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Genetic Characterization and Authentication of *Penthorum* Species Using RAPD and SCAR Markers

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ABSTRACT

Penthorum sedoides L. and *Penthorum chinense* Push is closely related aneuploids in genus *Penthorum* L. based on the previous data. However, *P. chinense* are treated as variety or subspecies of *P. sedoides* successively by botanists and little was known about their relationships on molecular level. An improved randomly amplified polymorphic DNA (RAPD) and Sequence Characterized Amplified Region (SCAR) were performed to obtain their genetic characterizations and species-specific DNA fragments. Obviously, five 10-mer random primers SBS-A3, SBS-I3, SBS-I18, SBS-M6 and SBS-Q9 demonstrated different fingerprints. Six candidate specific bands (I3-PS, I3-PC, I18-PC, M6-PS, Q9-PS and Q9-PC) displayed in two taxa were successfully cloned and sequenced. Based on these sequences, six pairs of SCAR primers from *P. sedoides* (I3PSF/I3PSR, M6PSF/M6PSR and Q9PSF/Q9PSR) and *P. chinense* (I3PCF/I3PCR, I18PCF/I18PCR and Q9PCF/Q9PCR) were designed, respectively. However, four of six primer pairs yielded amplicons common in the two taxa. The DNA amplification using Q9PSF/Q9PSR primers generated a single 555 bp band only in *P. sedoides* and Q9PCF/Q9PCR primers produced a 760 bp fragment unique to *P. chinense*. The results suggested that *P. sedoides* and *P. chinense* are still closely related, although there are a lot of variations in RAPD genetic sites. The developed RAPD and SCAR techniques are effective and useful in revealing genetic characterizations of *P. sedoides* and *P. chinense*. Also, the SCAR analysis based on the improved RAPD method is powerful in authentication of species with close relationships.

Key words: *Penthorum chinense* Push, *Penthorum sediodes* L., SCAR marker, genetic characterization, phylogeny

INTRODUCTION

Penthorum sedoides L. and *Penthorum chinense* Push are the only two species in genus *Penthorum* L. with a disjunct distribution, the two taxa are perennial rhizomatous herbs living in wet habitats (Haskins and Hayden, 1987; Wen, 1999; Pan *et al*., 2001). *P. sedoides* (ditch stonecrop) distributed in eastern North America is useful in making cough syrups (Foster and Duke, 1999) and *P. chinense* (ganhuangcao) located in eastern Asia is effective in protecting the liver cells (Cao *et al*., 2007; Zhou *et al*., 2008; Yu *et al*., 2011). They have similar morphological and anatomical features. Also, variations between them are detected. In which, *P. chinense* is slightly

different to *P. sedoides* in its longer and narrower leaf blades and shorter and thicker styles (Hara, 1962). Cytologically, these plants are aneuploids each other. *P. sedoides* is diploid with 2n = 18 chromosomes while the chromosomes in diploid *P. chinense* are 2n = 16 (Cronquist, 1981).

The familial position of *Penthorum* has fascinated botanists and biogeographers since the Linnaean era. *Penthorum* species are considered to have close relationships to species in Crassulaceae, Saxifragaceae or Haloragaceae (Morgan and Soltis, 1993; Pan, 1995; Jian *et al*., 2008). Due to their disjunct distributions perhaps, the two species are seldom analyzed simultaneously, especially on molecular level (Fishbein *et al*., 2001; Davis and Chase, 2004; Moody and Les, 2007; Jian *et al*., 2008). Besides, *P. chinense* has ever been treated as variety or subspecies of *P. sedoides*. Therefore, it is necessary to reveal their genetic characterizations and authenticate the two taxa.

The randomly amplified polymorphic DNA (RAPD) technique has been shown to be useful in analysis of different species on molecular level (Lakshmi *et al*., 2008; Al-Atiyat, 2009; Shaptadvipa and Sarma, 2009; Su *et al*., 2009; Ruzicka *et al*., 2009; Thangaraj *et al*., 2011). Thereafter, the Sequence Characterized Applied Region (SCAR) which is usually converted from RAPD by specific primers, is a locus-specific technology with more reliability and more reproducibility for molecular discrimination (Paran and Michelmore, 1993). Recently, 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 is to demonstrate the genetic characterizations and authenticate *Penthorum* species based on the RAPD and SCAR analysis.

MATERIALS AND METHODS

Plant materials: The taxa, accession numbers and geographic origins are listed in Table 1. *P. sedoides* in Richmond (Virginia, USA) is kindly provided by Dr. W. John Hayden and *P. chinense* is collected from Luzhou (Sichuan, China). The seeds were germinated and grown in the perennial nursery of Medicinal Botanical Garden, Luzhou Medical College. The mature plants were carefully identified by Dr. Haiqing Yu. All voucher specimens have been deposited at the Medicinal Botanical Association of Zhongshan Mountain (MBAZM), Luzhou Medical College.

Genomic DNA extraction: 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 by Doyle and Doyle (1990).

RAPD analysis: The PCR reaction was executed using SBS primers A3, I3, I18, M6 and Q9 (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

Table 1: Materials used in this study

Species	Common name	Geographic origin	Accession No.
Penthorum sedoides L.	Ditch stonecrop	Richmond, Virginia, USA	PSTY001
Penthorum chinense Push	Ganhuangcao	Luzhou, Sichuan, China	PCTY001

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 RAPD by prolonging the ramp time from annealing to extension and increasing the resolution and production was introduced by Fu *et al*. (2000).

RAPD bands 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. 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. The accession numbers of six sequences used in this study are listed in Table 2.

SCAR primer designing and detecting: Six pairs of candidate specific primers from six sequences 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, these 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 s, 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-A3, SBS-I3, SBS-I18, SBS-M6 and SBS-Q9 were initially investigated for genetic characterizations between the two taxa. The five primers produced clear and reproducible RAPD fingerprints ranging in size from about 250 bp to 2000 bp (Fig. 1). Obviously, all primers demonstrated different amplification band patterns. Six expected specific bands displayed by the latter four primers were successfully cloned and sequenced, respectively. These RAPD markers were named based on information of primers and species (Table 2). Among which, I3-PS, M6-PS and Q9-PS fragments were unique to *P. sedoides* while I3-PC, I18-PC and Q9-PC fragments were found only in *P. chinense* (Fig. 1).

Sequence alignment showed that the similarity of clones from each unique fragment of *P. sedoides* was 100% and all clones from each single band of *P. chinense* were consistent too. After blast, no significant similar sequence of all RAPD fragments except M6-PS was detected in the NCBI nucleotide collection (nr/nt) database. M6-PS is closely related to predicted hypothetical protein of *Vitis vinifera* (accession number XM_002285443) and full-length cDNA clone from *Zea mays* (accession number BT063499) with 80% and 77% identities in query sites, respectively (Fig. 2). Besides, no open reading frame was revealed in the other five RAPD markers.

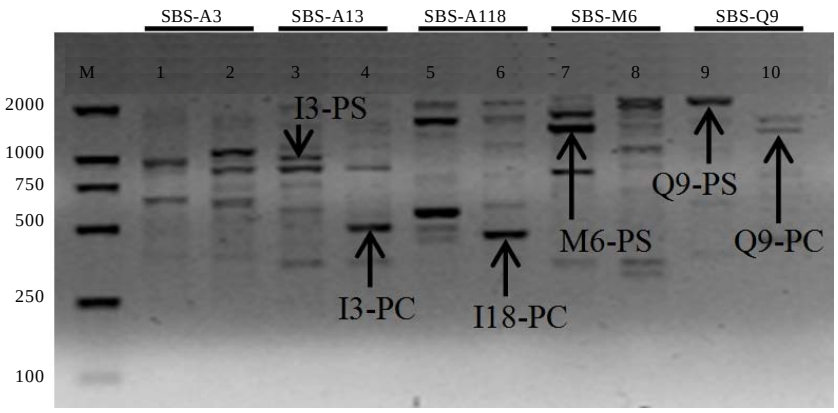

Fig. 1: RAPD fingerprints of *P. sedoides* and *P. chinense* with primers SBS-A3, SBS-I3, SBS-I18, SBS-M6 and SBS-Q9. Lanes 1, 3, 5, 7 and 9 = *P. sedoides*; Lanes 2, 4, 6, 8 and 10 = *P. chinense*. M, DL2000 DNA ladder (bp). Arrows indicate the cloned RAPD genetic characterization bands (I3-PS = 986 bp, I3-PC = 477 bp, I18-PC = 429 bp, M6-PS = 1347 bp, Q9-PS = 2047 bp and Q9-PC = 1210 bp) unique to the two species, respectively

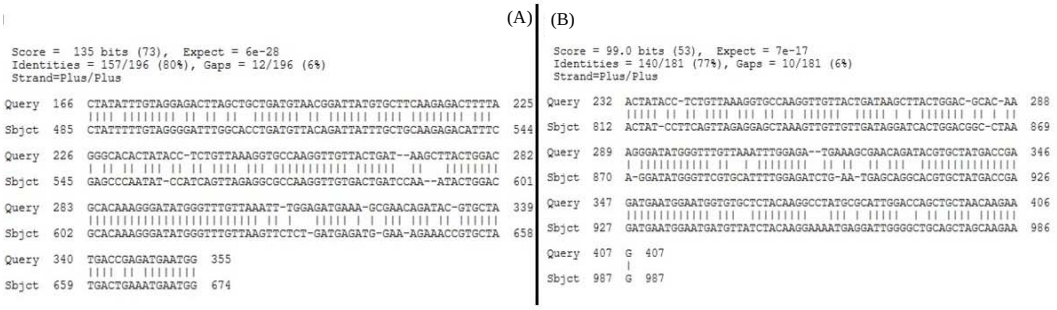

Fig. 2: Identical hits of M6-PS sequence blast to NCBI nucleotide collection (nr/nt) database. The predicted hypothetical protein of *Vitis vinifera* (accession number XM-002285443) (A) and full-length cDNA clone from *Zea mays* (accession number BT063499) (B) are close related to M6-PS with 80 and 77% identities in query sites, respectively

Six pairs of SCAR primers with longer lengths and higher annealing temperatures were designed according to six specific sequences (Table 2). These forward and reverse primers are located in interiors of six RAPD marker sequences, respectively. When using primers I3PSF/I3PSR, M6PSF/M6PSR and Q9PSF/Q9PSR to detect *P. sedoides*, the former two generated the expected 666 bp and 430 bp amplicons in the two taxa and primers Q9PSF/Q9PSR produced a 555 bp fragment unique to *P. sedoides* (Fig. 3). To diagnose *P. chinense*, I3PCF/I3PCR, I18PCF/I18PCR and Q9PCF/Q9PCR primers were used in analysis. In Q9PCF/Q9PCR amplification, the wanted 760 bp fragment was displayed only in *P. chinense*, however, the 186 bp and 238 bp bands were common in the two taxa when using primers I3PCF/I3PCR and I18PCF/I18PCR (Fig. 3).

Table 2: Primers used in RAPD and SCAR analysis

RAPD primer	RPAD marker	GenBank #	SCAR primer (5'-3')	Annealing (°C)	Amplicon size (bp)
SBS-A3 (agtcagccac)	-	-	-	-	-
SBS-I3 (cagaagccca)	I3-PS (986 bp)	JF750772	I3PSF/I3PSR	60	666
			tggatgaccgagacgat/tttatgcgactccacagc		
	I3-PC (477 bp)	JF750773	I3PCF/I3PCR	60	186
			agaatgacgaagacaaacgaaccg/gacgccagaagaagagcaaaaggg		
SBS-I18 (tgcccagcct)	I18-PC (429 bp)	JF750774	I18PCF/I18PCR		
			tgacctaatccattcatccgtgct/tgcggaacctcaatctggtctaat	60	238
SBS-M6 (ctgggcaact)	M6-PS (1347 bp)	JF750775	M6PSF/M6PSR		
			ctggacgcacaaagggat/tcgtatgagcgcagaggc	60	430
SBS-Q9 (ggctaaccga)	Q9-PS (2047 bp)	JF750776	Q9PSF/Q9PSR	60	555
			gcaacttggctcggctac/taacacccgcatactttt		
	Q9-PC (1210 bp)	JF750777	Q9PCF/Q9PCR	60	760
			ttcccacaaacctccaaact/aagcaatcaagagacccact		

-: No specific band is cloned

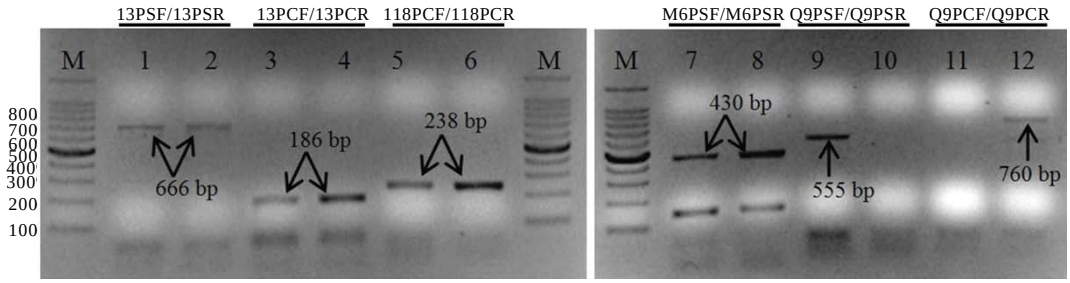

Fig. 3: SCAR analysis of *P. sedoides* and *P. chinense* using primers pairs I3PSF/I3PSR, I3PCF/I3PCR, I18PCF/I18PCR, M6PSF/M6PSR, Q9PSF/Q9PSR and Q9PCF/Q9PCR. Lanes 1, 3, 5, 7, 9 and 11 = *P. sedoides*; Lanes 2, 4, 6, 8, 10 and 12 = *P. chinense*. M, 100 bp DNA ladder (bp). Arrows indicate the exact sizes of amplified fragments

DISCUSSION

Five RAPD primers generated clear, reproducible and distinguishable band patterns in the current study. This indicated that it is effective to detect the genetic characterizations of two taxa via the improved RAPD technique. Based on the RAPD analysis, the genetic characterizations among different herbs were also revealed, respectively (Devaiah and Venkatasubramanian, 2008; Ruzicka *et al*., 2009). Due to the low annealing temperature conditions in RAPD analysis, the specific RAPD bands are usually cloned and converted into SCAR markers to improve the efficiency and stability of diagnosis (Liao *et al*., 2009; Ruzicka *et al*., 2009). Thus, six candidate fragments obtained by four RAPD primers were sequenced in two species respectively to reveal their RAPD genetic characterizations.

The published data indicated that RAPD markers often displayed specific sequences in introns (Lubbers *et al*., 1994; Pessino *et al*., 1997). When blasting six RAPD sequences in NCBI database, no similar query item and open reading frame were found except partial M6-PS fragment. This also suggested that most cloned sequences are likely located in non-coding regions. Introns in RAPD fragments could play an important role in positive or negative regulation of eukaryotic gene

expression (Salgueiro *et al.*, 2000; Hisatsune *et al.*, 2005). However whether these sequences participate gene expression needs to be detected further. Moreover, M6-PS in *P. sedoides* most likely belongs to one kind of unknown function gene.

In the two taxa, all RAPD primers generated obviously dissimilar fingerprints. However, four of six candidate specific primers subsequently demonstrated that the amplicons are common in *P. sedoides* and *P. chinense*, respectively. This suggested that although there are a lot of variations in RAPD genetic sites, *P. sedoides* and *P. chinense* are still closely related. Lee *et al.* (1996) also revealed their near relationships base on allozymes and ITS sequences analysis. According to SCAR analysis, four SCAR primers showed the identical bands among two species respectively while it was different in the amplification of Q9PSF/Q9PSR or Q9PCF/Q9PCR primers. Therefore, it is most likely that Q9-PS and Q9-PC sequences are unique to *P. sedoides* and *P. chinense* respectively while the other four cloned sequences are common in the two taxa.

To ensure the reproducibility and stability, each of amplification was repeated at least five times in SCAR detection and all the expected bands were identical. In the present study, the developed RAPD and SCAR techniques are effective and useful in revealing genetic characterizations of *P. sedoides* and *P. chinense*. Also, the SCAR analysis based on the improved RAPD method is powerful in authentication of species with close relationships.

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