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# Molecular Authentication of Medicinal *Penthorum chinense* Push from Different Localities in China by RAPD Analysis

[1,2,3]Haiqing Yu, [1,2,3]Chun Zhang, [1,2]Zhiqiang Mei, [1,2]Li Wang, [1,2]Juan Li, [1,2]Chenggang Duan, [1,2]Xiaoyan Liu, [1,2]Shu Gong, [1,2]Lin Gan, [1,2]Zhonghua Tao, [1,2]Fuli Yao, [1,2]Ye Yang, [1,2]Chunyan Duan, [1,2]Youping Liu, [1,2]Chunyan Zhang, [1,2]Chuanning Chen, [1,2]Xiaoli Zheng, [1,2]Hongxian Zhao, [1,2]Jiyan Cheng, [1,2]Xiaojun Tang, [1,2]Yong Zhang, [1,2]Yingxi Zhang and [1,2]Junjiang Fu
[1]Research Center for Preclinical Medicine, Luzhou Medical College, Luzhou 646000, Sichuan, China
[2]Medicinal Botanical Association of Zhongshan Mountain, Luzhou Medical College, Luzhou 646000, Sichuan, China
[3]Drug Discovery Research Center, Luzhou Medical College, Luzhou 646000, Sichuan, China

**Abstract:** As a traditional medicinal herb in China, *Penthorum chinense* Push from Gulin county is regarded as genuine regional drug with better clinical effects. To discriminate the geographical origin of six *P. chinense* samples cultivated in three provinces, Randomly Amplified Polymorphic DNA (RAPD) analysis was carried out with an improved method to increase the resolution and production using 10 mer-random primers. Similarity index was ranged from 0.61 to 0.97, which demonstrated that samples from different localities displayed similar band patterns. However, based on the analysis of selected 13 primers, primers SBS-I9, SBS-I20 and SBS-Q9 produced distinguishable bands among Sichuan, Yunnan and Hubei *P. chinense*. T-test of mean S.I. values of six accessions demonstrated the significant differences between Luzhou or Sichuan samples and others. Cluster analysis indicated that cultivars with close geographic distributions were clustered together consequently. This suggested that there are RAPD site variations and local specialized genotypes among the six samples. The results indicated that RAPD analysis is effective in distinguishing the geographical origin of genuine regional drug *P. chinense* grown in Luhzou, Sichuan. The approach is a valuable tool to authenticate other morphologically similar herbal medicinal materials.

**Key words:** Authentication, *Penthorum chinense* push, genuine regional drug, genetic characterization, specialized genotype

## INTRODUCTION

*Penthorum chinense* Push in genus *Penthorum* L. is a perennial rhizomatous herb with wet habitats native to far eastern Russia, China, Korea and Japan (Haskins and Hayden, 1987; Pan, 1992). As a traditional medicinal herb in China, *P. chinense* is effective in remedying jaundice, edema and injuries (Pan *et al*., 2001). Also, it has the curative effects such as declining the level of tansaminase, inhibiting hepatic stellate cell proliferation and protecing the liver (Wang *et al*., 1999; Cao *et al*., 2007; Zhou *et al*., 2008). With a common name Ganhuangcao, *P. chinense* grown in Gulin county, Luzhou city of Sichuan province, is recognized as genuine regional drug, which is generally considered to possess a more potent clinical effect than those from other localities (He *et al*., 2002; Song *et al*., 2007; Chi *et al*., 2009). Furthermore, there is a decreased risk of liver disease among Gulin people in relating to Ganhuangcao drinking.

Owing to the favorable therapeutic effects, an increasing quantity of Ganhuangcao is needed throughout China in recent years. In the market, the selling Ganhuangcao is cultivated widely in four regions in China, which are Luzhou (Gulin County) and Leshan cities in Sichuan province, Zhaotong city in Yunnan province and Enshi city in Hubei province. In the modernization of traditional Chinese medicine, permanent quality controls are essential to ensure a continuous efficacy and safety of the drug, and in which an important rule is the regular verification of the species being used as source material (Zhang *et al*., 1999; Ruzicka *et al*., 2009). Since the better therapeutic effects of Gulin Ganhuangcao in four cultivated regions, it is necessary to authenticate their localities.

Traditionally, approaches to herbal locality authentication are dependent on morphological, histological or chemical analyses. Recently, mass spectra analysis displayed an alternative approach for the chemical ingredients authentication of Gulin

**Corresponding Author:** Junjiang Fu, Research Center for Preclinical Medicine, Luzhou Medical College, Luzhou 646000, Sichuan, China Tel: 86-830-3160283 Fax: 86-830-3160283

Ganhuangcao (Zhang *et al*., 2007). However, the morphological and histological characteristics of herbs from different localities are similar in their features, and the chemical ingredients are variable during the herb harvest (Zhang *et al*., 1999). Therefore, it is difficult to determine the geographical origin with visible inspection as well as through analytical tools (Na *et al*., 2004). On the contrary, molecular markers are available in unlimited numbers, irrespective of environment and the development stage of the plant and can be accomplished in a relatively shorter period (Goulão *et al*., 2001; Manimekalai and Nagarajan, 2006). The randomly amplified polymorphic DNA (RAPD) technology has been shown to be useful in authentication of medicinal materials at the species and their varieties in molecular level (Zhang *et al*., 1999; Na *et al*., 2004; Devaiah and Venkatasubramanian, 2008; Ruzicka *et al*., 2009).

To identify the geographical origin of six cultivated *P. chinense* samples from three provinces, the RAPD analysis was executed, the aims of this study was to 1) authenticate Luzhou Ganhuangcao from others; 2) evaluate the RAPD site variations among them; and 3) discuss the local specialized genotypes.

## MATERIALS AND METHODS

The study was conducted from January 2008 to December 2010 at Luzhou city, Research Center for Preclinical Medicine of Luzhou Medical College.

**Plant materials:** The material used in this study is listed in Table 1. A total of six cultivated Ganhuangcao accessions were used. All the accessions were collected from Luzhou (Gulin county) and Leshan cities in Sichuan province, Zhaotong city in Yunnan province and Enshi city in Hubei province by the present authors (Fig. 1). 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 BotanicalAssociationof Zhongshan Mountain (MBAZM), Luzhou Medical College.

**DNA extraction and purification:** 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 a slight modification of the cetyltrimethylammonium bromide (CTAB) procedure outlined in (Doyle and Doyle, 1990).

**RAPD PCR amplification:** The PCR reaction was carried out using SBS primer sets A, I, M, N and Q (Beijing SBS Genetech Co., Ltd, China). 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 thermocycling profile consisted of an initial denaturation at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and 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 here (Fu *et al*., 2000).

**Data analysis:** Bands in the gel profiles were recorded as present (1) and absent (0). The similarity matrix and the similarity index (S.I.) were calculated using SM coefficient. The t-test was used to compare the statistic significance of the variations between the mean S.I. of intra-province

Table 1: Sources of six *P. chinense* samples used in RAPD analysis

| Sample | Common name | Locality | Accession No. |
|---|---|---|---|
| LZ1 | Ganhuangcao | Gulin, Luzhou, Sichuan | PCTY001 |
| LZ2 | Ganhuangcao | Gulin, Luzhou, Sichuan | PCTY002 |
| LZ3 | Ganhuangcao | Gulin, Luzhou, Sichuan | PCTY003 |
| LS | Ganhuangcao | Leshan, Sichuan | PCTY004 |
| ZT | Ganhuangcao | Zhaotong, Yunnan | PCTY005 |
| ES | Ganhuangcao | Enshi, Hubei | PCTY006 |

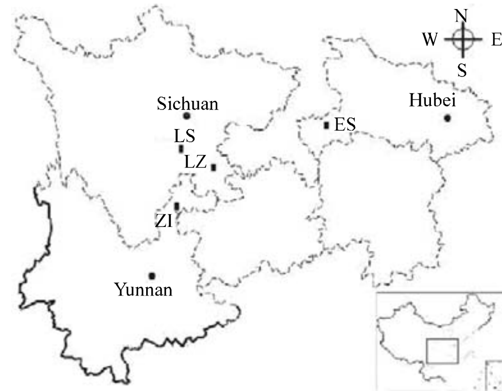


Fig. 1: Map showing localities of cultivated *P. chinense* sampled in China. LZ and LS refer samples from Luzhou and Leshan of Sichuan province. ZT and ES represent samples from Zhaotong of Yunnan province and Enshi of Hubei province. Directions are indicated in the upper right

or intra-city samples and that of inter-province or inter-city samples. The dendrogram based on UPGMA (unweighted pair group method with arithmetic mean) algorithm was generated using the SAHN module in NTSYS pc 2.1 package (Rohlf, 2002).

## RESULTS

A total of 100 primers from SBS primer sets were initially evaluated for polymorphism using a subset of six samples, in which 13 primers generated reproducible polymorphic RAPD amplification patterns. These 13 primers were selected for further PCR analysis of *P. chinense* from different localities (Table 2). The PCR reactions were repeated five times for each of the six samples and the distinct DNA banding patterns were also highly reproducible (data not shown). With an average of 11.69 per primer, the selected primers generated 152 clear and repeatable bands ranging in size from about 250 to 2000 bp. Among the six samples, 80 amplified bands were polymorphic and the whole polymorphism was 53%.

Overall, the selected primers displayed easily visible amplification patterns in the six cultivars. The RAPD genetic characterizations were achieved in Leshan, Zhaotong and Enshi samples respectively. Representative fingerprints including characteristic bands generated by primers SBS-I9, SBS-I20 and SBS-Q9 are shown in Fig. 2. As profiles showing, the SBS-I9 amplification patterns are similar in the six samples, while a band about 300 bp size is present in Leshan accession but absent in the other five samples (Fig. 2A). Among samples from Sichuan province, the SBS-I20 amplification patterns of three Luzhou accessions are similar to that of Leshan sample. Two specific bands about 350 and 1500 bp sizes are displayed in Zhaotong and Enshi cultivars respectively

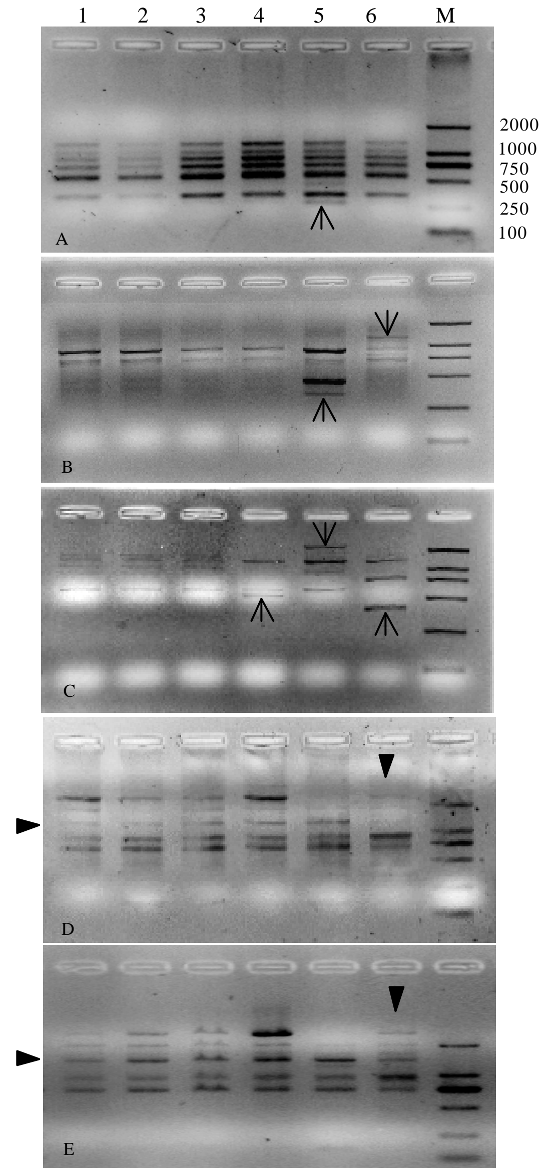


Fig. 2: RAPD profiles of the six *P. chinense* samples generated by primers SBS-I9 (A), SBS-I20 (B), SBS-Q9 (C) and SBS-Q12 (D) with a 3°C/s ramp, and SBS-Q12 and (E) with a 0.3°C/s ramp. Lanes 1-3, *P. chinense* from Luzhou of Sichuan province; Lanes 4-6, *P. chinense* from Leshan of Sichuan province, Zhaotong of Yunnan province and Enshi of Hubei province, respectively. M, DL2000 DNA ladder (bp). Arrows indicate the RAPD genetic characterization bands unique to samples from different localities. Black triangles indicate the increasing of the resolution and production, respectively

Table 2: Primers used in RAPD analysis

| Primer | Sequence | Primer | Sequence |
|---|---|---|---|
| SBS-A3 | AGTCAGCCAC | SBS-M6 | CTGGGCAACT |
| SBS-A20 | GTTGCGATCC | SBS-N3 | GGTACTCCCC |
| SBS-I3 | CAGAAGCCCA | SBS-N9 | TGCCGGCTTG |
| SBS-I9 | TGGAGAGCAG | SBS-N16 | AAGCGACCTG |
| SBS-I18 | TGCCCAGCCT | SBS-Q9 | GGCTAACCGA |
| SBS-I20 | AAAGTGCGGG | SBS-Q12 | AGTAGGGCAC |
| SBS-M2 | ACAACGCCTC | | |

Table 3: The similarity index of RAPD fingerprints generated by the 13 primers

| Sample* | LZ1 | LZ2 | LZ3 | LS | ZT | ES |
|---|---|---|---|---|---|---|
| LZ1 | - | | | | | |
| LZ2 | 0.9605 | - | | | | |
| LZ3 | 0.9539 | 0.9671 | - | | | |
| LS | 0.8882 | 0.9276 | 0.8947 | - | | |
| ZT | 0.7105 | 0.7500 | 0.7171 | 0.7829 | - | |
| ES | 0.6118 | 0.6250 | 0.6053 | 0.6316 | 0.7039 | - |

*Samples are consistent with those listed in Table 1. S.I. values of intra-province samples are underlined.

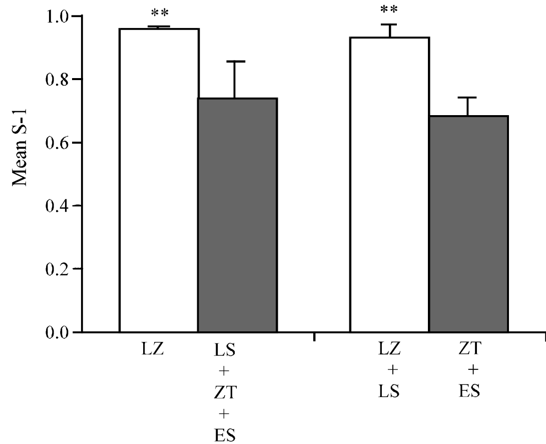


Fig. 3: T-test of mean S.I. values of six cultivated *P. chinense* samples. LZ refers the three samples from Luzhou (Sichuan). LS + ZT + ES, the three samples from Leshan (Sichuan), Zhaotong (Yunnan) and Enshi (Hubei). LZ + LS represent the four samples from Luzhou and Leshan (Sichuan). ZT + ES, the two samples from Zhaotong (Yunnan) and Enshi (Hubei). ** indicates the significant differences at level of p<0.01

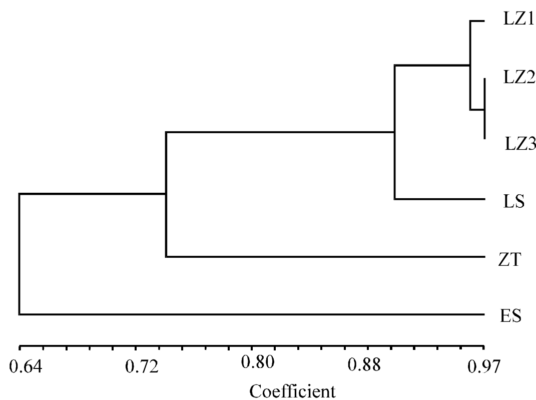


Fig. 4: Dendrogram of the six cultivated *P. chinense* samples based on 152 PCR bands amplified by 13 arbitrary 10-mer RAPD primers. LZ1, LZ2 and LZ3 refer three samples from Luzhou (Sichuan). LS, ZT and ES represent three samples from Leshan (Sichuan), Zhaotong (Yunnan) and Enshi (Hubei) respectively. Bar on the bottom indicates similarity index based on SM coefficient

(Fig. 2B). The SBS-Q9 amplification patterns within three Luzhou samples are consistent. The band numbers and positions of three Luzhou accessions are different to those of the other three samples. Three unique bands about 500 450 bp and 2000 bp sizes are found in Leshan, Zhaotong and Enshi cultivars respectively (Fig. 2C). All primers except SBS-Q12 demonstrated similar repeatable fingerprints when the ramp time from annealing to extension was adjusted from 3 to 0.3°C $sec^{-1}$. The resolution and production generated by primer SBS-Q12 with a 0.3°C $sec^{-1}$ ramp time were obviously increased in six *P. chinense* samples (Fig. 2D-E).

Based on the generated DNA fingerprints, banding patterns were scored in the form of a binomial matrix to calculate the S.I. values using SM coefficient. The S.I. values of three Luzhou cultivars are ranged from 0.95 to 0.97, which is higher than those of Leshan, Zhaotong and Enshi accessions (Table 3). For Leshan, Zhaotong and Enshi samples, the S.I. values are ranged from 0.89 to 0.93, 0.71 to 0.78 and 0.61 to 0.70, respectively (Table 3). The t-test analysis indicated that differences are very significant between the mean S.I. of three Luzhou samples and that of three cultivars from Leshan, Zhaotong and Enshi (Fig. 3). The similar results are also obtained when analyzing four intra-Sichuan (Luzhou and Leshan) samples and two inter-Sichuan (Zhaotong of Yunan and Enshi of Hubei) samples (Fig. 3).

The similarity matrix was also used to derive the genetic relationships among the six accessions based on the scoring of 152 bands. The derived dendrogram using UPGMA algorithm is shown in Fig. 4. The similarity coefficient ranged from 0.64 to 0.97. Three major clades (Sichuan, Yunnan and Hubei) were formed. The Sichuan clade consisted of Luzhou and Leshan groups, in which three accessions from Luzhou were clustered together with a 0.96 similarity, followed by one Leshan accession with a 0.90 similarity. The Yunnan clade including one Zhaotong accession formed the sister to the Sichuan clade with a 0.74 similarity. The Hubei clade comprised of one Enshi accession was in a basal polytomy with a 0.64 similarity.

## DISCUSSION

Ma *et al*. (2000) and Xu *et al*. (2008) indicated that there are abundant genetic variations and differentiations among different herb cultivars based on RAPD analysis. In the present study, the genetic differentiations among P. *chinense* cultivars were also detected. The S.I. indicated that the band numbers and positions of three Luzhou samples are nearly consistent. Their amplification patterns are more similar to those from Leshan than those from Zhaotong and Enshi. T-test analysis demonstrated that *P. chinense* cultivars from Luzhou or Sichuan are different significantly to those from other localities. This suggested that there are variations in RAPD genetic sites among the six cultivars, and it is helpful in discrimination Luzhou Ganhuangcao from the other three localities selling in the market.

According to the RAPD results, Zhang *et al.* (1999) and Na *et al.* (2004) found the distinguishable bands among cultivated herb populations respectively. In this study, no genetic characterization was found to distinguish three Luzhou accessions from Leshan, Zhaotong and Enshi cultivars. However, six unique bands accessing by three RAPD primers were obtained among Leshan, Zhaotong and Enshi cultivars. These genetic characterizations are effective to authenticate Ganhuangcao cultivars from four geographic regions. The obvious distinguishable amplification patterns indicated that *P. chinense* samples from four regions are most likely formed the local specialized RAPD genotypes. The specific bands obtained in them were useful as molecular markers for locality identification.

Cluster analysis and principal component analyses are valuable for determining relationships among populations of the same and different species (Crawford, 1990). In the derived dendrogram, cultivated *P. chinense* samples with close geographic distributions were clustered together consequently. The result indicated that Luzhou and Leshan cultivars from Sichuan province are closely related. Among inter-province cultivars, Zhaotong *P. chinense* from Yunnan province is more closely related to Sichuan samples than Enshi *P. chinense* from Hubei province. This suggested that the relationship of six samples is correlated to their geographic localities respectively. The similar results are also reported in analyzing genetic relationship among populations of different herbs (Feng *et al.*, 2002; Li *et al.*, 2005; Zhou *et al.*, 2005; Wang *et al.*, 2007; Yang *et al.*, 2008; Asif *et al.*, 2000)

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 the same species from different localities (Zhang *et al.*, 1999; Na *et al.*, 2004). It is well known that genuine regional drug, which has the specific growing locality, is more effective than those from other localities. A number of genuine regional drugs in Chinese medicine are reported, such as *Panax ginseng* C.A.Mey from Changbai Mountain region, *Atractylodes macrocephala* Koidz from Jiangning county of Jiangsu province, *Saposhnirovia divaricata* (Turcz.) Schischk. from Shandong province and so on (Zhang *et al.*, 1999). Ganhuangcao grown in Gulin County, Luzhou city of Sichuan province has been commonly considered as genuine regional drug with a higher price in the market. The present detecting demonstrated there are variations in RAPD genetic sites among Luzhou, Leshan, Zhaotong and Enshi Ganhuangcao cultivars, which possessed the local specialized RAPD genotypes respectively. Thus, the RAPD analysis is effective in authentication of medicinal *P. chinense* from different localities, especially when the ramp time is prolonged to increase the resolution and production.

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