materials: The taxa, accession numbers and geographic origins are listed in Table 1. The two accessions were collected from Luzhou (Sichuan province) and Dandong (Liaoning province), respectively by the present authors. The seeds were germinated and grown in the perennial nursery of Medicinal Botanical Garden, Luzhou Medical College. The mature plants were carefully identified by Haiqing Yu. All voucher specimens have been deposited at the Medicinal Botanical Associationof Zhongshan Mountain (MBAZM), Luzhou Medical College.

Genomic DNA isolation: The leaf samples for each accession were collected from mature plants in the perennial nursery of Medicinal Botanical Garden and ground in liquid nitrogen in a 1.5 mL microfuge tube. DNA was extracted and purified with the. Cetyltrimethylammonium bromide (CTAB) procedure outlined Doyle and Doyle (1990).

Table 1: Materials used in this study

Species	Common name	Geographic origin	Accession no.
Viola philippica Cav.	Diding	Luzhou, Sichuan, China	VPTY001
Viola mandshurica W. Beck.	Diding	Dandong, Liaoning, China	VMTY001

RAPD PCR amplification: The PCR reaction was carried out using SBS primers A8, I10 and Q6 (Beijing SBS Genetech Co., Ltd, China) (Table 2). RAPD was performed in a total volume of 20 μL containing 30 ng DNA, 1× reaction buffer, 2 mM $MgCl_2$, 0.25 μM of each primer, 200 μM of each dNTP (TakaRa Biotechnology (Dalian) Co., Ltd), 1 unit of rTaq DNA polymerase (TakaRa) and sterile water to the final volume. 1 drop of mineral oil was added in each reaction tube. The thermal profile consisted of an initial denaturation at 94°C for 4 min, 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, followed by final extension of 10 min at 72°C. PCR reactions of each accession were carried out in a Mastercycler 5331 (Eppendorf, Germany). The amplified PCR products were resolved by electrophoresis on 2% agarose gel in 1×TAE buffer. Gels were visualized by 0.5 μg mL^{-1} ethidium bromide staining and the images were documented using the ChemiDoc XRS (Bio-Rad, USA). An improved method for increasing the efficiency of the technique of RAPD by prolonging the ramp time from annealing to extension and increasing the resolution and production was introduced by Fu *et al*. (2000).

RAPD fragments cloning and sequencing: The expected species-specific RAPD bands were excised from the agarose gels and purified using the Gel Extraction Kit (50) (Omega, GA, USA). These amplification products were then linked into a pMD18-T Easy Vector Systems according to the manufacturer's instruction (TakaRa). The transformed competent *E. coil* DH5α (TakaRa) were plated on LB solid medium containing ampicillin (Sigma, USA) and cultured overnight at 37°C. The candidate clones were confirmed directly using specific M13F/M13R primers via PCR amplification. Three to five positive clones for each species were randomly selected and sequenced in both directions by Sunbiotech Co., Ltd (Beijing, China). The cloned sequences alignment was executed with Clustal X program (Thompson *et al*., 1997) to reveal their homology and confirm the presence of a unique amplified product in the RAPD marker band, respectively. Two sequences used in this study have been deposited in Genbank with accession numbers JF742608 and JF742609 (Table 2).

Table 2: Primers used in RAPD and SCAR analysis

RAPD primer	RAPD marker	GenBank #	SCAR primer*	Annealing (°C)	Ampliconsize(bp)
SBS-A8 (GTGACGTAGG)	A8-VP	JF742608	VPF1/VPR1	60	694
			VPF2/VPR2		403
	A8-VM	JF742609	VMF1/VMR1		574
			VMF2/VMF2		261
SBS-I10 (ACAACGCGAG)	-	-	-		-
SBS-Q6 (GAGCGCCTTG)	-	-	-		-

*Primer sequences (5'-3') are indicated with arrows in Fig. 2, respectively. No specific band is cloned

SCAR primer designing and detecting: Two pairs of candidate specific primers from each sequence were designed using Primer Premier 5.0 (Premier Biosoft, USA) and synthesized in SBS Genetech (China). Primers data were summarized in Table 2. Based on PCR amplification, all primers were used to detect specific DNA fragments between the two species. The SCAR reaction program was as follows: 94°C for 3 min, 30 cycles at 94°C for 40 sec, 60°C for 50 sec, 72°C for 1 min and final extension at 72°C for 8 min. This thermal profile was optimized and standardized according to the specific Tm of the primer pair. The gel electrophoresis and image documentation conditions were as described above.

RESULTS

Primers SBS-A8, SBS-I10 and SBS-Q6 were initially evaluated for polymorphism between the two taxa. The three primers generated clear and reproducible polymorphic RAPD amplification patterns ranging in size from about 250 to 2000 bp and the former two primers demonstrated different amplification band patterns obviously (Fig. 1). Two expected specific bands showed by SBS-A8 primer were successfully cloned and sequenced, respectively. VP fragment was unique to *V. philippica* with a 694 bp size, while VM fragment was found only in *V. mandshurica* with a 574 bp size (Fig. 1). The two RAPD markers were named A8-VP and A8-VM (Table 2).

Sequence alignment showed that the similarity of VP clones from *V. philippica* was 100% and all VM clones from *V. mandshurica* were identical too. After blast, no significant similar sequence of the two RAPD fragments was detected in the NCBI nucleotide collection (nr/nt) database. Besides, no open reading frame was revealed in the two RAPD markers. The A8-VP and A8-VM sequences were shown in Fig. 2.

Based on the two sequences, four pairs of SCAR primers with longer lengths and higher annealing temperatures were designed (Table 2). Primers VPF1/VPR1 and VPF2/VPR2 were designed from A8-VP, while primers VMF1/VMR1 and VMF2/VMR2 were obtained from A8-VM (Fig. 2). These forward and reverse primers are located either in ends or in interiors of two specific sequences respectively and primers in ends contain SBS-A8 sequences (Fig. 2). When using primers VPF1/VPR1 and VPF2/VPR2 to diagnose the two species, the wanted 694 bp and 403 bp amplicons generated only in *V. philippica* (Fig. 3). Primers VMF1/VMR1 produced a single 574 bp fragment unique to *V. mandshurica*, while the expected 261 bp band was common in the two taxa when using primers VMF2/VMR2 (Fig. 3). The similar

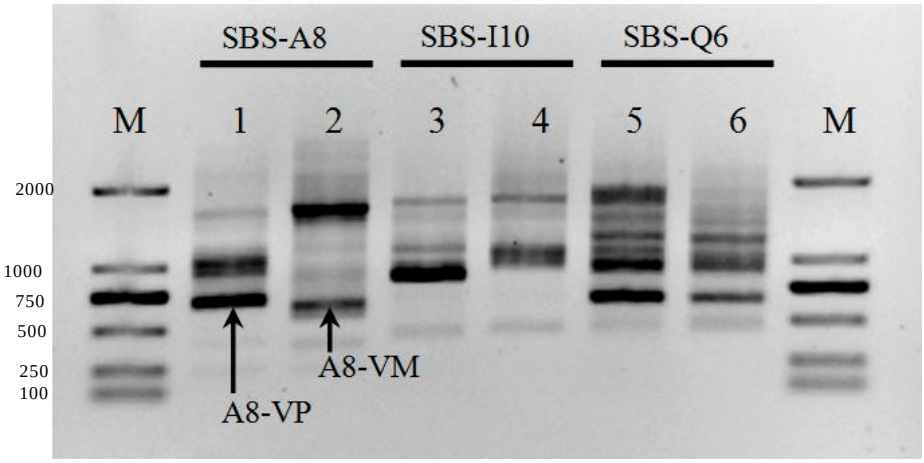

Fig. 1: RAPD profiles of *V. philippica* and *V. mandshurica* using primers SBS-A8, SBS-I10 and SBS-Q6. Lanes 1, 3 and 5 = *V. philippica*; Lanes 2, 4 and 6 = *V. mandshurica*. M, DL2000 DNA ladder (bp). Arrows indicate the cloned RAPD genetic characterization bands (A8-VP = 694 bp, A8-VM = 574 bp) unique to the two species, respectively

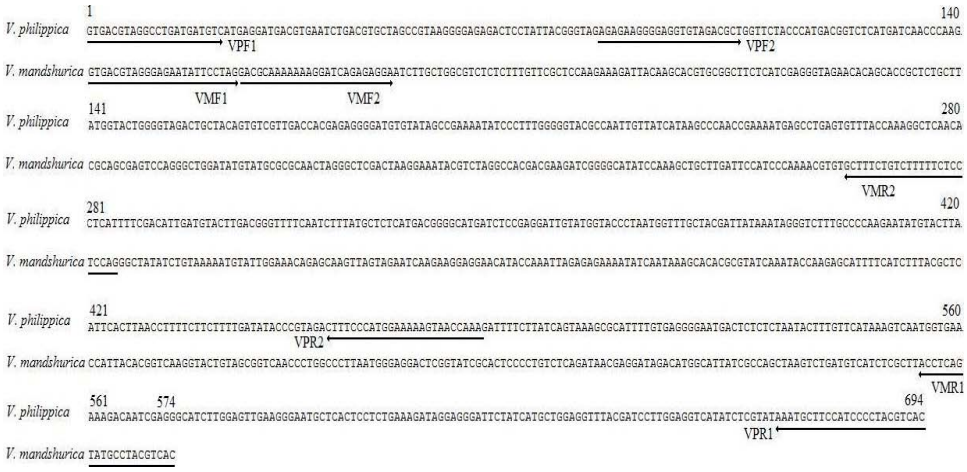

Fig. 2: The cloned A8-VP and A8-VM sequences from *V. philippica* and *V. mandshurica*. Arrows refer the forward and reverse positions of SCAR primer pairs VPF1/VPR1, VPF2/VPR2, VMF1/VMR1 and VMF2/VMR2. The expected amplicon sizes are 694, 403, 574 bp and 261 bp, respectively

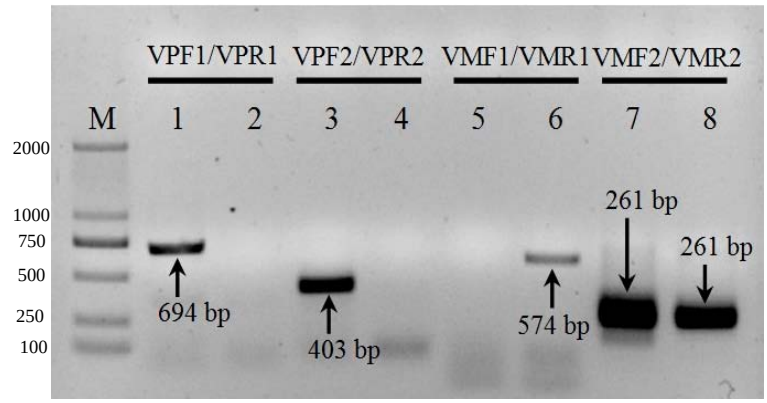

Fig. 3: SCAR analysis of *V. philippica* and *V. mandshurica* using primers pairs VPF1/VPR1, VPF2/VPR2, VMF1/VMR1 and VMF2/VMR2. Lanes 1, 3, 5 and 7 = *V. philippica*; Lanes 2, 4, 6 and 8 = *V. mandshurica*. M, DL2000 DNA ladder (bp). Arrows indicate the exact sizes of amplified fragments

results were obtained when the developed primers/markers were used to detected more individuals from wilds or markets (data not shown).

DISCUSSION

In the present study, two of three RAPD primers generated clear, reproducible and distinguishable bands. This indicated that it is effective to diagnose the two taxa via the improved RAPD technique. According to the RAPD results, the distinguishable bands among different herbs were also demonstrated respectively (Devaiah and Venkatasubramanian, 2008; Ruzicka *et al*., 2009). Since the sensitive reaction conditions in RAPD analysis, the specific bands are usually sequenced and converted into SCAR markers to improve the efficiency and stability of identification (Liao *et al*., 2009; Ruzicka *et al*., 2009). Therefore, two unique fragments accessing by SBS-A8 primer were obtained in *V. philippica* and *V. mandshurica*, respectively to make the discrimination more specific and stable.

No similar item was detected when blasting A8-VP and A8-VM sequences in NCBI database and no open reading frame was revealed in the two fragments. This

suggested that the two bands are most likely located in non-coding regions. The published data also claimed that RAPD markers often displayed specific sequence in intron (Lubbers *et al*., 1994; Pessino *et al*., 1997). Some of these introns could play an important role in positive or negative regulation of eukaryotic gene expression (Salgueiro *et al*., 2000; Hisatsune *et al*., 2005). However, whether A8-VP or A8-VM sequences participate gene expression needs to be investigated further.

In the two taxa, primer SBS-Q6 generated similar RAPD fingerprints. Although the patterns were obviously different when using primer SBS-A8, the amplicons subsequently produced by primers VMF2/VMR2 were common in *V. philippica* and *V. mandshurica*. The results suggested their close phylogenetic relationships. Liang and Xing (2010) also reported their near relationships base on molecular evidences from *trn*L-*trn*F, *psb*A-*trn*H, *rpb*L16 and ITS sequences. In SCAR analysis, primers designed from A8-VP sequence showed the identical result, while it was different in the amplification with A8-VM sequence primers. Considering the different locations of SCAR primers, this indicated that there are variations in RAPD genetic sites among the two species. It is most likely that A8-VP sequence is unique to *V. philippica* but A8-VM sequence is common in *V. philippica* and *V. mandshurica*.

To ensure the efficiency and stability in SCAR diagnosis, each of amplification was repeated at least five times and all the expected bands were consistent. In the modernization of Chinese medicine, quality control is one of the most important factors and the traditional morphological, histological or chemical approaches are limited in detecting genuine herbs (Na *et al*., 2004; Diao *et al*., 2009). In the current study, the developed SCAR markers, especially the designed VPF1/VPR1, VPF2/VPR2 and VMF1/VMR1 primers, are effective and powerful in identification of *V. philippica* from *V. mandshurica*. Therefore, the SCAR analysis based on the improved RAPD technique is effective in authentication of closely related medicinal herbs.

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