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RAPD Profile for Authentication of Medicinal Plant *Glycyrrhiza glabra* Linn.

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Abstract : Correct identification and quality assurance is indispensable to ensure reproducible medicinal quality of herbal drugs. Authentication is especially useful in case of those medicinal herbs that are frequently substituted or adulterated with other species or varieties morphologically and phytochemically indistinguishable. In this study, the RAPD (Random Amplified Polymorphic DNA) technique was employed for authentication of *Glycyrrhiza glabra* L. from its adulterant *Abrus precatorius* L. Fifty two decamer oligonucleotide primers were screened in the RAPD analysis for identification of genuine and adulterant samples. The DNA isolated from the dried root of the samples was used as templates in polymerase chain reactions with fifty two primers. Out of fifty two primers, sixteen primers gave species specific reproducible unique amplicons. The obtained unique amplicons in PCR amplification clearly distinguished genuine as well as adulterant samples having similar morphology and thus, RAPD helping to serve as a complementary tool for quality control.

Key words: *Glycyrrhiza glabra*, *Abrus precatorius*, authentication , RAPD, adulterant

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Introduction

Traditional herbal and herbo-mineral drugs have been used since the dawn of civilization to maintain and alleviate human sufferings from diseases. These medicinal herbs have been in use in one form or another, under indigenous systems of medicine like Ayurveda, Sidha and Unani. According to an estimate of the world health organization (WHO), about 80% of the world population still uses herbs and other traditional medicines for their primary health care needs (Agrawal, 2006). *Glycyrrhiza glabra*, is one of the most frequently employed botanicals in traditional medicine. The root of *G. glabra* is known as licorice and history of it, as a medicinal plant, is very old and has been used in many societies throughout the millennia (Wang, 2001). There are many useful compound in licorice root such as, glycyrrhizin (saponin- like glycoside -50 time sweeter than sugar) and its aglycone, glycyrrhetinic acid which are clinically used for hyperlipidemia (Tamir *et al.*, 2001). Licorice flavonoid constituents mainly include flavones, flavonals, isoflavones, chalcones, bihydroflavones and bihydrochalcones. Pharmacological investigations indicate that they have antioxidant, antibacterial and anti-inflammatory activities (Vaya *et al.*, 1997). The roots of *Abrus precatorius* are similar in morphology with roots of *G. glabra*. Its root is sweetish and mucilaginous, and is adulterated in roots of *G. glabra* in herbal market. On the basis of root morphology, it is difficult to distinguish between both plants. Several herbal drugs on the market still cannot be identified based on their morphological or histological characteristics. From the very beginning, herb authentication has presented a great challenge for people using them for medical purposes. Usage of a wrong herb may be ineffective or it may worsen the condition and may even cause death. Ideally, authentication should be done from the harvesting of the plant material to the final product. Morphological as well as biochemical markers used in the authentication of herbal drugs have many

limitations due to the impact of environmental conditions. DNA based molecular markers however, are important tool in quality assurance and preservation of germplasm of medicinal plant species in the plant kingdom. Our major objective therefore, was to develop DNA based molecular tools for accurate identification of *G. glabra* in local market. Random amplified polymorphic DNA (RAPD) involves the use of a single 'arbitrary' primer in a polymerase chain reaction (PCR) and results in the amplification of several discrete DNA fragments (Kirtikar and Basu, 1990). Each fragment is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands that are complementary to the primer and sufficiently close together for the amplification to work (Hon et al., 2003).

Materials and Methods

The genuine sample of *Glycyrrhiza glabra* L. was provided by Central Council Research for Unani Medicine (CCRUM) Hyderabad. The samples for authentication were purchased from local markets of Khari Baobli, Delhi, India. The material was identified at National Institute of Science Communication and Information (NISCAIR) by Dr. H. B. Singh and voucher (NISCAIR/RHMD/consult/-2007-08/937/121) was deposited in Herbarium.

Reagents and chemicals

The stock solution concentration were: CTAB 3% (w/v), 1M Tris–Cl (pH 8), 0.5M EDTA (pH 8), 5M NaCl, absolute ethanol (AR grade), chloroform-IAA (24:1 [v/v]), polyvinylpyrrolidone (PVP) (40 000 mol wt) (Sigma), β -mercaptoethanol. All the chemicals used in the experiments were of analytical grade. The extraction buffer consisted of CTAB 3% (w/v), 100 mM Tris–Cl (pH 8), 25 mM EDTA (pH 8), and 2M NaCl respectively. The polyvinylpyrrolidone and β-mercaptoethanol were added freshly prepared.

DNA Extraction

DNA was isolated from dried root powder using a modified CTAB method (cetyl trimethyl ammonium bromide (Salim et al., 2007). The fine powder was transferred into the microcentrifuge tube containing freshly prepared 600 µl of extraction buffer (100 mmol/L Tris buffer pH 8.0, 25 mmol/L Na2 EDTA, 2.0 mol/L NaCl, 3% CTAB, 3% polyvinyl pyrrolidone). The suspension was gently mixed and incubated at 65 ℃ for 20 min with occasional mixing. The suspension was then cooled to room temperature and an equal volume of chloroform : isoamyl alcohol (24:1) was added. The mixture was centrifuged at 12,000 rpm for 10 min. The clear upper aqueous phase was then transferred to a new tube and added 2/3 volume of ice-cooled isopropanol and incubated at –20 ℃ for 30 min. The nucleic acid was collected by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 80% ethanol. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE (10 mmol/L Tris buffer pH 8.0, 1 mmol/L Na2 EDTA) at room temperature and stored at 4 ℃ until used. The RNA from crude DNA was eliminated by treating the sample with RNase A (10mg/ml) for 30 min at 37 ℃. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide.

RAPD reaction and amplification

The RAPD reaction was performed according to the method developed by (McClelland and Welsh, 1995).The reaction was carried out in 25 µl volume in a tube using sixteen random decanucleotide primers, OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, OPC-8, OPC-13, OPC-14, OPC-15, OPC-16, OPC-17, OPC-18, OPC-19 and OPC-20 (Operon Technologies Inc., USA and Genex PVT, India). Each reaction tube contained 30 ng template DNA, 1.5 mmol/L $MgCl_2$, 300 µmol/L of dNTPs, 2.5 µl 1×*Taq* DNA polymerase buffer, 30 pmol decanucleotide primer and 1.5 units of *Taq* DNA polymerase (Genei, India). Amplification was performed in a DNA thermal cycler (Techne Thermal cycler, England) using the following conditions: 94 ℃ for 3 min; 36 cycles at 94 ℃ for 1 min, 35.6 ℃ for 30 s and 72 ℃ for 1 min; final extension at 72 ℃ for 2 min. The amplified products were resolved on 1.2% agarose gel in 1×TAE buffer. The DNA was stained with 0.5 mg/ml ethidium bromide, visualized and photographed under a UV transilluminator. A sample without template DNA was included as a negative control in each experiment to check contamination. Electrophoretic profile was visualized under UV radiation and photographed with UV transilluminator, U.K. The sizes of DNA fragments were estimated by comparison with standard DNA Ladder 1kb and 100 bp (Banglore Genei, India).

Results & Discussion

Medicinal plants constitute an effective source of traditional and modern medicine. Adulterations and substitutions are common in raw material trade of medicinal plants. Adulteration in market samples is one of the greatest drawbacks in promotion of herbal products. Due to this adulteration and altered efficacy, the faith in crude drug promotion has declined (Dubey *et al*., 2004). Development of RAPD markers that can correlate DNA fingerprinting data with quantity of selected phytochemical markers associated with that specific medicinal herb, would have extensive applications in quality control of raw materials. These

designed markers would act as a qualitative/quantitative diagnostic tool for identification of medicinal herbs from harvesting to finished product. The RAPD, PCR based assay described here is fast, reliable, and easy to conduct in any laboratory. It can be carried out in very short period using unknown genomic DNA from any developmental stages and body parts of herbs (Kethidi *et al*., 2003). In the present article *Glycyrrhiza glabra* L. was chosen to test the reliability of the quality control using RAPD technique. In the local market samples, *Abrus precatorius* L. was found as adulterant when samples of local market identified at NISCAIR, New Delhi (voucher no, NISCAIR/RHMD/consult/-2007-08/937/121). The high quality and purity of genomic DNA free from secondary metabolites was isolated from dried root powder of genuine and adulterant samples by modified CTAB method for RAPD-PCR (Salim *et al*., 2007). When DNA was isolated using methods described by (Doyle and Doyle, 1987) and (Murray and Thompson, 1980), DNA of desired quality and quantity could not be isolated. For RAPD reaction, it was necessary to standardize the following variables for successful amplification using PCR. RAPD amplification is not reproducible below a certain concentration of genomic DNA and produces ‘smears’ or results in poor resolution if DNA concentration is high. The concentration of $MgCl_2$ is important for genomic DNA amplification. The $MgCl_2$ of 1.5mM concentration was proved best in 25 µl reaction volume. For most amplification reactions 0.5 unit of the enzyme was used in amplification. Primer annealing in most reactions was carried out at 35 ℃ to 60 ℃. In all PCR trials the annealing temperature of 35.5 ℃ has been used which was determined by several trials of PCR. Different concentrations of dNTP ranging from 100 mM to 250 mM of each dNTP per 25 µl reaction volume were tried. Finally 100 mM of each dNTP proved to be the best. In our study, out of 52 RAPD primers, 16 primers OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, OPC-8, OPC-13, OPC-14, OPC-15, OPC-16, OPC-17, OPC-18, OPC-19, and OPC-20 (Operon Technologies Inc., USA and Genex PVT, India) gave reproducible results and further selected to simultaneously identify *G. glabra* and its adulterant *A. precatorius.* The total number of unique amplicons specific to genuine as well as adulterant samples with primers OPC-1(1), OPC-2 (2), OPC-3(3), and OPC-4(6) were found in PCR amplification (Figure 1). With primers OPC-5, OPC-6, OPC-7, and OPC-8, the number of unique amplicons were found 0, 6, 7 and 8 respectively (Figure 2). With primers OPC-13, OPC-14, OPC-15, and OPC-16, the number of unique amplicons were found 6, 5, 5 and 4 respectively (Figure 3). Similarly, the total number of unique amplicons with primers OPC-17, OPC-18, OPC-19, and OPC-20 were found 4, 4, 6 and 6 respectively (Figure 4). The primer OPC-8 gave highest number of unique amplicons than the other primers used in the amplification. Each unique amplicon differentiate between genuine as well as adulterant sample (Table 1).

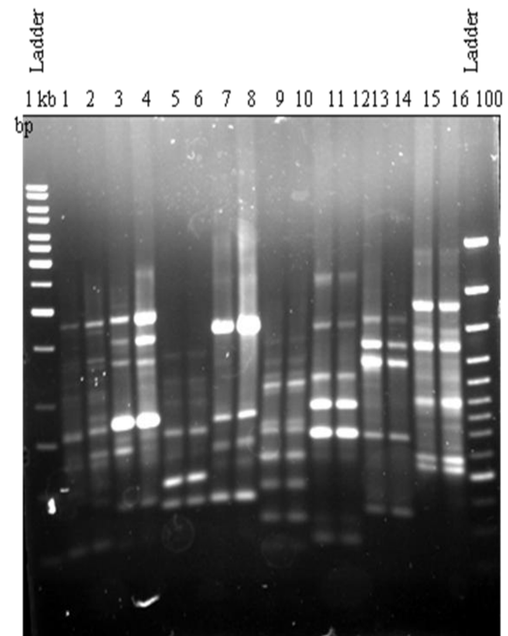

Figure1. RAPD analysis carried out with primer: OPC-1(lanes-1-4), OPC-2 (lanes-5-8), OPC-3(lanes-9-12), OPC-4(lanes-13-16) on genomic DNA from *Glycyrrhiza glabra* L. (lanes-1,2,5,6, 9,10, 13,14) and *Abrus precatorius* (lanes-3,4,7,8,11,12,15,16). 1kb and 100 bp are DNA ladder.

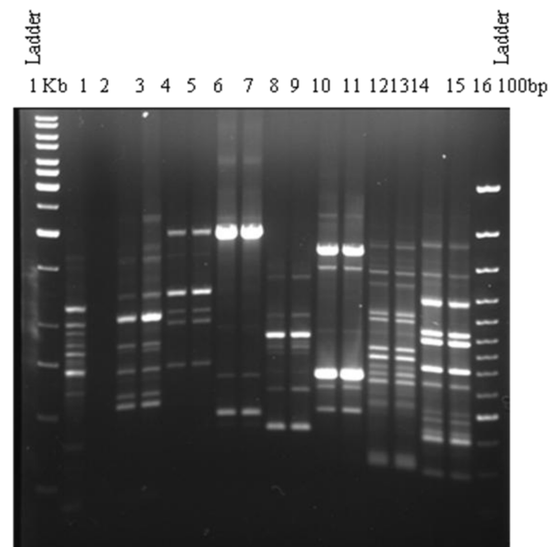

Figure 2. RAPD analysis carried out with primer: OPC-5(lanes-1-4), OPC-6(lanes-5-8), OPC-7(lanes-9-12), OPC-8(lanes-13-16) on genomic DNA from *Glycyrrhiza glabra* L. (lanes-1,2,5,6, 9,10, 13,14) and *Abrus precatorius* (lanes-3,4,7,8,11,12,15,16). 1kb and 100 bp are DNA ladder.

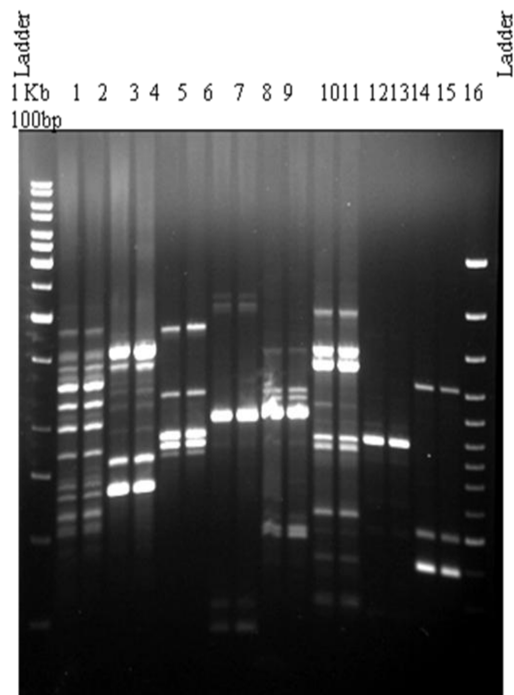

Figure 3. RAPD analysis carried out with primer: OPC-13(lanes-1-4), OPC-14(lanes-5-8), OPC-15(lanes-9-12), OPC-16(lanes-13-16) on genomic DNA from *Glycyrrhiza glabra* L. (lanes-1,2,5,6, 9,10, 13,14) and *Abrus precatorius* (lanes-3,4,7,8,11,12,15,16). 1kb and 100 bp are DNA ladder.

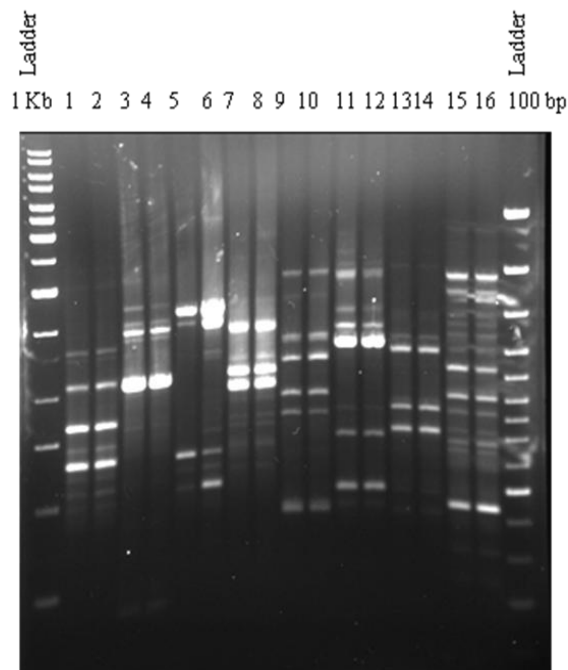

Figure 4. RAPD analysis carried out with primer: OPC-17(lanes-1-4), OPC-18(lanes-5-8), OPC-19(lanes-9-12), OPC-20(lanes-13-16) on genomic DNA from *Glycyrrhiza glabra* L. (lanes-1,2,5,6, 9,10, 13,14) and *Abrus precatorius* (lanes-3,4,7,8,11,12,15,16). 1kb and 100 bp are DNA ladder.

Name of Plant Species	Fig. 1 Size of unique amplicons (bp)				Fig.2 Size of unique amplicons (bp)				Fig.3 Size of unique amplicons (bp)				Fig. 4 Size of unique amplicons (bp)			
	OPC-1	OPC-2	OPC-3	OPC-4	OPC-5	OPC-6	OPC-7	OPC-8	OPC-13	OPC-14	OPC-15	OPC-16	OPC-17	OPC-18	OPC-19	OPC-20
G. glabra	-	700 500	600	1200 700	-	1400 1200 1031 800	1500 1200 950 1150	1600 1200 1031 750 700	1900 1400 1200 1031 600	2000 1200 1031 900	1200 1031	900	1200 800 600	1500 650	1200 1037 800	1200
A. precatorius	1400	-	1500 900	1800 900 600 500	- -	700 550	1700 700 500	1180 1000 900	850	1100	2000 1500 900	1200 500 400	1400	1031 1000	1500 700 500	1900 1800 1031 900 550
Total unique amplicons /primer	1	2	3	6	0	6	7	8	6	5	5	4	4	4	6	6

Table1. The unique amplicons specific to *G. glabra* and *A. precatorius* samples with sixteen decamer oligonucleotides OPC primers obtained in PCR amplification.

RAPD analysis has been widely used for differentiation of a large number of medicinal species from their close relatives or adulterants, including *Panax* species (Shaw and But, 1995), *Coptis* species (Cheng *et al*., 1997), *Astragalus* species (Cheng *et al*., 2000) and turmeric (Sasikumar *et al*., 2004). The advantages of RAPD techniques include their simplicity, rapidity, the low amount of genomic DNA required and the fact that isotopes and prior genetic information are not required (Micheli *et al*., 1994). The advantages of this approach are its rapidity, simplicity and the absence of any need for prior genetic information of the plant. RAPD fingerprints patterns obtained are consistent irrespective of the plant source or age (Welsh and McClelland, 1990; Kethidi *et al*., 2004). These characters are especially advantageous for the identification of herbal medicine because little DNA exist in the dried material and also because sequence data are difficult to obtain. PCR amplifications were tested with RAPD primers from kits (Operon Technologies, CA and USA). Thus RAPD markers, which are specific to *G. glabra* and *A. precatorius*, were obtained through the primers screening. The most of the primers gave unique amplicons specific to genuine as well as adulterant sample which were selected for further study. Significance of present work is that single primer can differentiate genuine as well as adulterant samples. More high quality linked DNA marker, sequenced characterized amplified regions (SCAR) for the herbal medicines will be developed in further studies, which can provide an alternative tool to monitor the quality of herbal medicines.

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