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Full Length Research Paper

DNA isolation, optimization of ISSR-PCR system and primers screening of *Scutellaria baicalensis*

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To estimate genetic diversity and to authenticate the medicinal materials of *Scutellaria baicalensis* Georgi, the present work including DNA isolation, optimization of PCR assay of inter-simple sequence repeat (ISSR) and primers screening were investigated. Among three DNA isolation methods, improved CTAB, improved SDS and isolation kit, the improved CTAB was the best if restricted by funding. Based on selection design and protocols of reports, the optimal ISSR-PCR action was carried out in a volume of 20 µl containing 20 ng of DNA template, 1.0 U of *Taq* DNA polymerase, 1 × buffer, 200 µM dNTPs, 0.2 µM of primer and 2.25 µM Mg^{2+}. According to this PCR system, fifteen out of one hundred primers were chosen for their high clarity and repetition.

Key words: DNA isolation, ISSR, optimal PCR condition, primer screening, *Scutellaria baicalensis.*

INTRODUCTION

Radix Scutellariae, dry root of *Scutellaria baicalensis* Georgi, is a well-known Traditional Chinese Medicine widely used in China and other Asian countries. It has been officially listed in the China Pharmacopoeia for treating various ailments such as fever, ulcer, bronchitis, hepatitis, tumor and inflammatory disease (National Committee of China Pharmacopoeia, 2005). However, with the quick growth of commercial demands for this medicine in recent years, excessive exploitation has shrunk the natural resources of *S. baicalensis* to a narrow distribution and its survival has been seriously threatened based on our field survey (Su et al., 2008). Due to over digging natural resources turn out to be rather sparse and fragmented, cultivated populations become the main source to produce this drug. To date, previous studies mainly focus on the resources distribution, components extraction and pharmacological properties (Hong et al., 1983; Liu et al., 2009) and few efforts have been made to its genetic analysis of wild and cultivated populations by only using randomly amplified polymorphic DNA (RAPD) markers (Feng et al., 2002; Shao et al., 2006; Su et al., 2008). In order to formulate effective conservation strategies of *S. baicalensis*, assessment of its genetic diversity and population structure is urgent.

Among various molecular markers, inter-simple sequence repeats (ISSRs) use repeat-anchored primers to amplify DNA sequences between two inverted SSRs (Zietkiewicz et al., 1994). Because of high annealing temperature and longer sequence of ISSR primers, they can yield reliable and reproducible bands, and the cost of the analysis is relatively lower than that of some other markers, that is, AFLP (amplified fragment length polymorphism). Therefore, ISSRs have established wide applications in genetic diversity studies in a wide range of medicinal plant species (Yao et al., 2008).

In present study we aimed to: (1) estimate optimal DNA isolation method from *S. baicalensis*; (2) form an efficient protocol of PCR condition and (3) screen one hundred primers and select those with high clarity and repetition.

MATERIALS AND METHODS

Plant material

Plant samples were collected from various sites including Juxian,

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Table 1. Selection design for optimization of ISSR-PCR reaction condition.

Code	Taq DNA polymerase (U/20µl)	Mg^{2+} (mM)	dNTP (mM)	Primer (µM)
1	1.0	2.0	0.25	0.10
2	1.0	2.25	0.20	0.20
3	1.0	2.5	0.15	0.30
4	1.0	3.0	0.10	0.40
5	1.25	2.0	0.20	0.40
6	1.25	2.25	0.25	0.30
7	1.25	2.5	0.10	0.20
8	1.25	3.0	0.15	0.10
9	1.5	2.0	0.25	0.20
10	1.5	2.25	0.20	0.10
11	1.5	2.5	0.15	0.40
12	1.5	3.0	0.10	0.30
13	2.0	2.0	0.15	0.30
14	2.0	2.25	0.10	0.40
15	2.0	2.5	0.25	0.10
16	2.0	3.0	0.20	0.20

Jiaonan, Rizhao, Shandong province, Chengde of Hebei, Yonghe of Shanxi and Chifeng of Inner Mongolia. These provinces are both main distribution regions and cultivated drug producing areas. Young leaf tissues (wild and cultivated individuals) were stored in zip-lock bags with silica gel and transported back to laboratory for DNA extraction.

DNA isolation and detection

The dried leaf tissues of *S. baicalensis* were used for DNA isolation and their total DNA were extracted to obtain optimal protocol by evaluating three isolation methods, improved CTAB (cetyl trimethyl ammonium bromide), improved SDS (Sodium dodecyl sulfate) and isolation kit. The improved CTAB was followed by Guo et al. (2007). The improved SDS was consulting with Su et al. (2008) and the third was according to “Instruction for User” provided by isolation kit (manufactured by Biocolor Bioscience and Technology Company, Shanghai).

DNA quantifications were performed by UV-spectrophotometer at 260 and 280 nm and the purity was then determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (OD_{260}/OD_{280}). DNA concentration and purity was also determined by electrophoresis on 1.0% agarose compared with Lambda DNA digested by Hind β/EcoR ā as marker. The re-suspended DNA was then diluted in sterile distilled water to the concentration of 20 ng/µL for use.

Optimization of ISSR-PCR system

Based on our ISSR analysis of *Glycyrrhiza uralensis* Fisch. (Yao et al., 2008), four factors including *Taq* DNA polymerase, dNTP, primer concentration and Mg^{2+} were investigated with four treatments in each factor (Table 1). The PCR was performed in a PTC100 thermocycler (MJ Research Inc, Watertown, Mass.). A denaturation period of 5 min at 94^0C was followed by 40 cycles of 1 min at 94^0C, 60 s at different annealing temperature for each primer (Table 2) and 2 min at 72^0C and then 10 min at 72^0C for final extension. An 8.5 µL aliquot of the amplification products was separated by electrophoresis in 2.0% agarose gel in 0.5 × TBE buffer and the DNA fingerprints were photographed by an automatically imaging system.

Primers screening

For the optimization of ISSR – PCR system using the DNA extracted from *S. baicalensis*, one-hundred ISSR primers from University of British Columbia, Canada (UBC set No.9) were used for amplification to standardize the PCR conditions based on the high clarity and repetition. For each primer four annealing temperatures (51, 52, 53 and 54^0C) were established due to their differences among them.

RESULTS

DNA isolation and detection

White deposit pellet could be obtained in most samples by using both CTAB and isolation kit, while most of samples were brown in SDS method. Nucleic acid detection results showed that all ratios of OD_{260}/OD_{280} ranged from 1.7 to 2.0 when detecting purity of DNA extracted by three methods. Through electrophoresis neither lagging and unbinding band nor impurity was found in each lane (Figure 1).

ISSR-PCR system

Among sixteen groups (Table 1), group 2 exhibited most clarity and repetition. The eighteen DNA samples extracted by three methods (Figure 1) were amplified by primer 836 (Table 2) and their electrophoresis result was displayed in (Figure 2.)

Primers screening

Fifteen primers out of one hundred were selected for their

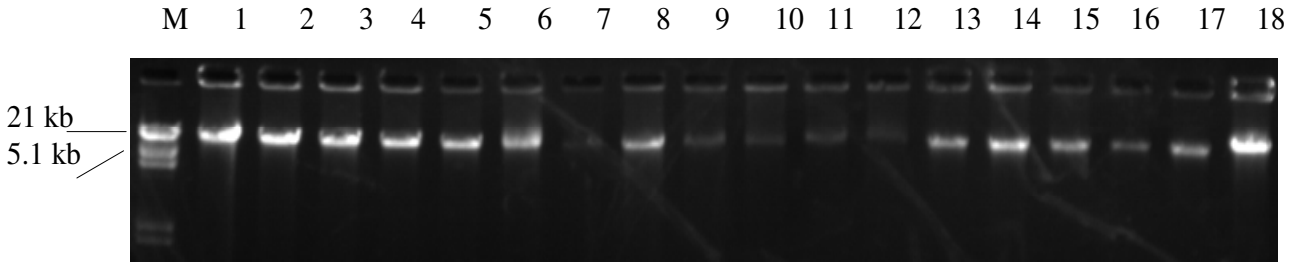

Figure 1. Electrophoresis result of genomic DNA extracted from *S. baicalensis* by using three isolation methods. M represented lambda DNA Hind β/EcoR ¯marker; Lane 1 – 6: DNA extracted by improved CTAB; Lane 7 – 12: DNA extracted by improved SDS; Lane 13 – 18: DNA extracted by isolation kit.

Table 2. Code, sequence and annealing temperature of selected fifteen ISSR primers.

Primer code	Sequence (5'→ 3')	Annealing temperature (°C)	Total bands	Polymorphic bands	Percentage of polymorphism (%)
807	AGA GAG AGA GAG	52	13	4	30.8
811	AGA GT	51	11	9	81.8
812	GAG AGA GAG AGA	51	10	4	40.0
813	GAG AC	51	8	5	62.5
830	GAG AGA GAG AGA	51	8	5	62.5
834	GAG AA	51	9	5	55.6
835	CTC TCT CTC TCT	53	10	7	70.0
836	CTC TT	53	12	6	50.0
840	TGT GTG TGT GTG	51	11	9	81.8
851	TGT GG	53	9	4	44.4
854	AGA GAG AGA GAG	51	10	6	60.0
856	AGA GYT	51	12	7	58.3
857	AGA GAG AGA GAG	52	8	5	62.5
868	AGA GYC	51	10	2	20.0
880	AGA GAG AGA GAG AGA GYA GAG AGA GAG AGA GAG AYT GTG TGT GTG TGT GTG TYG TCT CTC TCT CTC TCT CRG ACA CAC ACA CAC ACA CYA ACA CAC ACA CAC ACA CYG GGA GGA GGA GGA GGA GGA GGA GAG GAG AGG AGA	51	13	10	76.9

clarity, repetition and relatively high polymorphism (Table 2). Six samples from different regions were used to detect the amplification of these primers. A total of 154 bands were amplified, among which 88 were polymorphic (57%). Mean 10.3 bands were amplified by each primer.

DISCUSSION

Among all dominant markers, ISSR has its advantages with high annealing temperature and repetition and lower cost, this has been widely used to medicinal plant species.

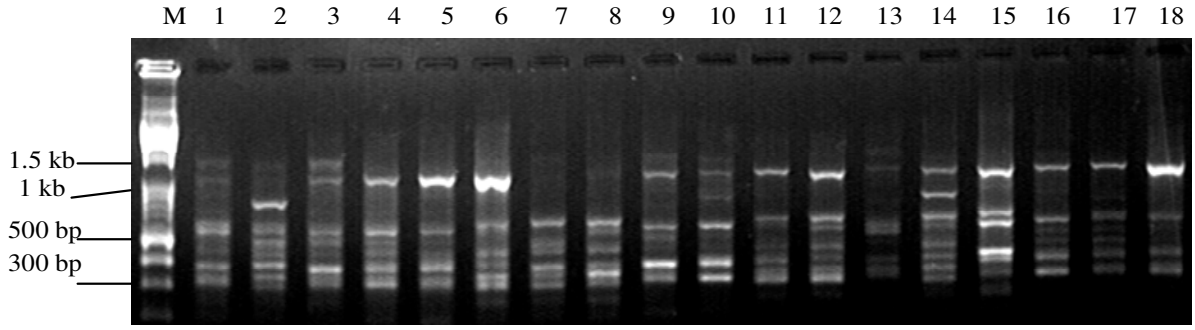

Figure 2. PCR results of eighteen DNA samples in Figure 1 amplified by primer 836. M was 1 kb DNA ladder marker.

Although we may obtain qualified genomic DNA by following those reported isolation methods, primers still need to be screened if their screening has not been reported because the same primer may exhibit different amplification results in different species. Therefore, primers screening and optimal PCR system based on each selected primer are necessary after obtaining qualified DNA with high purity and yield.

Both CTAB and SDS are two universal DNA isolation methods, but they are time-cost with so many steps. In recent years isolation kit is welcomed for their concise steps and shortening time in which all necessary buffer or solution were provided. Our results showed that all three isolation methods could effectively extract DNA with higher quality and yield, but the isolation kit was the best choice if speediness was foremost in spite of high cost (ca. 1 US dollar per sample in China). On the other hand, improved CTAB may be the best when restricted by funding, but it often need to be slightly modified even if it has been reported in other laboratory. This may be attributed to compounds difference of leaf tissue in different regions, chemicals and solutions manufactured by different company and manipulation criteria difference. In order to obtain optimal DNA isolation method, those protocols reported by references were re-acted and our steps on other medicinal plant species were modified based on past experience. The results exhibited that our improved CTAB was better than both CTAB methods reported by Feng et al. (2002) and Shao et al. (2006).

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