Electronic Journal of Biotechnology 30 (2017) 6–11

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Genetic analysis of *Penthorum chinense* Pursh by improved RAPD and ISSR in China

Zhiqiang Mei [a,1], Xianqin Zhang [a,1], Md. Asaduzzaman Khan [a], Saber Imani [a,b], Xiaoyan Liu [a], Hui Zou [a,c], Chunli Wei [a,d], Junjiang Fu [a,c,d,e,*]

[a] Key Laboratory of Epigenetics and Oncology, the Research Center for Preclinical Medicine, Southwest Medical University, Luzhou, Sichuan, China
[b] Chemical Injuries Research Center, Baqiyatallah Medical Sciences University, Tehran, Iran
[c] Hunan Normal University Medical College, Changsha, Hunan 410081, China
[d] State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Macau, China
[e] Judicial Authentication Center, Southwest Medical University, Luzhou, Sichuan 646000, China

ARTICLE INFO

Article history:
Received 13 April 2017
Accepted 25 August 2017
Available online 7 September 2017

Keywords:
Alleles
Chinese medicine
Coefficient
Genetic diversity
Inter-simple sequence repeat (ISSR)
Molecular marker
Polymorphism
Random amplification of polymorphic DNA (RAPD)
Traditional medicine
Treatment of liver diseases

ABSTRACT

Background: Penthorum chinense Pursh (*P. chinense*) is a well-known traditional Chinese medicine (TCM) plant, which has long been used for the prevention and treatment of hepatic diseases. This study aimed to genetically characterize the varieties of *P. chinense* from different geographic localities of China by random amplification of polymorphic DNA (RAPD)-PCR technique and verified with inter-simple sequence repeat (ISSR) markers.
Results: The *P. chinense* samples were collected from nine different geographic localities. Previously improved RAPD and ISSR markers were utilized for genetic analysis using DNA amplification. The genetic relationship dendrogram was obtained by conducting cluster analysis to the similarity coefficient of improved RAPD and ISSR markers. Improved RAPD yielded 185 scorable amplified products, of which 68.6% of the bands were polymorphic, with an average amplification of 9.25 bands per primer. The ISSR markers revealed 156 alleles with 7.8 bands per primers, where 59.7% bands were polymorphic. Furthermore, the similarity coefficient ranges of RAPD and ISSR markers were 0.71–0.91 and 0.66–0.89, respectively.
Conclusions: This study indicated that improved RAPD and ISSR methods are useful tools for evaluating the genetic diversity and characterizing *P. chinense*. Our findings can provide the theoretical basis for cultivar identification, standardization, and molecular-assisted breeding of *P. chinense* for medicinal use.

1. Introduction

Penthorum chinense Pursh (*P. chinense*), known as "Gan huang cao" in traditional Chinese medicine (TCM), is a wild-growing plant that is widely found in several regions of China, including Sichuan, Jiangxi, Hunan, and Guizhou [1,2]. It has a long history of being used as medicine in the region of the Miao ethnicity. *P. chinense* has a special role in protection of the liver and treatment of liver diseases including hepatitis B, hepatitis C, chronic liver injury, and alcoholic liver damage [3,4].

Recently, DNA markers of *P. chinense* have been used for evaluating genetic diversity and identifying cultivars. However, the difficulties in quality control and standardization restrict its clinical application worldwide [2]. Moreover, the chemical constituents among different cultivars of *P. chinense* have excessive variations. To achieve better pharmaceutical quality of this plant's extracts, it is necessary to genetically characterize *P. chinense*. Nevertheless, to date, very few studies investigated the polymorphism and genetic relationship of *P. chinense*, which made it difficult for cultivar identification and breeding [2].

DNA markers have several advantages over typical phenotype makers. Usually, DNA markers uncover genetic polymorphisms and distinguish each species because of their unique genetic compositions. Moreover, genetic polymorphisms detected by DNA markers are not affected by physiological conditions or environmental factors. Several types of DNA-based molecular techniques have been developed for species characterization of medicinal plants, including random amplification of polymorphic DNA (RAPD), simple sequence repeats (SSRs), inter-simple sequence repeats (ISSRs), sequence-characterized amplified region (SCAR), and amplified fragment length polymorphism (AFLP). Among these, improved RAPD analysis is a widely used technique for differentiating many species, e.g., *Scutellaria* [5], *Cuscuta reflexa* [6], and *Dendrobium officinale* [7,8]. ISSR marker is also an

* Corresponding author.
E-mail addresses: fujunjiang@hotmail.com, fujunjiang@swmu.edu.cn (J. Fu).
[1] These authors contributed equally to this study.
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2017.08.008

efficient and reliable tool in the genetic identification of medical plants. Moreover, ISSR markers are very stable and have been used in several studies, e.g., to identify *D. officinale* [7], *Tribulus terrestris* [9], *Podophyllum hexandrum* [10], etc.

Regarding the importance of *P. chinense* species in modern alternative medicine and TCM, research on the genetic characterization and authentication of *P. chinense,* especially using DNA-based technologies, is necessary. Here we have distinguished the different cultivars and geographic localities of *P. chinense* to genetically characterize the polymorphisms by using a previously developed RAPD-PCR technique [11]; in addition, we verified the results by using ISSR markers. This may provide a valuable understanding of the genetic and molecular diversity of this promising TCM plant.

2. Materials and methods

2.1. Sample collection

In this study, nine fresh young leaves of different cultivar of *P. chinense* were collected from different regions of China (three samples of each species), which are shown in Fig. 1. The plant specimens were authenticated in the Medicinal Botanical Association of Zhongshan Mountain (MBAZM), Southwest Medical University, Luzhou, Sichuan, China, and were preserved at the source bank of Southwest Medical University. The collected fresh tissues were powdered in liquid nitrogen and stored at −70°C until further processing. The characteristics of the different sample source localities are given in Table 1.

Table 1
Sources of the samples used in this study.

Sample	Common name	Sources	No.
YT	*Penthorum chinense* Pursh	Yingtan, Jiangxi	001
AQ	*Penthorum chinense* Pursh	Anqing, Anhui	002
AK	*Penthorum chinense* Pursh	Ankang, Shanxi	003
CZ	*Penthorum chinense* Pursh	Chenzhou, Hunan	004
LZ	*Penthorum chinense* Pursh	Luzhou, Sichuan	005
XC	*Penthorum chinense* Pursh	Xichang, Sichuan	006
ZY	*Penthorum chinense* Pursh	Zunyi, Guizhou	007
YC	*Penthorum chinense* Pursh	Yichang, Hubei	008
XZ	*Penthorum chinense* Pursh	Xinzhou, Shanxi	009

2.2. DNA extraction and purification

Modified cetyl trimethyl ammonium bromide method was performed to extract the genomic DNA from fresh leaves, following previously described protocols [11,12,13]. Briefly, fresh leaf tissues (1–1.5 g) were ground in liquid nitrogen with polyvinyl pyrrolidone (PVP). Freshly prepared extraction buffer (containing 0.3% [v/v] β-mercaptoethanol) was added to adjust the pH to 7.5–8. The suspension was incubated at 65°C for 30 min with intermittent pH monitoring. After cooling at room temperature for 5 min, an equal volume of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 665 × g, 25°C, for 10 min. Then, the plant genomic DNA from the aqueous layer was precipitated by adding 10 volumes of 3 M sodium acetate and 2 volumes of chilled ethanol. The mixture was centrifuged at 665 × g, 4°C, for 5 min. The DNA pellet was washed with 70% (v/v) ethanol and dissolved in 1× Tris-Cl-EDTA (pH 8.0) buffer after being air-dried. DNA quality was determined by electrophoresis on 1% agarose gels, and DNA concentration was measured by NANODROP™ 2000 spectrophotometry (Thermo, Delaware, USA) at 260 and 280 nm [13]. DNA quality was assessed by 0.8% [w/v] agarose gel electrophoresis and spectrophotometry. The DNA concentrations of all the samples were adjusted to 10 ng/μl for PCR analysis and were stored at −20°C for future analysis [11,13].

2.3. Improved RAPD/RAMP-PCR amplification

RAPD amplification was performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, California, USA), with 2.5 μmol of specific primers (Table 2), 15 ng of DNA template, and 5 μl of 2 × PCR Taq Mastermix (Tiangen Biotech, Beijing, China). All PCR reactions were repeated thrice. PCR amplification for previously improved RAPD (also called RAMP-PCR) was performed as follows: initial denaturation at 95°C for 90 s; followed by 40 cycles of 94°C for 40 s, 36°C for 90 s, and 72°C for 90 s; and final extension at 72°C for 5 min. The RAMP time from annealing to extension was adjusted from 2.5°C/s (100%)

Fig. 1. Sampling localities of *P. chinense* cultivars. Black dots indicate cities and light blue lines indicate the Yellow River (above) and the Yangtze River (below).

Table 2
Primers used for improved RAPD.

Primer	Sequence (5′-3′)	Primer	Sequence (5′-3′)
SBS-A1	AGGGGTCTTG	SBS-A10	GTGATCGCAG
SBS-A11	CAATCGCCGT	SBS-A12	TCGGCGATAG
SBS-A19	CAAACGTCGG	SBS-I10	ACAACGCGAG
SBS-I18	TGCCCAGCCT	SBS-M9	GTCTTGCGGA
SBS-M12	GGGACGTTGG	SBS-M15	GACCTACCAC
SBS-N2	ACCAGGGGCA	SBS-N7	CAGCCCAGAG
SBS-N19	GTCCGTACTG	SBS-Q3	GGTCACCTCA
SBS-Q5	CCGCGTCTTG	SBS-Q6	GAGCGCCTTG
SBS-Q7	CCCCGATGGT	SBS-Q9	GGCTAACCGA
SBS-Q18	AGGCTGGGTG	SBS-Q19	CCCCCTATCA

(default time) to 0.125°C/s (5%) to compare the resolution and production of the two methods by RAMP-PCR. The RAMP rate from annealing to extension was adjusted to 0.125°C/s (5% RAMP rate), and the steps were conducted in triplicate for all samples [11,14]. The PCR conditions for ISSR were as follows: initial denaturation at 95°C for 90 s; followed by 35 cycles of 94°C for 40 s, 50°C for 30 s, and 72°C for 90 s; and final extension at 72°C for 5 min.

2.4. ISSR amplification

ISSR amplifications were also performed in 10-μl reaction volumes consisting of 1 μl of 2.5 μmol/l primers, 1 μl of DNA templates of *P. chinense* species or cultivars, 5 μl of 2× PCR Taq Mastermix, and 3 μl of ddH_2O. The PCR conditions were as follows: initial denaturation at 95°C for 90 s; followed by 35 cycles of 94°C for 40 s, 50°C for 30 s, and 72°C for 90 s; and final extension at 72°C for 5 min. PCR was executed in the abovementioned PCR machine "Applied Biosystems Veriti® 96-Well Thermal Cycler" (Life Technology, NY, USA). All ISSR primers were tested initially, and 10 primers (Table 3) amplified the DNA well with valuable polymorphic bands.

2.5. Agarose electrophoresis

The amplified PCR products were resolved by electrophoresis on 1.5% agarose gels in 0.5× TAE buffer. Gels were visualized by 0.5 μg/ml ethidium bromide staining, and the images were documented using the Chemi Doc XRS (Bio-Rad, USA) [13]. Bands that were unambiguous and reproducible in successive amplifications were selected for scoring.

2.6. Statistical analysis

Bands in the gel profiles were recorded as present (1) or absent (0). The similarity matrix (SM) and the similarity index (SI) were calculated from the SM coefficient and the dendrograms were based on unweighted pair group method with arithmetic mean (UPGMA) algorithm were generated using the SAHN module in NTSYS pc 2.1 packages, as previously described in detail [13,15].

Table 3
Primers used for ISSR.

Primer	Sequence (5′-3′)	Primer	Sequence (5′-3′)
UBC807	AGAGAGAGAGAGAGAGT	UBC808	AGAGAGAGAGAGAGAGC
UBC810	GAGAGAGAGAGAGAGAT	UBC813	CTCTCTCTCTCTCTCTT
UBC814	CTCTCTCTCTCTCTA	UBC816	CACACACACACACACAT
UBC819	GTGTGTGTGTGTGTGTA	UBC821	GTGTGTGTGTGTGTGTT
UBC824	TCTCTCTCTCTCTCTCG	UBC829	TGTGTGTGTGTGTGTGC
UBC834	AGAGAGAGAGAGAGAGYT	UBC843	CTCTCTCTCTCTCTCTRA
UBC845	CTCTCTCTCTCTCTCTRG	UBC848	CACACACACACACACARG
UBC851	GTGTGTGTGTGTGTGTYG	UBC866	CTCCTCCTCCTCCTCCTC
UBC873	GACAGACAGACAGACA	UBC876	GATAGATAGACAGACA
UBC880	GGAGAGGAGAGGAGA	UBC892	TAGATCTGATATCTGAATTCCC

R = (A, G), Y = (C, T).

3. Results

3.1. Establishment of improved RAPD technique for the analysis of P. chinense

To obtain the optimized condition for improved RAPD in *P. chinense*, the primer SBS-Q19 was used. RAMP-PCR time was adjusted from 2.5°C/s to 0.125°C/s for annealing to extension in nine samples. Similar and repeatable 3 bands were found with the RAMP time 2.5°C/s in each sample (Fig. 2a). PCR products and number of DNA bands clearly increased when the RAMP time was adjusted to 0.125°C/s (Fig. 2b). Only three bands were found in sample no. 2 (Fig. 2a), whereas the number of bands increased to six with the improved RAPD (Fig. 2b), revealing that the RAPD production was significantly optimized. These results suggest that the improved RAPD technique, by prolonging the RAMP time to 0.125°C/s from annealing to extension, could be applied for *P. chinense* sample characterization.

3.2. DNA amplification with improved RAPD analysis

Twenty primers (Table 2) were used for amplification in improved RAPD analysis. The representative fingerprints are shown in Fig. 3. The band sizes of PCR products ranged from 150 to 2500 bp. In total, 185 bands were obtained, with an average amplification number of 9.25 per primer, among which polymorphic fragments accounted for 68.6%. These results clearly show the high genetic diversity among the nine experimental materials used in improved RAPD amplification. The highest number of amplified bands, i.e., 12, was found when the primer SBS-Q6 was used, while the primer SBS-N19 could amplify only 7 bands. These results implied that these primers have good potential for identifying DNA polymorphisms and analyzing the genetic relationship among *P. chinense* samples.

3.3. Genetic distance and cluster analysis by improved RAPD

The genetic relationship dendrogram was obtained by conducting cluster analysis to the similarity coefficient by improved RAPD (Fig. 4). Nine samples were separated into two groups. Samples AK and XZ clustered together forming one group, which was independent from other seven samples. The similarity coefficients of nine cultivars ranged from 0.71 to 0.91 (Fig. 4a). The similarity coefficient of samples LZ and ZY was the highest (0.91), whereas the similarity coefficient of samples YT and XZ was the lowest (0.71). Thus, these findings indicate a large genetic distance between cultivars of *P. chinense,* as determined by improved RAPD.

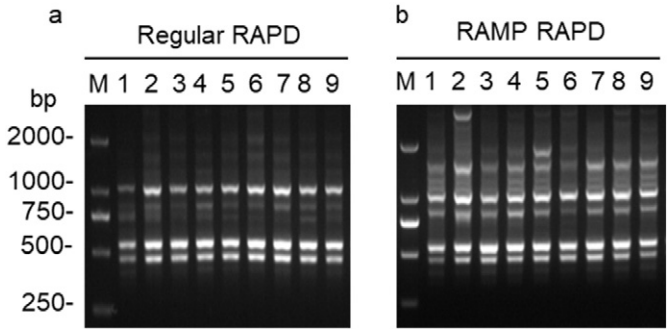


Fig. 2. Comparison between regular RAPD and improved RAPD using the primer SBS-Q19. Regular RAPD was amplified at 100% RAMP rate, while improved RAPD was amplified at 5% RAMP rate. (a) The results of regular RAPD amplification and (b) results of improved RAPD amplification. Lanes 1–9 represent the different samples listed in Table 1. "M" represents DL2000 DNA marker with indicated molecular weight size (bp).

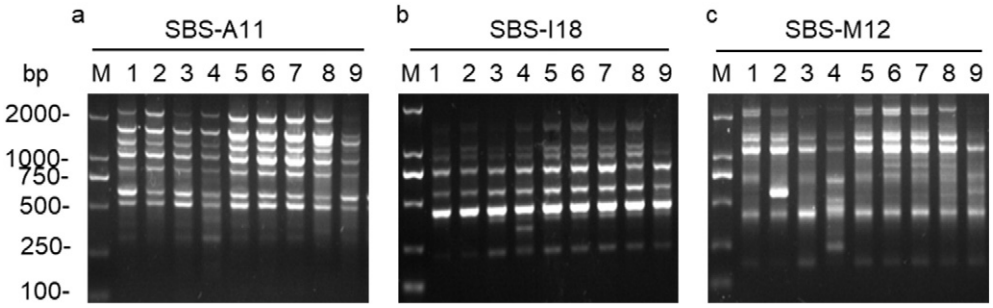


Fig. 3. Results of different RAPD banding patterns of *P. chinense* samples obtained with represented primers. SBS-A11 (a), SBS-I18 (b), and SBS-M12 (c). Lanes 1–9 represent the different samples listed in Table 1. "M" represents DL2000 DNA marker with indicated molecular weight size (bp).

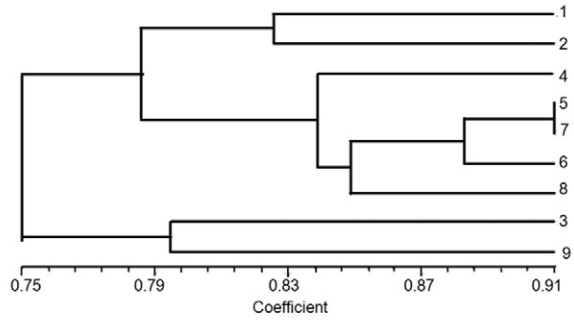
3.4. Amplification results of ISSR analysis

Twenty primers were used to conduct ISSR analysis; the primers are listed in Table 3. The representative fingerprints of ISSR analysis are shown in Fig. 5. The band sizes of PCR products ranged from 250 to 2000 bp. In total, 156 bands with an average amplification number of 7.8 per primer were obtained, among which polymorphic fragments accounted for 59.7%. Fig. 5 presents the high genetic diversity among nine experimental materials for ISSR analysis. The highest number of amplified bands, i.e., 14, was found when primer UBC899 was applied, while primer UBC845 amplified only 6 bands. In general, these findings indicate the vital roles of these primers in identifying DNA polymorphisms and analyzing the genetic relationship among *P. chinense* samples.

3.5. Genetic distance and cluster analysis by ISSR

The genetic relationship dendrogram was obtained by conducting cluster analysis to the similarity coefficient of the abovementioned nine cultivars used in ISSR analysis (Fig. 6). Similar to improved RAPD results, the nine samples were separated into two groups, with samples AK and XZ clustering together. The similarity coefficient of the samples ranged from 0.66 to 0.89 (Fig. 6a). Among the cultivars, the similarity coefficient of samples LZ and ZY was the highest (0.89), whereas the similarity coefficient of samples AQ and CZ was the lowest (0.36), which indicated a large genetic distance (Fig. 6a and b).

a



b

Sample	1	2	3	4	5	6	7	8	9
1	1								
2	0.83	1							
3	0.76	0.72	1						
4	0.72	0.80	0.79	1					
5	0.81	0.84	0.77	0.84	1				
6	0.79	0.79	0.80	0.88	0.88	1			
7	0.80	0.80	0.81	0.82	0.91	0.89	1		
8	0.72	0.79	0.75	0.82	0.88	0.83	0.84	1	
9	0.71	0.71	0.80	0.72	0.76	0.72	0.72	0.76	1

Fig. 4. Dendrogram of nine *P. chinense* cultivar samples by improved RAPD. (a) Dendrogram of *P. chinense* cultivars based on improved RAPD-PCR amplification files. Bar at the bottom indicates similarity index based on similarity matrix coefficient. (b) Genetic distance dendrogram of *P. chinense* cultivars by improved RAPD. Lanes 1–9 represent the different samples listed in Table 1.

4. Discussion

Our data present the first report on genetic variability among *P. chinense* cultivars, detected by using developed RAPD/ISSR molecular markers. In the present study, RAPD/ISSR was applied to analyze the polymorphism and genetic relationship of *P. chinense* samples from different regions of China. In total, 185 distinguishable bands were obtained with 68.6% of polymorphic fragments by improved RAPD and 156 apparent bands were achieved with 59.7% of polymorphic fragments in ISSR analysis. The results showed that the two methods were effective for genetic polymorphism investigation of *P. chinense*.

RAPD [16], AFLP [17], sequence related amplified polymorphism [18], and SSR [19] are the most developed molecular marker techniques used for TCM plant characterizations. The combination of RAPD-PCR and ISSR has been used before for the genetic analysis of *Litchi chinensis* Sonn., *Saccharina germplasm*, etc. [20,21,22]. However, few studies focused on the genetic information of *P. chinense* using RAPD-PCR and ISSR. In this study, first an improved RAPD technique was developed and applied by prolonging the RAMP time from 2.5°C/s to 0.125°C/s to obtain optimized effectiveness of the RAPD method in *P. chinense*, which is consistent with our previous study [23]. By the improved RAPD technique, the production of RAPD fragments and the band numbers were enhanced. Specifically, the band numbers and productions are improved obviously from three to six in both samples (sample no. 3 and 5, respectively). Therefore, the improved RAPD conducted here can increase the stability and accuracy of genotype fingerprinting and molecular-assisted breeding of *P. chinense*.

Despite the discrimination of PCR-amplified profiles in different marker assays [24,25], combination of ISSR and RAPD resulted in more information on the phylogeny of *P. chinense*. The number of bands and polymorphic fragments detected by ISSR (156, 59.7%) were lower than those detected by improved RAPD (185, 68.6%). This indicates that improved RAPD might be a more useful method to study the phylogeny of *P. chinense* than ISSR. However, different molecular marker techniques are still needed to obtain more systematic genetic information for cultivar identification and molecular-assisted breeding of *P. chinense*. Furthermore, development of SCAR markers [26,27] should also provide high level of authenticity for the genetic identification of *P. chinense* using specific PCR primers designed according to the sequences of these improved RAPD amplicons.

In our study, sample no. 3 (AK) and 9 (XZ) were clustered together, revealing that they have a close genetic relationship; however, the remaining seven samples exhibited high genetic similarity but low

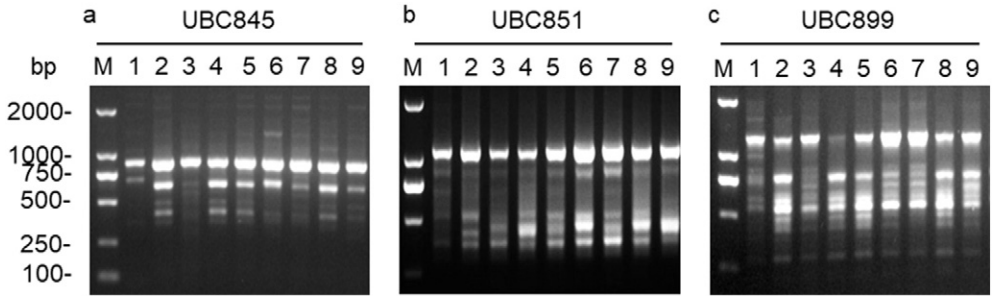
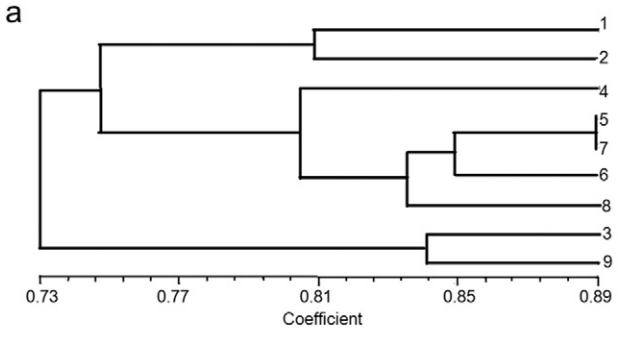


Fig. 5. Results of different ISSR maker patterns of *P. chinense* cultivars obtained by ISSR analysis. (a) Primer UBC845, (b) Primer UBC851, and (c) Primer UBC899. Lanes 1–9 represent the different samples listed in Table 1. Lane "M" represents DL2000 DNA marker with indicated molecular weight size (bp).

genetic distance. These genotype differences are probably due to the geographical and climatic distribution. Interestingly, sample no. 3 (AK) and 9 (XZ) are located along the Yellow River in China, which belongs to the temperate monsoon climate; subtropical monsoon climate may provide a different growth environment for other samples. The understanding of genetic differences between geo-authentic habitats and other areas can provide important information for cultivar identification and breeding of *P. chinense*, which will help in further understanding the influence of environment on the genotype and medical efficiency.

5. Conclusions

The result of this work provides the evidence that improved RAPD and ISSR markers are valuable tools for distinguishing genetic polymorphisms among *P. chinense* genotypes. Studying the genetic diversity among different samples selected from various areas is very useful in cultivar identification and breeding. Our study also provides a theoretical basis for quality control and standardization of *P. chinense* for medicinal use.



b

Sample	1	2	3	4	5	6	7	8	9
1	1								
2	0.81	1							
3	0.77	0.71	1						
4	0.69	0.63	0.66	1					
5	0.81	0.77	0.74	0.85	1				
6	0.77	0.71	0.74	0.82	0.84	1			
7	0.79	0.76	0.79	0.77	0.89	0.85	1		
8	0.74	0.77	0.77	0.76	0.84	0.81	0.85	1	
9	0.74	0.71	0.84	0.69	0.71	0.68	0.69	0.77	1

Fig. 6. Dendrogram of nine *P. chinense* cultivar samples with ISSR primer. (a) Dendrogram of *P. chinense* cultivars based on ISSR-PCR amplification files. Bar at the bottom indicates similarity index based on similarity matrix coefficient. (b) Genetic distance dendrogram of *P. chinense* cultivars using ISSR primers. Lanes 1–9 represent the different samples listed in Table 1.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Financial support

The research was supported in part by the National Natural Science Foundation of China (81672887), the Research Foundation of the Science and Technology Department of Sichuan Province (14JC0797, 2015JY0038) and the Joint Program of the Science & Technology Office of Luzhou and the Science & Technology Department of Sichuan (14JC0154).

Acknowledgments

The authors thank all individuals who provided plant leaves.

References

[1] Feng CG, Wang HW, Ren QS. Analysis of the volatile components of *Penthorum chinense* by gas chromatography-mass spectrometry. Chin Pharm J 2003;38:340–1.
[2] Lin SF, Yuan Y, Shao AJ, et al. Analysis on genetic diversity of *Penthorum chinense* Pursh from separate sources based on ISSR markers. Res Pract Chin Med 2012;26: 18–21.
[3] Zhao JQ, Yang M, Zhao LS, et al. Inhibitory effect of *Penthorum chinese* Pursh on hepatitis B virus in vitro. Chin J Integr Tradit West Med Liver Dis 2002;12:26–7.
[4] Cao YW, Jiang Y, Zhang DY, et al. Protective effects of *Penthorum chinense* Pursh against chronic ethanol-induced liver injury in mice. J Ethnopharmacol 2015;161: 92–8. http://dx.doi.org/10.1016/j.jep.2014.12.013.
[5] Hosokawa KZ, Minami MY, Kawahara KH, et al. Discrimination among three species of medicinal Scutellaria plants using RAPD markers. Planta Med 2000;66(3):270–2. http://dx.doi.org/10.1055/s-2000-8557.
[6] Khan S, Mirza KJ, Abdin MZ. Development of RAPD markers for authentication of medicinal plant *Cuscuta reflexa*. Eurasian J Biosci 2010;4(1):1–7. http://dx.doi.org/10.5053/ejobios.2009.4.0.1.
[7] Ding G, Li X, Ding X, et al. Genetic diversity across natural populations of *Dendrobium officinale*, the endangered medicinal herb endemic to China, revealed by ISSR and RAPD markers. Genetika 2009;45:375–82.
[8] Xue DW, Feng SG, Zhao HY, et al. The linkage maps of dendrobium species based on RAPD and SRAP markers. J Genet Genomics 2010;37(3):197–204. https://doi.org/10.1016/S1673-8527(09)60038-2.
[9] Sarwat M, Das S, Srivastava PS. Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in *Tribulus terrestris*, a medicinal herb. Plant Cell Rep 2008;27(3):519–28. http://dx.doi.org/10.1007/s00299-007-0478-5.
[10] Naik PK, Alam MA, Singh H, et al. Assessment of genetic diversity through RAPD, ISSR and AFLP markers in *Podophyllum hexandrum*: A medicinal herb from the Northwestern Himalayan region. Physiol Mol Biol Plants 2010;16(2):135–48. http://dx.doi.org/10.1007/s12298-010-0015-9.
[11] Fu JJ, Yang LQ, Khan MA, et al. Genetic characterization and *authentication of Lonicera japonica* Thunb. by using improved RAPD analysis. Mol Biol Rep 2013;40(10): 5993–9. http://dx.doi.org/10.1007/s11033-013-2703-3.
[12] Sharma P, Joshi N, Sharma A. Isolation of genomic DNA from medicinal plants without liquid nitrogen. Indian J Exp Biol 2010;48:610–4.
[13] Fu JJ. Short protocols in medical molecular biology. Beijing: China Medical Science Press; 2012.
[14] Mei ZQ, Fu SY, Yu HQ, et al. Genetic characterization and authentication of *Dimocarpus longan* Lour. using an improved RAPD technique. Genet Mol Res 2014; 13(1):1447–55. http://dx.doi.org/10.4238/2014.March.6.3.
[15] Rohlf FJ. NTSYS-pc: Numerical taxonomy system, version 2.1. New York: Exeter Publishing. S. Ltd; 2002.

[16] Sheng L, Wang D, Si HJ. Analysis of germplasm resources of *Angelica sinensis*(Oliv.) Diels in Gansu province with RAPD. J Gansu Agric Univ 2007;43:60–3.
[17] Yang J, Li XD, Li XW, et al. Analysis of AFLP variation of the endemic and rare species *Psilopeganum sinense* in Central China. J Wuhan Bot Res 2007;25:226–34.
[18] Qian D, Huang LQ, Cui GH, et al. Study on genetic relationship of *Astragalus membranaceus* var mongholicus in different producing area using SRAP. Zhongguo Zhong Yao Za Zhi 2009;34:382–5.
[19] Yang WZ, Jin H, Cui XM, et al. Analysis on EST-SSR of three species in genus *Panax* and the application in *Panax notoginseng*. Genomics Appl Biol 2011;30:Q939.5.
[20] Long Y, Cheng J, Mei Z, et al. Genetic analysis of litchi (*Litchi chinensis* Sonn.) in southern China by improved random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR). Mol Biol Rep 2015;42(1):159–66. http://dx.doi.org/10.1007/s11033-014-3755-8.
[21] Cui C, Yan L, Liu Y, et al. Determination of genetic diversity among Saccharina germplasm using ISSR and RAPD markers. C R Biol 2017;340(2):76–86. http://dx.doi.org/10.1016/j.crvi.2016.11.005.
[22] Desai P, Gajera B, Mankad M, et al. Comparative assessment of genetic diversity among Indian bamboo genotypes using RAPD and ISSR markers. Mol Biol Rep 2015;42(8):1265–73. http://dx.doi.org/10.1007/s11033-015-3867-9.
[23] Fu JJ, Li LY, Xu X, et al. An improved method for increasing the efficiency of the technique of random amplified polymorphic DNA (RAPD). Hereditas 2000;22: 251–2.
[24] Ray T, Roy SC. Phylogenetic relationships between members of Amaranthaceae and Chenopodiaceae of lower gangetic plains using RAPD and ISSR markers. Bangladesh J Bot 2007;36(1):21–8.
[25] Poyraz I. Comparison of ITS, RAPD and ISSR from DNA-based genetic diversity techniques. C R Biol 2016;339(5–6):171–8. http://dx.doi.org/10.1016/j.crvi.2016.04.001.
[26] Cheng J, Long Y, Khan MA, et al. Development and significance of RAPD-SCAR markers for *Litchi chinensis* Sonn. variety authentication by improved RAPD amplification and molecular cloning. Electron J Biotechnol 2015;18(1):35–9. http://dx.doi.org/10.1016/j.ejbt.2014.11.004.
[27] Fu S, Cheng J, Wei C, et al. Development of diagnostic SCAR markers for genomic DNA amplifications in breast carcinoma by DNA cloning of high-GC RAMP-PCR fragments. Oncotarget 2017;8(27):43866–77. https://doi.org/10.18632/oncotarget.16704.