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Full Length Research Paper

Optimization of DNA isolation for RAPD-PCR analysis of selected (*Echinaceae purpurea* L. Moench) medicinal plants of conservation concern from Turkey

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Genetic analysis of plants relies on high yields of pure DNA samples. Here we present the optimization of DNA isolation protocols and PCR conditions for RAPD analysis of selected medicinal plants of conservation concern from Turkey, containing high levels of polysaccharides, polyphenols and secondary metabolites. These methods involve a modified CTAB extraction employing polyvinyl pyrrolidone while grinding, successive long-term Chloroform -Isoamylalcohol extractions, and EZ1 Nucleic Acid isolation protocols. The yield of DNA ranged from 1 - 2 g/ l per gram of the leaf tissue and the purity (ratio) was between 1.7 - 1.8 indicating minimal levels of contaminating metabolites. EZ1 nucleic acid isolation technique is ideal for isolation of DNA from different plant species and the DNA isolated was used for randomly amplified polymorphic DNA (RAPD) analysis. RAPD protocol was optimized based on the use of 50 ng of template DNA and annealing temperature of 37°C, resulted optimal amplification. Thus, the results indicate that the optimized protocol for DNA isolation and PCR was amenable to plant species belonging to different genera which is suitable for further work on diversity analysis. Furthermore, here we used suitable DNA isolation protocol for RAPD analysis to study the genetic variation in the future in Echinaceae sp. grown in Turkey.

Key words: *Echinaceae purpurea,* RAPD-PCR, medicinal plant.

INTRODUCTION

Medicinal plants utilization and conservation has attracted global attention (Parrotta, 2001). Echinaceae is a true medicinal plant and was used for a variety of medicinal purposes by various tribes. Echinaceae entered mainstream herbal medicine over a century ago, multi billion dolar natural products industry. Current focus on chemotype-driven fingerprinting and related techni-ques requires integration with genotype-driven molecular techniques so that an optimal characterization of botani-cal materials is possible. This review provides a brief account of various DNA-based technologies that are use-ful in genotyping and quick identification of botanicals with suitable examples. DNA-based techniques have been widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable. Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism. These are hybridization-based methods, polymerase chain reaction (PCR)-based methods and sequencing-based methods. PCR-based markers involve *in vitro* amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide pri-mers and the thermostable DNA polymerase enzyme. PCR-based techniques where random primers are used, include random amplified polymorphic DNA (RAPD). RAPD analysis and improper priming of DNA templates during thermal cycle sequencing. Different plant taxa often may not permit optimal DNA yields from one isola-tion protocol. For example, some closely related species of the same genus require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required.

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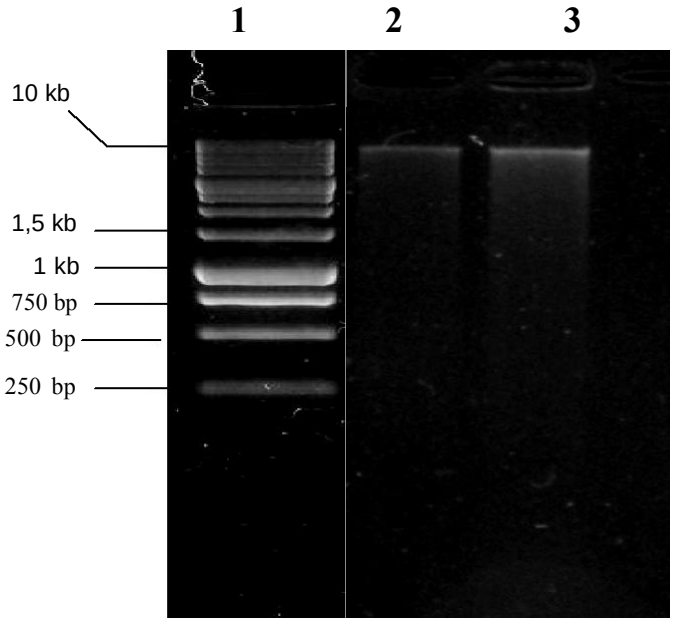

Figure 1. Genomic DNA s were loaded in a %1 agarose gel and separated by electrophoresis for 90 min at 100 V, then visualised by ethidium bromide staining with transillumination.
Lane 1: 1 kb ladder size standard marker
Lane 2 – 3: Genomic DNAs isolated from *(Echinaceae Purpurea* L. Moench) DNA with manuel DNA isolation method.

Various protocols for DNA extraction have been success-fully applied to many plant species (Doyle and Doyle, 1987; Ziegenhagen and Scholz, 1993), which were fur -ther modified to provide DNA suitable for several kinds of analyses (Wang and Taylor, 1993; Ziegenhagen and Scholz, 1998). We have tested previously established DNA isolation protocols but these methods resulted in DNA with lot of impurities and not very suitable for RAPD analysis.

Therefore, we report here a total genomic DNA isolation protocol derived from a method originally developed for other plants (Doyle and Doyle, 1987). Modifications were made to minimize polysaccharide coisolation and to simplify the procedure for processing large number of samples. The protocol optimized for RAPD proved to be inexpensive with relation to the use of primer, quantity of DNA, usage of dNTPs*, Taq* polymerase and the reaction volume. Thus the protocol derived for both genomic DNA isolation and RAPDs, is genus independent, efficient, inexpensive, simple, rapid and yields pure DNA amplifiable by PCR as indicated by the results of the RAPD technique. The isolated DNA would be suitable for further downstream applications.

MATERIALS AND METHODS

Plant material

Echinacea purpurea was used in this study. Plant samples were collected from various forests in the state of stanbul, Konya and Karadeniz Agricultural Research Institute in Turkey and grown in experimental site. After acclimatization 1 g of young leaves were harvested fresh for DNA isolation.

DNA isolation

DNA was isolated from fresh as well as commercial samples using modified cetyl trimethyl ammonium bromide (CTAB) extraction method. In brief, fresh leaf tissue (1 - 1.5 g) or dried fruit powders (0.5 g) was ground in liquid nitrogen. The method involves a modified CTAB extraction employing polyvinyl pyrrolidone while grinding, successive long-term Chloroform-lsoamylalcohol extractions, EZ1 Nucleic Acid isolation protocols. Furthermore, seeds were germinated in 25 - 30 cm diameter pots containing soil, sand and organic manure in glass house and field. Plant tissue samples were collected from field and glass house, frozen in liquid nitrogen and stored at -80°C or in pots for future use. DNA was extracted from both fresh and stored tissue samples. The yield of DNA ranged from 1 - 2 g/ l per gram of the leaf tissue and the purity (ratio) was between 1.7 - 1.8 indicating minimal levels of contaminating metabolites. EZ1 nucleic acid isolation technique is ideal for isolation of DNA from different plant species and the DNA isolated was used for randomly amplified polymorphic DNA (RAPD) analysis (Figure 1).

Amount and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a UV Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 1% agarose gel based on the intensities of band when compared with the Lambda DNA marker (used to determine the concentration). The nucleic acid concentration was calculated following Sambrook et al. (1989).

DNA digest by restriction endonuclease

The purity of the DNA was confirmed by means of complete *Eco*RI /*Hin*dIII digestion and monitoring the banding profile of the digested DNA after incubating the reaction mixture at 37°C for 3.5 h. This indicated that isolated DNA was amenable for further downstream applications. Protocol containing; 1 l 10 X Buffer, 0.25 l Lambda EcoRI/Hind III, 15.75 l water and 3 l genomic DNA (Figure 2 and Figure 3).

Optimization of RAPD reaction

For the optimization of RAPD reaction using DNA extracted from Echinaceae plant species, oligonucleotide primers from A and C series (Operon Technologies Inc. Almeda CA, USA) were used for amplification to standardize the PCR conditions. The reactions were carried out in a DNA Thermocycler (Bio Rad Research, Archeometry Lab., Turkey). Reactions without DNA were used as negative controls. Each 15 l reaction volume contained about 50 ng of template DNA, 1 X PCR Buffer (10 mM Tris HCl pH 8.3; 50 mM KCl), 3 mM $MgCl_2$ *(*Invitrogen Life Technologies, Brazil), 0.2 mM dNTP Mix, 0.5 M of single primer, 0.2 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Brazil). The thermocycler was programmed for an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 37°C, extension was carried out at 72°C for 1 min and final extension at 72°C for 7 min and a hold temperature of 4°C at the end. PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TBE Buffer at 50 V for 3 h and then stained with ethidium-bromide (0.5 g/ml). Gels with amplification fragments were visualized and photographed

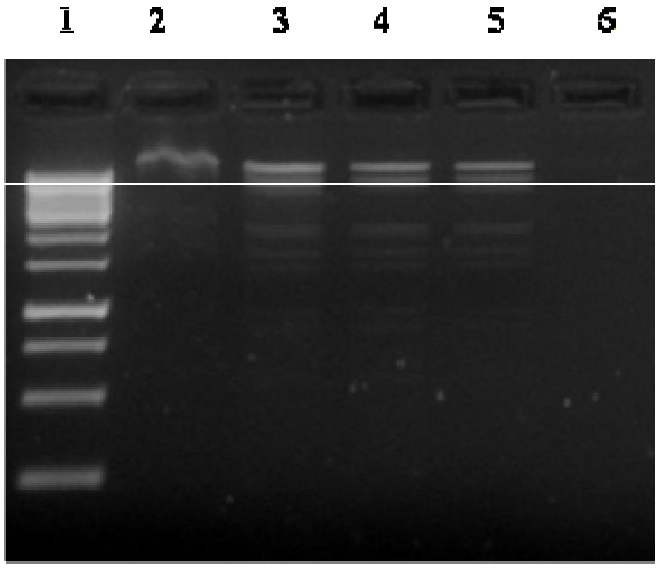

Figure 2. Screening of agarose gel electrophoresis of *Echinaceae purpurea* DNA digested with restriction endonuclease (EcoRI/Hind III).
Lane 1: 1 kb ladder size standard marker.
Lane 2 - 5: digested with restriction endonuclease of DNA isolated from Echinacea purpurea with manuel DNA isolation method.
Lane 6: negative control (None DNA).

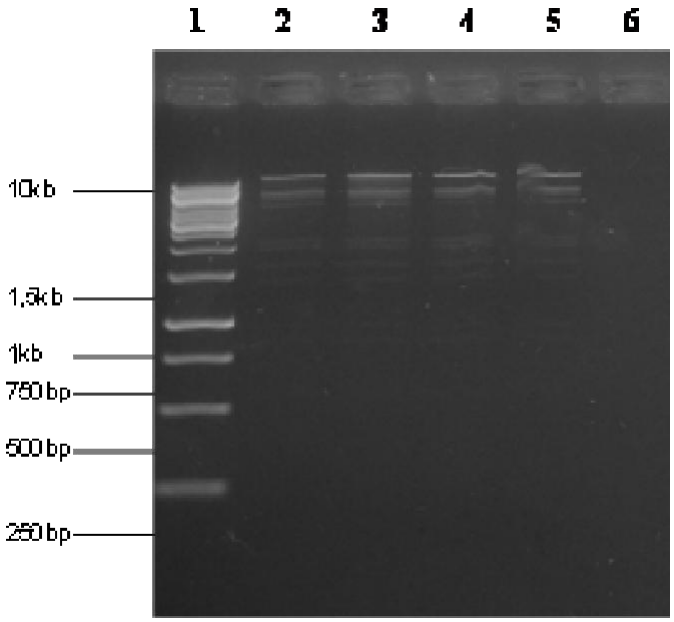

Figure 3. Screening of agarose gel electrophoresis of Echinaceae purpurea DNA digested with restriction endonuclease (EcoRI/Hind III).
Lane 1: 1 kb ladder size standard marker.
Lane 2 - 4: digested with restriction endonuclease of DNA isolated from Echinacea with manuel DNA isolation method.
Lane 5: digested with restriction endonuclease of DNA isolated from Echinaceae with EZ1 Nucleic acid isolation method.
Lane 6: negative control (None DNA).

under UV light. Lambda DNA *EcoR*1- *Hin*dIII double digest was used as molecular marker (Invitrogen Life Technologies) to know the size of the fragments. The reproducibility of the amplification products was tested twice for each experiment at different times using similar reaction conditions.

RESULTS AND DISCUSSION

Echinaceae, also known as the purple coneflower, is an herbal medicine that has been used for centures, customarily as a treatment for the common cold, coughs, bronchitis, upper respiratory infections, and some inflammatory conditions. More information is needed before a definitive statement about the efficacy of Echinaceae can be made. Future work needs to clearly identify the species of it and distinguish between the efficacy of the different plant parts. Traditionally, pharmacognosy mainly addressed quality related issues using routine botanical and organoleptic parameters of crude drugs. Extensive research on DNA-based molecular markers is in progress in many research institutes all over the world. These techniques have been widely used for authentication of plant species of medicinal imporance in plant genome research with its applications in pharmacognostic identification and analysis. Chinese researchers have applied DNA markers extensively for characterization of botanicals from the Chinese materia medica. These markers have been useful in other investigations of genetic variation among geographically distant populations *Echinaceae* sp. Isolation of good-quality DNA suitable for analysis from semi-processed or processed botanicals is also a challenge (Li et. al., 2003). Another important issue is that DNA fingerprint will remain the same irres-pective of the plant part used, while the phytochemical content will vary with the plant part used, physiology and environment. DNA fingerprinting ensures presence of the correct genotype but does not reveal the contents of the active principle constituents (Yang et. al., 2001).

We found these modified steps necessary to standardize and increase the quality and quantity of genomic DNA. The degree of purity and quantity varies between applications. The extracted DNA was of high quality as it showed a reading of between 1.6 to 1.7 after calculating the 260/280 nm absorbance (Figure 1). The DNA yield obtained ranged from 1 to 2 g/ l. The conditions described in the present work, modified for use in RAPD analysis, consistently amplified DNA fragments of plant species belonging to different genera with various medicinal and aromatic properties, which are highly recalcitrant. The present optimized protocol for DNA isolation and RAPD technique may serve as an efficient tool for further molecular studies. Thus, we conclude that present protocol describes a reliable, rapid, simple and consistent DNA isolation method for Echinaceae.

DNA isolation for *Echinacea* genotypes

Samples analysed included dried *Echinaceae* sp. seeds

were used directly for DNA extraction as they were found to yield DNAs comparable in quality and quantity to that obtained using EZ1 Nucleic acid isolation analyser (QIagen, 2007; first author's laboratory or Archeometry and Biotechnology Lab., Turkey) and by the using of the CTAB method, Chloroform-Isoamylalcohol extraction method and DNA extraction with phenol purification and liquid nitrogen method. A *E. purpurea* seed bulk sample was ground to fine powder. This experiment was repeated twice under repeatability conditions resulting in all DNA samples. Furthermore, *E. purpurea* seeds were germinated then these plantlets was ground to powder by liquid nitrogen treatment. Genomic DNA solutions from Echinaceae genotypes by both extraction methods were of adequate purity and yield for applying PCR. Genomic DNA was extracted by CTAB method and EZ1 Nucleic Acid DNA isolation method and Chloroform-Isoamyl-alcohol extraction from *Echinaceae* sp. Isolated DNA were found that of material *Echinaceae* sp. can be extracted by rapid and simple methods were of a sufficient purity (A_{260}/A_{280} = 1.7–1.8) (Figure 1).

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