ASSESSMENT OF GENETIC DIVERSITY OF *REHMANNIA GLUTINOSA* LIBOSCH BASED ON ISSR MARKERS

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Key words: *Rehmannia glutinosa* Libosch; Inter-simple sequence repeat (ISSR); Genetic diversity; Genetic similarity.

Abstract: In order to assess the genetic diversity of *Rehmannia glutinosa* Libosch cultivars (lines) in Huai zone, Inter-simple sequence repeat (ISSR) was performed. Ten appropriate ISSR primers were selected from a total of 44 ISSR ones for ISSR PCR amplification. The ten primers could amplify one hundred and ten bands. Based on them, A Jaccard's genetic similarity matrix and a dendrogram for these ten cultivars were established using SPSS 10.0 software. In this dendrogram, they could be divided into two groups : Group1 contained six individuals such as Zupei 85.5, Datian 85.5, Zupei 9302, Jinbai, Jinzhuangyuan and Datian9302; Group2 consisted of four ones such as Beijing No.1, Dahongpao, Dihuang9104 and wild dihuang. Furthermore, Principal coordinate analysis (PCA) supported the above cluster analysis; Shannon's Information index (I) is 0.3577, effective number of alleles (Ne) is 1.4037, the percentage of polymorphic loci is 71.82 % by means of POPGENE32 software; A DNA fingerprint was developed with a single primer, ISSR6, in which each of ten individuals tested had its unique fingerprint pattern and was distinguished from each other. The results revealed that ISSR method is suitable for DNA fingerprinting, identification and genetic diversity analysis of *Rehmannia glutinosa* in Huai zone.

INTRODUCTION

Up to now, DNA markers have provided some valuable tools for plant genetic researches such as heredity, physical mapping construction, gene mapping and cloning, the inheritance of quantitative trait locus, assessment of genetic diversity, species origin, marker-assisted selection breeding and so on. Inter-simple sequence repeat (ISSR) among many DNA markers has made success in the research on plant genetic diversity. ISSR markers were put forward by Zietkiewicz *et al* (1994).This technique with some advantages including stability and reproducibility, rich polymorphism, reliability and so on involves amplification of genomic segments flanked by inversely oriented and closely spaced microsatellite sequences by a single primer or a pair of primers based on SSRs anchored 5' or 3'end with 1-4 purine or pyramodine residues (Li,et al.,2003; Qian, et al., 2001). So far, ISSR marker has been used in field crops including wheat, rice, etc. and fruit trees such as apple, orange and so forth but seldom in herbs. There are only two reports about the authentication of *Lilium species* (Masumi, et al., 2002) and *Schisandra* varieties (Sun, et al., 2003). To our knowlege, now there is no report about the application of ISSR marker in the identification and assessment of genetic diversity of *Rehmannia glutinosa* germplasm.

Rehmannia glutinosa belongs to *Rehmannia, Scrophulariaceae.* There are six species of *Rehmannia* all over the

world. It is commonly used in clinics in the orient and is called di-huang, or "yellow earth." .It is used to replenish vital force and helps with diabetes, constipation, urinary tract problems, anemia, dizziness, and regulating menstrual flow. It is protective to the liver and helps prevent the depletion of stored glycogen, which can make it beneficial for hypoglycemia. Di huang has an antifungal effect and has been used for candida. It can lower glucose levels and helps to reduce blood pressure while increasing circulation to the brain. It is a blood tonic and diuretic. It helps strengthen the bones and tissue and can enhance fertility. Chinese doctors have used it with licorice for the treatment of hepatitis. Rehmannia helps disperse heat from the body and alleviates night sweats and fevers (Zhou, 2002). Its year demand is more than 15000 tons (Wen, et al.,2002). *Rehmannia glutinosa* can be planted in all parts throughout China, but Henan provincial Huai di huang, i.e. *Rehmannia glutinosa Libosh .f. hueichingensis (Chao et Schih)* Hsiao, has better quality and curative effects than others. It is a member of *Rehmannia glutinosa*, one of world-famous “Four famous huai herbs ” living in ancient huaiqingfu (called Jiaozuo City now), Henan Province. Every year a lot of Di huang is sold in Chinese market and exported into Southeast, Korea and Japan. These years it has become part of economic income, which people can get in its growing areas. Therefore, domestic and overseas markets have made higher demands than ever for the quality, purity and special producing area of *Rehmannia glutinosa .f. hueichingensis Hsiao.* On the other hand, current Huai Dihuang products originate from different places of production, so its good varieties and bad ones are mixed together; its classification and genetic analysis are still conducted by means of traditional morphologic traits and kin. The genetic diversity of ten *Rehmannia glutinosa* cultivars (lines) in Huai zone was analyzed using ISSR markers to exactly identify and use its cultivars, to overcome the limitations of traditional identification methods, and to provide molecular basis for the seed selection of new cultivars and the protection of breeding specialists’ intellectual property right.

MATERIALS AND METHODS

1.1 Instrument and reagents

Beckman Avanti^{TM}J-25 centrifuge; TG16 - W micro high-speed centrifuge (Xiang Yi centrifuge instrument CO.LTD); Beckman Du530 DNA/Protein Analyzer ; PolaroidMP-4 Land Cameraand Transilluminator UV; DYY - lll8A constant voltage and current electrophoresis meter (Beijing Liuyi instrument factory) JY2002 electronic balance (Shanghai precision and scientific instrument CO.LTD) ;Taq DNA polymerase and NTPs (Pharmacia company) ; ISSR Primers (Shanghai Sangon biological engineering technology &service CO.LTD) ; Biowest agarose (Shanghai Yito Enterprise Company); CTAB BRO361/100g and EB (Sino-American biotechnology company) .

1.2 Materials: the young leaves of eight *Rehmannia glutinosa* cultivars grown in field and 2 virus-free *Rehmannia glutinosa* lines micropropagated by tip tissue culture and cultured in plant tissue culture room, were used in this study. They were 1.Zupei85.5; 2.Datian85.5; 3.Zupei9302; 4.Datian9302; 5.9104; 6.Jinzhuangyuan; 7.Wild Dihuang; 8.Beijing No.1; 9.Dahongpiao and 10.Jinbaidihuang,which were provided by Wenxian Institute of Agricultural Sciences, Henan, P R C and identified by its senior agronomist Wang Qian-ju. In each cultivar or line population, 10 young plants were randomly selected and their leaves were equally mixed. These leaves were washed by tap water, put on clean bumf to be

dried in the air, loaded in spotless 50-ml plastic tubes, which were immediately stored in liquid nitrogen to be frozen, and taken back to the laboratory.

1.3 Methods

1.3.1 Total DNA extraction and concentration determination：DNA was extracted from 3-5g leaf tissue (as treated above) for each cultovar or line by CTAB method (Wang and Fang, 1998) after they were thoroughly mashed by a glass rod. The DNA was re-suspended in TE buffer with proper concentration of 10mg/ml RNase A. DNA concentration was determined by Beckman Du530 DNA/Protein Analyzer. DNA molecular weight was analyzed by electrophoresis on 0.8% (W/V) agarose gel by means of DNA molecular weight standard, λDNA /Hind□。

1.3.2 ISSR assay: one material total DNA was randomly selected from ten materials to conduct preliminary experiment, through which ten primers with distinct and reproducible fragments and reliability were screened for further analysis from a total of 44 ISSR primers (Table1). PCR total volume of 25 μL was composed of 1.5~1.0 U of Taq DNA polymerase, 3.0 mmol.L^{-1} Mg^{2+}, 1 x Taq DNA polymerase buffer (10mmol.L Tris-HCl, 50mmol.L^{-1} KCl, 0.1% Trion X-100, pH 9.0), 60 ng of template DNA, 0.4 μ mol.L of primer, 0.4 m mol.L^{-1}each of dATP、dGTP、dCTP and dTTP and 2% Formamide deionized. PCR was performed for one initial step of 7-min denaturation at 94°, followed by 45 cycles of 45 s denaturation at 94°, 90 s annealing at 50°or 53°or 55°, 120 s extension at 72°, and a finial extension step of 7 min at 72 °. Amplification products were loaded on 1.4% (w/v) agarose gel plus 0.5 μg/ml EB for 1.5-2.5 h with constant voltage of 70 volume and DNA molecular weight standard, λDNA /*Eco*RI + *Hin*d□ or 100bp ladder. Gels were photographed under UV light with Polaroid MP-4 Land Camera.

1.3.3 Data statistics and analysis

Because ISSR markers are dominant markers, PCR Amplified fragments were scored for the presence (1) or absence (0) or missing or failure (9) .0-1-9 data set was entered into SPSS VERSION 10.0 to calculate genetic similarity (GS) for all pairs of individuals with Jaccard method, which were used to construct dendrograms using Within-group linkage method; In addition, POPGENE32 software was used to compute the number of effective loci, the percentage of polymorphic loci, Shannon's polymorphism information content (PIC=1-$\sum p_i^2$,pi stands for the phenotype of amplified band) and effective number of alleles (Ne= Ne=1/$\sum p_i^2$) for ISSR markers.

RESULTS AND DISCUSSIONS

2.1 Polymorphism of PCR-amplified Products

These ten primers generated a total of 110 bands ranging from 200 to 1600bp (Fig.1～3).Based on the analyses of these bands by SPSS10.0 and POPGENE32 softwares. The number of polymorphic loci was 79, The percentage of polymorphic loci was 71.58 %, Shannon's Information index (I) was 0.3775, and effective number of alleles (Ne) was 1.4037,PPB was 71.82%(Table 1).

Table 1. List of ISSR primers and their sequences and analysis of ISSR-generated banding patterns

Primer	Sequence (5'-3')	Amplified band (No.)	Polymorphic band (No.)	Size of band	PPB (%)	I	NE
ISSR1	BDB$(CA)_6$	10	7		70		
ISSR6	$(CT)_8$RC	18	16		90%		
ISSR11	CCA$(GTG)_4$	12	6		50		
ISSR14	GGA$(GTG)_4$	10	4		60		
ISSR19	$(TC)_8$G	7	6		85.7		
ISSR20	$(GA)_8$YT	10	7		70		
ISSR28	$(AG)_8$G	9	5		55.6		
ISSR29	$(GA)_8$C	10	8		80		
ISSR33	T$(GA)_8$	15	-		-		
ISSR34	$(GA)_8$C	9	7		77.8		
Total		110		200-1600bp	71.82	0.3775	1.4037

Note: B= C, G, T; D= A, G, T; R= A, G; Y= C, T. - : missing one individual's bands replaced by DNA standard molecular weight marker; I: Shannon's Information index; Ne: effective number of alleles; PPB: the percentage of polymorphic band

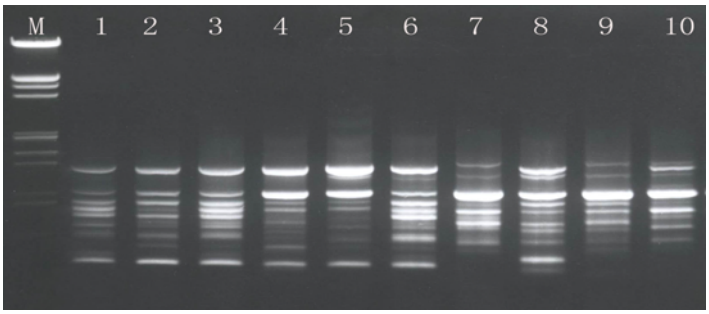

Fig.1. PCR- amplified patterns by Primer ISSR6 in ten *Rehmannia glutinosa Libosch .f. hueichingensis*(*Chao et Schih*) Hsiao.

M: λ DNA *Eco*RI + *Hin*dIII fragment sizes (bp) : 21227, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125 from the top to the bottom. 1. Zupei 85.5; 2. Datian 85.5; 3 Zupei 9302; 4. Datian9302; 5.Dihuang9104; 6.Jinzhuangyuan; 7. wild dihuang; 8. Beijing No.1; 9. Dahongpao ; 10. Jinbai

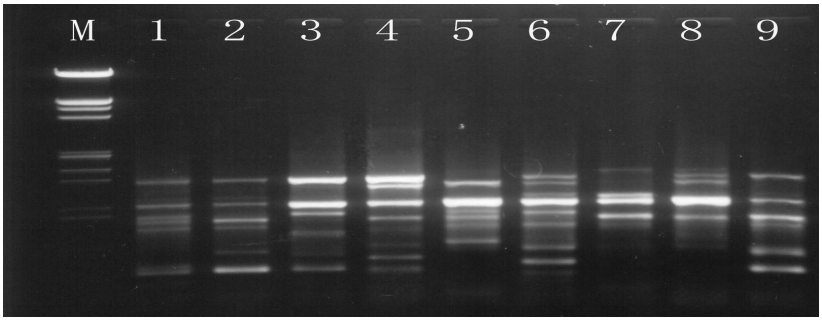

Figure 2. ISSR fingerprints of nine cultivars (lines) of *Rehmannia glutinosa* in Huai zone generated by Primer ISSR11.

1 - Datian85.5; 2 - Zupei9302; 3 - Datian9302; 4 - Jinzhuangyuan; 5 - 9104; 6 - Wild

Dihuang; 7 - No.1Beijing; 8 - Dahongpao; 9 - Jinbaidihuang; M: λ DNA *Eco*RI + *Hin*dIII

fragment sizes (bp) : 21227, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125 from the top to the bottom.

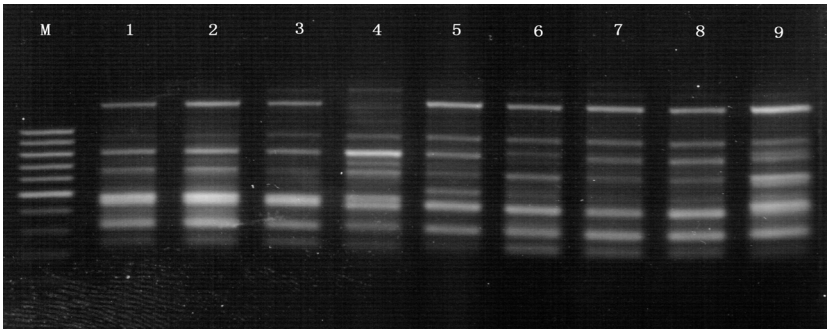

Figure 3. ISSR fingerprints of 9 cultivars (lines) of *Rehmannia glutinosa* in Huai zone generated by Primer ISSR33.

1 - Datian85.5; 2 - Zupei85.5; 3 - Datian9302; 4 - Jinzhuangyuan; 5 - 9104; 6 - Wild Dihuang;

7 - No.1 Beijing 8 - Dahongpao 9 - Jinbaidihuang M: 100bp ladder, fragment sizes (bp) : 1000, 900, 800, 700, 600, 500, 400, 300, 200,100 from the top to the bottom.

In addition, Jaccard's similiarity matrix for ten cultivars (lines) of *Rehmannia glutinosa* in Huai zone was obtained based on 110 bands generated by ISSR-PCR.(Table 2).It was seen from Table 2 that the GS between cultivar Datian85.5 and Zupei 85.5 was the biggest,0.979; the GS between Wild Dihuang and cultivar Datian9302 was the smallest,0.557; Mean GS was 0.665.

Table 2. Jaccard's similiarity matrix for ten cultivars (lines) of *Rehmannia glutinosa* in Huai zone

Cultivar or line	Zupei 85.5	Datian 85.5	Zupei 9302	Datian 9302	Jinzhuangyuan	Dihuan 9104	WildDihuang	Beijing No.1	Dahongpao	Jinbai
Zupei 85.5	1.000									
Datian 85.5	0.979	1.000								
Zupei 9302	0.900	0.918	1.000							
Datian 9302	0.649	0.661	0.638	1.000						
Jinzhuangyuan	0.650	0.661	0.695	0.678	1.000					
Dihuang 9104	0.629	0.613	0.619	0.603	0.606	1.000				
WildDihuan	0.610	0.593	0.627	0.557	0.613	0.619	1.000			
Beijing No.1g	0.621	0.603	0.610	0.567	0.571	0.772	0.696	1.000		
Dahongpao	0.607	0.589	0.596	0.607	0.583	0.732	0.685	0.915	1.000	
Jinbai	0.746	0.729	0.705	0.585	0.588	0.642	0.651	0.717	0.678	1.000

2.2 Establishment of ISSR fingerprint: DNA fingerprints were established after the DNAs of ten materials as templates were amplified by PCR technique with the ten ISSR primers selected （Fig.2-3）. Furthermore, a DNA fingerprint was developed with a single primer, ISSR6, in which each of ten individuals tested had its unique fingerprint pattern and was distinguished from each other.(Fig.1).This DNA fingerprint of ten materials generated with this primer provided scientific basis for the identifications of different cultivars and their young plants.

2.3 Cluster analysis

Dendrogram for 10 cultivars (lines) of *R. glutinosa* based on ISSR markers shown as in Fig.4. In this dendrogram, they could be divided into two groups : Group1 contained six individuals such as Zupei 85.5, Datian 85.5, Zupei 9302, Jinbai, Jinzhuangyuan and Datian9302;

Group2 consisted of four ones such as Beijing No.1, Dahongpao, Dihuang9104and wild dihuang.

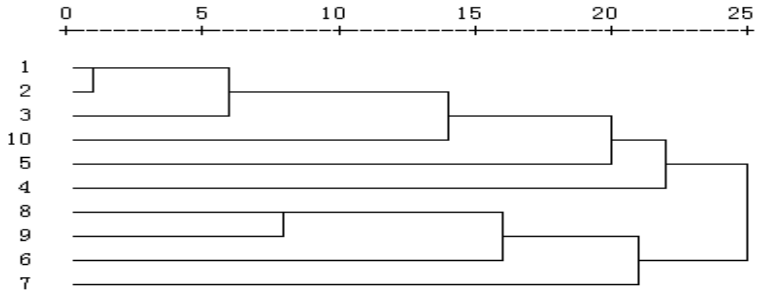

Figure 4. Dendrogram for 10 cultivars (lines) of *R. glutinosa* based on ISSR markers
No.1-10 on the left were the same as that of ten materials in 1.2 Materials

2.4 Principal coordinate analysis (PCA)

Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO)showed that KMO of ISSR markers was 0.806 , bigger than 0.5 and suitable for Principal coordinate analysis (PCA). The results of PCA supported the above cluster analysis of ten materials (Fig.5).

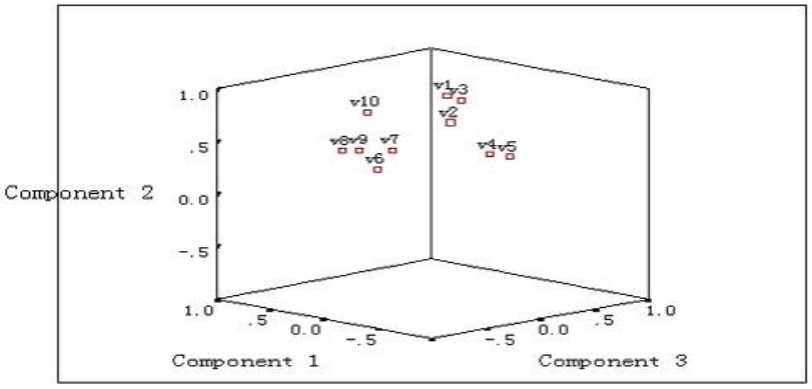

Figure 5. Principal coordinate analysis using ISSR markers and Equamax （Component plot in rotated space）

v1 - Zupei85.5; v2 - Datian 85.5; v3 - Zupei 9302; v4 - Datian 9302; v5 - Jinzhuangyuan;

v6 - 9104; v7 - Wild dihuang; v8 - No.1Beijing ;v9- Dahongpao; v10- Jinbaidihuang.

CONCLUSIONS

3.1 In dendrogram of cluster analysis, the result that Beijing No.1 and Dahongpao were not divided into the same group with 85 types but into the same group with each other is consistent with that of RAPD analysis reported before (Masumi, et al., 2002).This indicated that ISSR technique is consistent with RAPD one.

3.2 ISSR technique is less sensitive than RAPD one to PCR amplification, but, like RAPD technique, based on PCR, its stability is influenced by impact factors on PCR reaction such as Mg^{2+} concentration, annealing temperature, Taq polymerase dosage, primer concentration, dNTP concentration and so forth. Thus, it is necessary to optimize its PCR reaction system (Chen, et al.,2002) and to use constant PCR reaction conditions during the normal experiments. In addition, the slide and unequal crossing over of the target sequences in genomic DNA, which can anneal with ISSR primers containing repeat sequences, make them repeated different times among different varieties or individuals and are prone to bring about the changes of primer-annealing sites and the fragments between any two annealing sites (Qian, et al., 2001).However , the slide also led the indistinct background and pulling bands on PCR products' electrophoretic patterns to occur easily. The addition of two percent of formamide deionized to PCR system could reduce the indistinct to some extent so that the bands were more distinct. This was similar to the previous report (Zhou, et al., 2004; Qin,et al., 2002).

3.3 ISSR marker technique could detect more DNA polymorphisms. and Furthermore, a set of ISSR primers can be commonly used in the studies of many different plant species, so their utilization ratios can be improved, they can be broadly applied in genetics. Charters et al. (1996) generated fifty-six polymorphic bands with two ISSR primers that could identify twelve cultivars from each other. Huang et al. (2000) amplified 2071 fragments from forty sweetpotato cultivars with fifteen ISSR primers, 62.2 percent of which were polymorphic. In the present study, polymorphism was revealed based on ISSR markers. The ten ISSR primers generated one hundred and ten fragments among ten materials, the percentage of polymorphic bands was 71.82%.one ISSR primer (ISSR6) could identify these ten cultivars (lines) of *Rehmannia glutinosa* from each other.

To sum up, ISSR markers is a practical and promising molecular marker tool for the

identification and assessment of genetic diversity of *Rehmannia glutinosa*. The cultivar-specific fragments generated by these markers will significantly help distinguish the cultivars or lines of *Rehmannia glutinosa*, which are the same variety with different names or different varieties with the same name, from each other; select proper crossing parents in *Rehmannia glutinosa* crossing breeding; put an end to the harm to users by false and forged varieties of *Rehmannia glutinosa*; improve the protection of intellectual property rights and make the most use of important germplasm resources of *Rehmannia glutinosa*.

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