Genetic diversity of different cultivars in *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao☆

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Abstract

Two types of molecular markers, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR), were assayed to determine the genetic diversity of ten experimental materials in *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao, including eight cultivars and two virus-free lines micropropagated by tip tissue culture from Wenxian county. A high level of polymorphism was found with both RAPD and ISSR markers, and Shannon's Information index (I) were 0.3135 and 0.3577 for RAPD and ISSR markers, respectively. In RAPD analysis, 109 out of 177 bands (61.58%) were polymorphic effective number of alleles (Ne) was 1.3641. The RAPD-based genetic similarity (RAPD-GS) ranged from 0.63 to 0.93, with the mean of 0.7545. In ISSR analysis, a total of 110 alleles were detected, among which 79 alleles (71.82%) were polymorphic effective number of alleles (Ne) was 1.4037. The ISSR-derived genetic similarity (ISSR-GS) ranged from 0.55 to 0.98, with the mean of 0.659. ISSR was better than RAPD to detect genetic diversity among these cultivars. A significant correlation (r = 0.648) was found between both sets of genetic similarity data, suggesting that both sets of markers revealed related estimates of genetic relationships. Cluster analysis indicated that all ten cultivars (lines) could be distinguished by both RAPD and ISSR markers. PCA analysis was employed to evaluate the resolving power of the markers to differentiate among them. This laid the foundation of the identification of *Rehmannia* cultivars and the efficient use of its germplasm resources. [Life Science Journal. 2007; 4(2): 69 – 75] (ISSN: 1097 – 8135).

Keywords: *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao; RAPD; ISSR; identification; genetic diversity

1 Introduction

Recently random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers among dozens of DNA markers have made success in the research on plant genetic diversity as reported by Chen, *et al*[1]. RAPD analysis was established as reported by Williams and Welsh, *et al* [2–3], amplifying organisms' genomic DNA with random primers containing 10 oligonucleotides; ISSR marker is an another kind of DNA markers as reported by Zietkiewicz, *et al*[4], with reliability and advantages of SSR. In herbs, RAPD marker has been used for the construction of fingerprinting, the authentication of official and unofficial plants, kin identification, the assessment of germplasm genetic diversity and the truth and fake of medical materials in many medicinal plants including Panax ginseng as reported by Ma, *et al*[5], *Panax quinquefolium, Angelica sinensis, Dendrobium nobile, Magnolia officinalis, Glycyrrhiza uralensis, Atractylodes chinensis* as reported by Wu, *et al*[6], *Codonopsis pilosula* as reported by Fu, *et al*[7], *Rheum officinale* as reported by Yang, *et al*[8], *Rabdosia amethystoides* as reported by Fang, *et al*[9], *Rehmannia glutinosa* as reported by Chen, *et al*[10], Choi[11] and Hatano[12], *Lilium brownii var.viridulum* as reported

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by Choi, *et al*[13], *Lilium species*, etc; ISSR marker has been used in the authentication of *Lilium species* as reported by Masumi[14] and *Schisandra* cultivars as reported by Sun, *et al*[15]. *Rehmannia glutinosa* Libosh is an herb of the Scrophulariaceae family, a perennial herb with reddish-violet flowers native to China, Japan and Korea. Its common name is Chinese Foxglove. Rehmannia's root is used medicinally in Oriental medicine to replenish vitality, to strengthen the liver, kidney and heart, to lower glucose levels and helps to reduce blood pressure while increasing circulation to the brain and to strengthen the bones and tissue as well as enhance fertility as reported by Zhou, *et al*[16] and for treatment of a cultivar of ailments like diabetes, constipation, anemia, urinary tract problems, dizziness, and regulation of menstrual flow. Chinese doctors have used it with licorice for the treatment of hepatitis. Its year demand is more than 15,000 tons as reported by Wen, *et al*[17] around world. *Rehmannia glutinosa* Libosh can be planted in all parts throughout China, but Henan provincial *Rehmannia glutinosa* Libosh, called *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao and one of world-famous "Four famous huai herbs", has better quality and curative effects than others. Every year a lot of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao is sold in Chinese market and exported into Southeast, Korea and Japan. Therefore, domestic and overseas markets have made higher demands than ever for the quality, purity and special producing area of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao. On the other hand, current *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao cultivars originate from different places of production, whose good and bad cultivars are mixed together; Furthermore, its classification and genetic analysis are still conducted by means of traditional morphologic traits and kin. In this paper, we analyzed the Genetic Diversity in *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao cultivars from Wenxian County using RAPD and ISSR markers to exactly identify and use them, 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.

2 Materials and Methods

2.1 Plant materials

A total of 10 cultivars (lines) of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao, including 8 cultivars growing in field and 2 virus-free lines, micropropagated by tip tissue culture and cultured in plant tissue culture room, were used in this study. They were provided by Wenxian Institute of Agricultural Sciences, Henan, China, identified by its senior agronomist Wang Qian-ju, and named as Zupei85.5; Datian85.5; Zupei9302; Datian9302; 9104; Jinzhuangyuan; Wild Dihuang; Beijing No.1; Dahongpiao and Jinbaidihuang.

2.2 Total DNA extraction

Genomic DNA was extracted from a bulk sampling of a minimum of ten individuals for each cultivar according to Chen[10] and by CTAB method as reported by Wang, *et al*[18]. DNA concentration was determined by Beckman Du530 DNA/Protein Analyzer. DNA molecular weight was analyzed by electrophoresis on 0.6% (W/V) agarose gel by means of DNA molecular weight standard, λDNA/*Hind* III Finally, the DNAs were stored at –20ºC.

2.3 RAPD and ISSR analysis

A total of 80 RAPD primers initially used in RAPD analysis, 17 of which were screened for further analysis (Table 1). A total of 44 ISSR primers initially used in ISSR analysis, 10 of which were screened for further analysis (Table 1) as reported by Zhou, *et al*[19], The PCR reaction mixture consisted of 60 ng genomic DNA, 1×PCR buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 0.1% Trion X-100, pH 9.0), 3.0 mmol/L $MgCl_2$, 0.4 mmol/L of each dNTP, 10.4 pmol/L 10-mer oligonucleotide primer (for RAPD analysis) or 0.4 μmol/L primer (for ISSR analysis) and 1 – 1.5 U Taq polymerase in a 30 μl (for RAPD analysis) or 25 μl (for ISSR analysis). The amplification protocol was 94ºC for 7 minutes to pre-denature, followed by 45 cycles of 94ºC for 60 seconds to denaturation, 36ºC (for RAPD analysis) or 50ºC or 53ºC or 55ºC (for ISSR analysis) for 90 seconds to annealing, 72ºC for 120 seconds to extension, with a finial extension at 72ºC for 7 minutes. Amplification products were fractionated on 1.4% (w/v) agarose gel plus 0.5 μg/ml EB.

2.4 Data analysis

RAPD and ISSR data were scored for presence (1), absence (0) or as a missing observation (9), and each band was regarded as a locus. Two matrices, one for each marker, were generated. The genetic similarities (GS) were calculated according to the formula: GS = 2Nij/(Ni+Nj), where Nij is the number of bands present in both genotypes i and j, Ni is the number of bands present in genotypes i, and Nj is the number of bands present in genotype j. Based on the similarity matrix, a dendrogram showing

Table 1. List of RAPD and ISSR primers

Primer	Sequence	Primer	Sequence	Primer	Sequence
RAPD		RAPD		ISSR	
MQ8082	GGGAGGCAAA	MQ8273	GGCTGCAGAA	ISSR1	$BDB(CA)_6$
MQ8085	AGTGCACACC	MQ8285	AGGGGTCTTG	ISSR6	$(CT)_8RC$
MQ8090	CCAGGCTGAC	MQ8288	GTGACGTAGG	ISSR11	$CCA(GTG)_4$
MQ8093	GAGCACTGCT	MQ8290	GTGATCGCAG	ISSR14	$GGA(GTG)_4$
MQ8096	GTCTGTGCGG	MQ8292	TCGGCGATAG	ISSR19	$(TC)_8G$
MQ8098	GTGTGCTGTG	MQ8295	TTCCGAACCC	ISSR20	$(GA)_8YT$
MQ8264	GGTGATCAGG	MQ8298	AGGTGACCGT	ISSR28	$(AG)_8G$
MQ8265	CCGAATTCCC			ISSR29	$(GA)_8C$
MQ8267	CCGATATCCC			ISSR33	$T(GA)_8$
MQ8269	CCAAGCTTCC			ISSR34	$(GA)_8C$

S = G,C; W = A,T; B = C, G, T; D = A, G, T; R = A, G; Y = C, T; H = A, C, T; V = A, C, G

the genetic relationships between genotypes, was constructed using Within-group linkage method through the software SPSS version11.0. POPGENE32 software was used to compute the number of effective loci, the percentage of polymorphic loci, Shannon's Information index (I) and effective number of alleles (Ne) for RAPD and ISSR markers. Finally, Bivariate test between genetic similarity matrices by RAPD and ISSR marker was done to show their correlation.

3 Results and Analysis

3.1 Polymorphism of PCR-amplified products

RAPD polymorphism. Because RAPD PCR is sensitive to reaction parameters, 80 primers were initially screened against one cultivar under the optimized condition. 17 out of 80 primers generated strong amplification products. A total of 177 bands from 200 to 2,500 bp were scored. The number of polymorphic loci was 109. The percentage of polymorphic loci was 61.58%, Shannon's Information index (I) was 0.3135, and effective number of alleles (Ne) was 1.3641. Figure 1 shows an example of the polymorphic bands of primer MQ8085 in nine cultivars (lines) in *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao.

ISSR polymorphism. A total of 44 ISSR primers were screened as described in 2.3. After optimizing the PCR reaction condition, 10 primers which produced distinct and reproducible fragments were selected for future analysis. A total of 110 bands ranging from 200 to 1,600 bp were scored. The number of polymorphic loci was 79. The percentage of polymorphic loci was 71.58%, Shannon's Information index (I) was 0.3577, and effective number of alleles (Ne) was 1.4037. Figure 2 shows an example of the polymorphic bands of primer ISSR6 in ten cultivars (lines) in *Rehmannia glutinosa Libosch .f. hueichingensis*(*Chao et Schih*) Hsiao

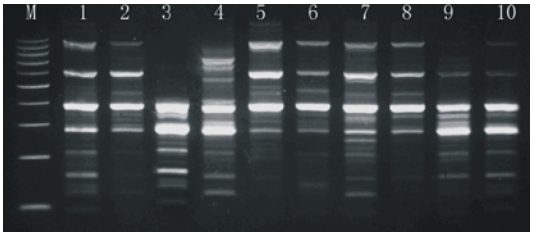

Figure 1. PCR amplified patterns by Primer MQ8085 in ten cultivars (lines) in *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao. M: 200 bp ladder; Zupei 85.5; Lane 2: Datian 85.5; Lane 3: Zupei 9302; Lane 4: Datian9302; Lane 5: Dihuang9104; Lane 6: Jinzhuangyuan; Lane 7: wild dihuang; Lane 8: Beijing No.1; Lane 9: Dahongpao; Lane 10: Jinbai.

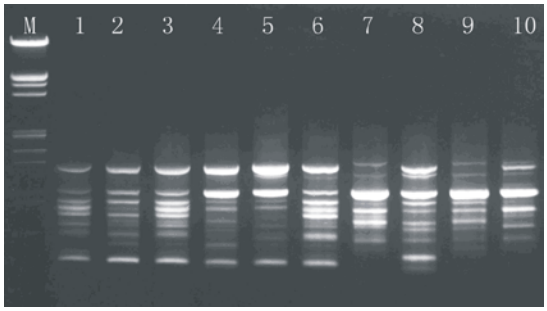

Figure 2. PCR-amplified patterns by Primer ISSR6 in ten *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao. M: λDNA/*Hind* III; Lane 1: Zupei 85.5; Lane 2: Datian 85.5; Lane 3: Zupei 9302; Lane 4: Datian9302; Lane 5: Dihuang9104; Lane 6: Jinzhuangyuan; Lane 7: wild dihuang; Lane 8: Beijing No.1; Lane 9: Dahongpao; Lane 10: Jinbai.

3.2 Genetic relationship among samples tested

Genetic similarity analysis. Genetic similarity matrices based on Jaccard' coefficient of genetic similarity were obtained for RAPD and ISSR markers. Comparison of the GS on RAPD and ISSR markers was shown in Table 2. It indicated that the GS range of 0.55 to 0.98 for ISSR markers was slightly bigger than that of 0.63 to 0.93 for RAPD markers, but mean GS for ISSR markers was a little smaller than that for RAPD markers. Therefore, by contrast, ISSR markers and RAPD markers could show similar genetic differences between the cultivars or lines.

3.3 Cluster analysis

Based on the data of RAPD and ISSR PCR-amplified products, two dendrograms for 10 cultivars (or lines) of *R. glutinosa Libosh* were formed using Within-group linkage and Hierarchical cluster analysis (Figure 3 and Figure 4). These two dendrograms were very similar to each other

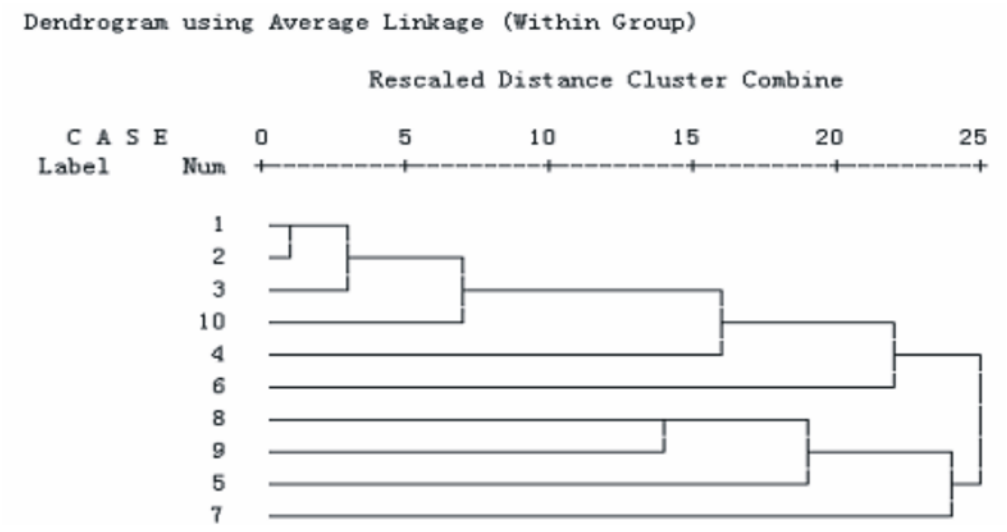

Figure 3. Dendrogram for 10 cultivars (lines) of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao based on RAPD markers.
1. Zupei 85.5; 2. Datian 85.5; 3. Zupei 9302; 4. Datian9302; 5.Dihuang9104; 6.Jinzhuangyuan; 7. Wild dihuang; 8. Beijing No.1; 9. Dahongpao; 10. Jinbai.

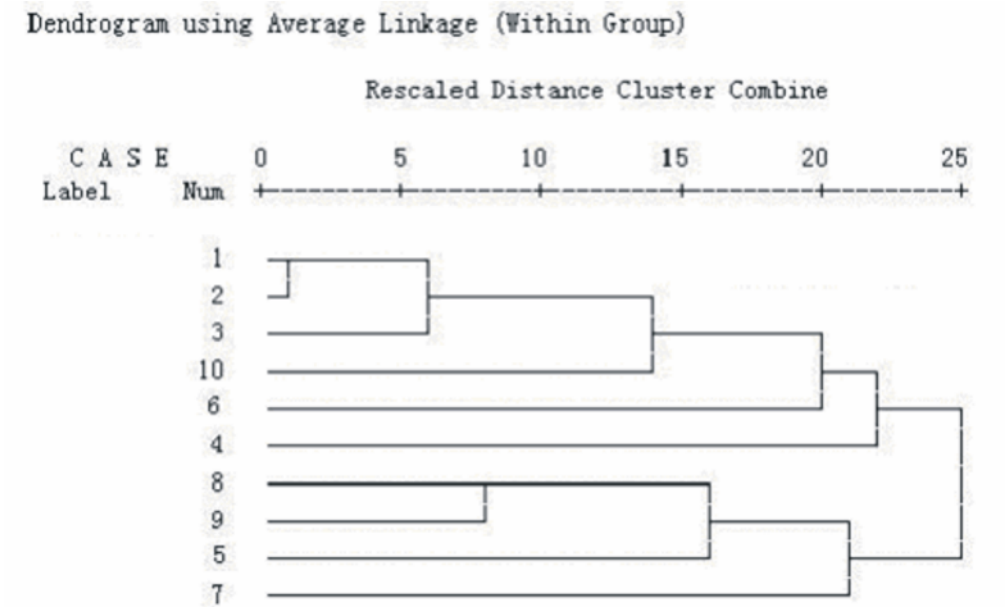

Figure 4. Dendrogram for 10 cultivars (lines) of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao based on ISSR markers
1. Zupei 85.5; 2. Datian 85.5; 3. Zupei 9302; 4. Datian9302; 5. Dihuang9104; 6. Jinzhuangyuan; 7. Wild dihuang; 8. Beijing No.1; 9. Dahongpao; 10. Jinbai.

but not the same. In dendrograms, these 10 cultivars or lines could be divided into two groups: one group containing six individuals such as Zupei 85.5, Datian 85.5, Zupei 9302, Datian9302, Jinzhuangyuan and Jinbai; the other composed of 4 such as Beijing No.1, Dahongpao, Dihuang9104 and wild dihuang. What is more, they could be distinguished from each other. It was showed that genetic variations and similarities existed between cultivars or lines. However, RAPD and ISSR markers showed some difference in the grouping of two cultivars – Datian9302 and Jinzhuangyuan.

Table 2. Comparison of the genetic similarities (GS) on RAPD and ISSR markers

Marker system	Total number	Maximum	Minimum	Mean	S.D
RAPD	45	0.93	0.63	0.7545	7.51E – 02
ISSR	45	0.98	0.55	0.659	9.906E – 02

S.D: Std. Deviation

3.4 Correlation analysis of RAPD and ISSR markers

To test the correlation of RAPD and ISSR markers' assessments of genetic diversity of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao, Bivariate test between genetic similarity matrices based on RAPD and ISSR markers was done by means of SPPS 10.0 software. The test results were shown in Table 3. It demonstrated that Pearson correlation coefficient (*r*) was 0.648 and correlation was significant at the 0.01 level (2-tailed).

Table 3. Bivariate test between genetic similarity matrices based on RAPD and ISSR markers

Marker system	ISSR	RAPD	*n*
ISSR	1.000	.648**	45
RAPD	.648**	1.000	45

n refers to the total number of genetic similarities per matrix. **: $P = 0.000$. Correlation is significant at the 0.01 level (2-tailed).

4 Conclusions

4.1 The evaluation of genetic diversity

In this study, RAPD and ISSR markers could generate their own polymorphic band patterns, identify different cultivars and assess genetic diversity of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao. For RAPD marker, the number of effective loci, the percentage of polymorphic loci, Shannon's Information index (I) and effective number of alleles (Ne) is 109, 61.58%, 0.3135, 1.3641; For ISSR marker, the number of effective loci, the percentage of polymorphic loci, Shannon's Information index (I) and effective number of alleles (Ne) is 79, 71.82%, 0.3577 and 1.4037. The results indicated that their polymorphism and test ability were different from each other, although RAPD and ISSR markers could produce their own specific polymorphic bands; that the genetic variations existed between the different cultivars or lines, which provided a basis for their identification and assessment of genetic diversity ;and that the percentage of polymorphic loci, Shannon's Information index (I) and effective number of alleles (Ne) of ISSR markers were superior to that of RAPD markers. The reason for their differences was maybe that 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 cultivars or individuals and are prone to bring about the changes of primer-annealing sites and the fragments between any two annealing sites as reported by Bruford, *et al*[20]. These results were in accordance with that as reported by Charter[21], Huang[22] and Qian[23]. 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. In addition, in the present research, one ISSR primer (ISSR6) and two RAPD primers (MQ8050 and MQ8090) were selected, which could identify these ten cultivars (lines) of *Rehmannia glutinosa Libosh .f. hueichingensis* (*Chao et Schih*) Hsiao from each other.

4.2 The evaluation of genetic similarity

First, mean GS value was 0.7545 for RAPD markers, whereas it was 0.659 for ISSR markers. Therefore, the former was bigger than the latter, comparatively speaking, ISSR markers could test more genetic differences than RAPD ones among the individuals mentioned above. It was seen from cluster analysis that the two dendrograms for the 10 cultivars (lines) based on RAPD and ISSR markers were not completely identical but similar, and that these ten ones could be divided into two groups: one group containing six individuals such as Zupei 85.5, Datian 85.5, Zupei 9302, Datian9302, Jinzhuangyuan and Jinbai; the other composed of 4 such as Beijing No.1, Dahongpao, Dihuang9104 and wild dihuang. Nevertheless, the GS of zupei9302 and Datian9302 and that of Jinbai and Jinzhuangyuan had difference in RAPD and ISSR

dendrograms. In RAPD dendrogram, the former is bigger than the latter. This is right, but in ISSR one, the former is smaller than the latter, which is wrong. That's because zupei9302 is the clone of Datian9302 while Jinbai is the cross offspring of Jinzhuangyuan X Yinzhuangyuan. The possible reasons are the following: a. the number (10) of the primers used in ISSR analysis is less, so fewer PCR-amplified fragments were generated; b. In the experiment, zupei9302 and Datian9302 often as missing value were removed to make room for DNA molecular weight standard; c. Test error; and so on. Maybe the result can remind people of something important.

Second, Pearson Correlation coincidence (r) between genetic similarity matrices based on RAPD and ISSR markers was 0.648, so correlation is significant at the 0.01 level (2-tailed).

Third, our result that Beijing No.1 and Dahongpao were not divided into the same group with 85 types is consistent with that of RAPD analysis reported before as reported by Chen, *et al*[10]; one of our conclusions that HuaiDihuang lines micro-propagated by tip tissue culture could be distinguished from was identical to that of RAPD analysis as stated by Choi[11,13] and Hatano[12]. However, these molecular markers' results showed some differences. Maybe these difference were made by the following reasons: a. ISSR and RAPD techniques tested different gene loci, respectively, because RAPD technique tested random sequences, and ISSR technique did the inter fragments between simple sequence repeats; b. Primer differences used. RAPD primers are randomly combined 10 mers while ISSR primers are 15 – 24 mers consisting of SSRs and anchored bases. Because different primers can test different polymorphism, and the number of polymorphic fragments can increase with the increase of primer amount so that genetic distances can be changed.

4.3 The reliability and reproducibility of RAPD and ISSR markers

The reliability and reproducibility of experimental reaction system is very important for the study of genetic diversity. Although the primers and their annealing temperature are longer and higher for ISSR marker than that for RAPD marker so that ISSR marker is less sensitive to PCR reaction conditions than RAPD marker, which like RAPD marker, based on PCR, 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, there was need to optimize their PCR reaction systems before the beginning of their normal experiments, and to use constant PCR reaction conditions during the normal experiments. Two ways to increase the reliability and reproducibility of RAPD and ISSR markers were used in the present study. a. Some good primers were screened from initially selected primers, which could generate distinct, reproducible, instense and polymorphic DNA fragments by PCR amplification. A total of 80 RAPD primers initially used in RAPD analysis, 17 of which were screened for further analysis (Table 1). A total of 44 ISSR primers initially used in ISSR analysis, 10 of which were screened for further analysis (Table 1) as reported by Zhou *et al*[19]. b. The PCR reaction systems for these markers were optimized in advance. The optimization results[15] revealed that their reliability and reproducibility were greatly affected by the factors mentioned above, especially those including Mg^{2+} concentration, annealing temperature, Taq polymerase dosage and primer concentration. Nevertheless, once PCR reaction conditions were strictly determined, and the loaded volume of PCR products was not changed, as long as only distinct bands were scored after electrophoresis, these two markers' reliability and reproducibility were quite high. This result was of the same as reported by Mattioni, *et al*[24]. Based on these two ways mentioned above, the reliability and reproducibility of RAPD and ISSR markers could amount up to 91.5% and 97.8%, respectively. However, it is believed that one of the most efficient ways to improve their reliability and reproducibility is the conversion of RAPD and ISSR markers to SCAR or STS markers by classical PCR. Therefore, cloning and sequencing of constant amplified fragments using RAPD and ISSR markers will be needed in the near future.

To sum up, RAPD and ISSR markers are practical and promising molecular marker tools for the identification and assessment of genetic diversity of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao. The cultivar-specific fragments generated by these two markers will significantly help distinguish the cultivars of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao, which are the same cultivar with different names or different cultivars with the same name, from each other; select proper crossing parents in *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao crossing breeding; put an end to the harm to users by false and forged cultivars of *Rehmannia glutinosa Libosh*; improve the protection of intellectual property rights and make the most use of important germplasm resources of *Rehmannia glutinosa Libosch .f. hueichingensis* (*Chao et Schih*) Hsiao.

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