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# An Alternative Strategy in Rapid DNA Extraction Protocol for High Throughput RAPD Analysis in Chickpea and Its Wild Related Species

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**Abstract**

The first step of RAPD fingerprinting is the preparation of the target DNA template. The extraction of DNA from plants has been described by numerous authors. Each author described a different method to overcome the problems for isolation of genomic DNA. Some species like *Cicer* have certain metabolites like polysaccharides and RNA which interfere with DNA isolation and polymerase chain reaction (PCR) amplification. The objective of this project was to develop high-throughput DNA extraction procedure without the need for greenhouse space or growing *Cicer* plants. Seeds were germinated of paper filter in Petri dish, and 4-day old seedling tissue was used to extract DNA by modified high salt CTAB protocol. Approximately 50-150 ng/µ of genomic DNA was isolated from 2 g of leaf tissue. DNA derived using this method was tested electrophoretically and then was examined with a spectrophotometer: A260/280 (Protein contamination). Polymerase chain reaction amplification was performed using different RAPD primers and *EcoRI, MseI* and *HindIII* were used for restriction enzyme reaction. The isolated DNA proved amenable to PCR amplification and restriction digestion. This method does not require expensive reagents and modern laboratory equipments and this technique allows one person to extract nearly 200 storage-stable DNA samples daily, while keeping costs at a minimum. This technique is fast, reproducible, and can be applied for RAPD analysis and most of genetic assays.

**Key words:** DNA extraction, chickpea, *Cicer* species, RAPD, restriction enzyme

## INTRODUCTION

A reliable method for plant DNA isolation is required for polymerase chain reaction (PCR) analysis in plant breeding, plant gene mapping and plant genetic diversity. Classical approaches to plant DNA isolation have aimed to produce larger quantities of DNA and more highly purified DNA than that needed for polymerase chain reaction (PCR) [1, 2]. Many simple procedures have been developed for use in preparing plant DNA for PCR [3,4,5]. However, these procedures may only work for a limited number of tissue or plant species, and most are still multi step procedures. In many cases, the presence of polysaccharides (e.g. starch) and secondary metabolites (e.g. phenolic compounds) often inhibited restriction endonuclease digestion and/or PCR amplification [6,7]. Many DNA isolation procedures also yielded large amounts of RNA, especially 18s and 25s rRNA. RNase treatment is the generally accepted method to remove RNA; however, degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problems including suppression of PCR amplification [8].Mini-prep methods require only a small amount of tissue, and use minimal numbers and amounts of chemicals. Furthermore, by this method high-quality DNA in large quantities is extracted. Many mini-prep methods for obtaining DNA have been developed, including such modifications as no grinding and no centrifugation [9]. Currently, there is no simple effective solution for high throughput extractions of plant leaf DNA. Many methods require multiple steps and often more than one expensive micro titer dish. Traditional hexadecyltrimethylammonium bromide (CTAB) based methods are laborious, and kits based on spin columns are expensive and not designed for high throughput. These methods also produce DNA samples that are likely to contain polyphenolic compounds and polysaccharides, which can cause the DNA to be unsuitable for PCR amplification. The first step of RAPD fingerprinting is the preparation of the target DNA template. Intuitively, minimal DNA template preparation should be necessary for RAPDs since, theoretically, PCR may amplify a single DNA molecule. It seems one would simply homogenize tissue and allow the PCR to "find" and amplify the target DNA. Indeed, many rapid DNA isolation methods designed for use with PCR actually involve little isolation of DNA. Rather, they employ a "grind and use" process [10] or minimal purification [11]. These methods are very fast, require little tissue and amplify well with plants that are amenable to DNA extraction and that contain few interfering secondary metabolites. However, the resulting DNA templates are not very pure and may not be stable for long periods of time. Recently, DNA purity has been implicated as one of the most important factors in RAPD reproducibility [12]. The assay they suggest consists of replicate RAPD-PCRs in which the DNA concentration is titrated over two orders of magnitude. If the DNA is of adequate quality the replicates should yield identical RAPD fingerprints. Thus, it seems-likely that there could be several interactive factors involving DNA purity or lack thereof in RAPD reproducibility. It seems prudent to researchers performing RAPD-PCR to assure them that the isolated DNA is of sufficient quality to ensure reproducible RAPD fingerprints. The goal of this research was to develop a low cost, high throughput DNA extraction procedure that need

for greenhouse space and isolates DNA of substantial quality for use in repeatable PCR reaction.

## MATERIALS and METHODS

***Plant Materials***: This study was conducted in 2006 at college of agriculture, Islamic Azad University of Sanandaj, Iran. Nineteen different genotypes including four cultivated chickpea (*Cicer arientinum* L.) and fifteen genotypes of other six wild annual *Cicer* species were used in this study (Table 1). Seeds were germinated in 7 cm Petri dish containing germinating paper Plates were placed in the dark at 25°C for 24h, followed by a germination period with 16-h day length at 27°C for 72 h. One set of tissue (seedling and coleoptiles) was extracted immediately.

**Table 1.** Accession, source and origin/collection site of the annual *cicer* species used for RAPD analysis.

| NO | Species | Accession | Source [a] | Origin |
|---|---|---|---|---|
| 1 | *C. echinospermum* | ILWC35 | ICARDA | Turkey |
| 2 | | ILWC181 | ICARDA | Turkey |
| 3 | | ILWC288 | ICARDA | Turkey |
| *4* | *C. reticulatum* | ILWC36 | ICARDA | Turkey |
| 5 | | ILWC231 | ICARDA | Turkey |
| 6 | | ILWC114 | ICARDA | Turkey |
| *7* | *C. pinnatifidum* | ILWC49 | ICARDA | Syrian |
| 8 | | ILWC225 | ICARDA | Turkey |
| 9 | | ILWC212 | ICARDA | Syrian |
| *10* | *C.judacium* | ILWC38 | ICARDA | Lebanon |
| 11 | | ILWC46 | ICARDA | Syrian |
| *12* | *C.cuneatum* | ILWC37 | ICARDA | Ethiopia |
| *13* | *C. yamashitae* | ILWC55 | ICARDA | Afghanistan |
| 14 | | ILWC215 | ICARDA | Afghanistan |
| 15 | | ILWC214 | ICARDA | Afghanistan |
| *16* | *C.arietinum* | Jam | ICARDA | Iran |
| 17 | | Kaka | ICARDA | Iran |
| 18 | | Pirooz | ICARDA | Iran |
| 19 | | FLIP97-111C | ICARDA | Syrian |

***Solutions***:

- Extraction Buffer [120 mM Tris-HCl (pH 8.0), 80 mM EDTA (pH 8.0) , 0.3% β-mercaptoethanol (v/v)]
- SDS 20%
- NaCl 5 M
- CTAB solution [0.7 M NaCl, 2% Cetyltrimethyl-ammonium bromide (CTAB)]
- Chloroform: phenol (24:1)
- Isopropanol.
- Ethanol 70%
- TE (1X) : 10 mM Tris-HCl, ImM EDTA, pH 8.0

***DNA isolation***:

- Grind about 0.2 g of germinated seedling and coleoptiles to a fine powder by using liquid nitrogen. Transfer the powder to an Eppendorf 2 mL tube.
- Add 550µL of extraction buffer and placed the tube on ice for 20-30 min vortex the tube vigorously 5 min intervals.
- Add 30-50µL SDS(20%) and incubated the tube at 60°C for 30 min.
- Add 92µL NaCl 5M and vortex vigorously for 2 min.
- Add 75µL CTAB solution and incubated 65°C water bath for 15 min.
- Add 300µl chloroform: phenol (24:1) and after several inversion, centrifuge it at 12000 g for 10 min (Repeated twice).
- Transfer the upon phase (aqueous) to an clean tube and add 600µL chloroform, centrifuge it at 12000 g for 3 min.
- Transfer the upon phase (aqueous) to an clean tube and add 600µL cold isopropanol, mix well and incubated for 30 min in room temperature
- Centrifuge at 12000 g for 10 min. Discard the supernatant and wash the pellet with 500µL 70% ethanol and let dry.
- Resuspend the pellet in 100µL buffer TE by incubating the tube in 65°C water bath for 30 min.

Quantify the DNA spectrophotometrically at 260 nm and electrophoretically in 0.7% agarose gel to verify the DNA purity and quantity.

***PCR amplification:*** A set of twenty random decamer oligonucleotides purchased from MWG Company (Germany) was used as single primers for the amplification of RAPD fragments. For each isolated DNA samples, PCR reaction were carried out in a 25 µl reaction mix containing approximately 15 ng template DNA, 2.5 pmol of each dNTP (Cinnagen Co, Iran), 5 pmol of a single 10-mer primer (MWG Co, Germany), 1 unit of Taq DNA polymerase (Cinnagen Co, Iran). The buffer [10mM Tris-Hcl, PH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 ] used as per instructions of the manufacturer. The PCR reaction were run in a Eppendorf thermal cycler (Eppendorf, Germany) programmed for an initial denaturation step of 3 min at 94°C followed by 35 cycle of 30 s at 94°C , 30 s at 34°C and 1.5 min at 72°C . A final elongation step of 5 min at 72°C was included. The PCR products were separated on 1.5% agarose gel run in 1X TBE and stained with ethidium bromide, observed under UV light and photographed using gel documentation unit (Vilber-Lourmat, France).

***Endonuclease digestion***: Isolated genomic DNA of each genotypes were incubated in 5 unit each of *EcoRI, MseI* and *HindIII* (Fermentas Co, Lithuania) in the Tango buffer at 37°C for 3 h. DNA digestion was assayed by visual inspection after agarose gel electrophoresis.

## RESULTS and DISCUSSION

The protocol was developed to take advantage of the ability to extract DNA from seedlings, which avoids greenhouse use and provide a quick turnaround from seed to DNA. Germination dishes were evaluated for ease of use and space utilization. Seeds can be germinated on filter paper to produce sufficient tissue for numerous extractions. The extraction is not completely destructive, and the seedling of interest can be grown later in soil for further study, progeny test, or to obtain large quantities of DNA. We have modified the previous DNA extraction procedure using CTAB [13, 14]. Polyphenols and other secondary plant compounds, cause damage to DNA and/or inhibit restriction enzymes and Taq polymerases. These compounds bind to DNA when cells are lysed [15]. Cell disruption by grinding in liquid nitrogen and incubation in extraction buffer results in lysis of both the cells containing the phenolics and the organelles. In a

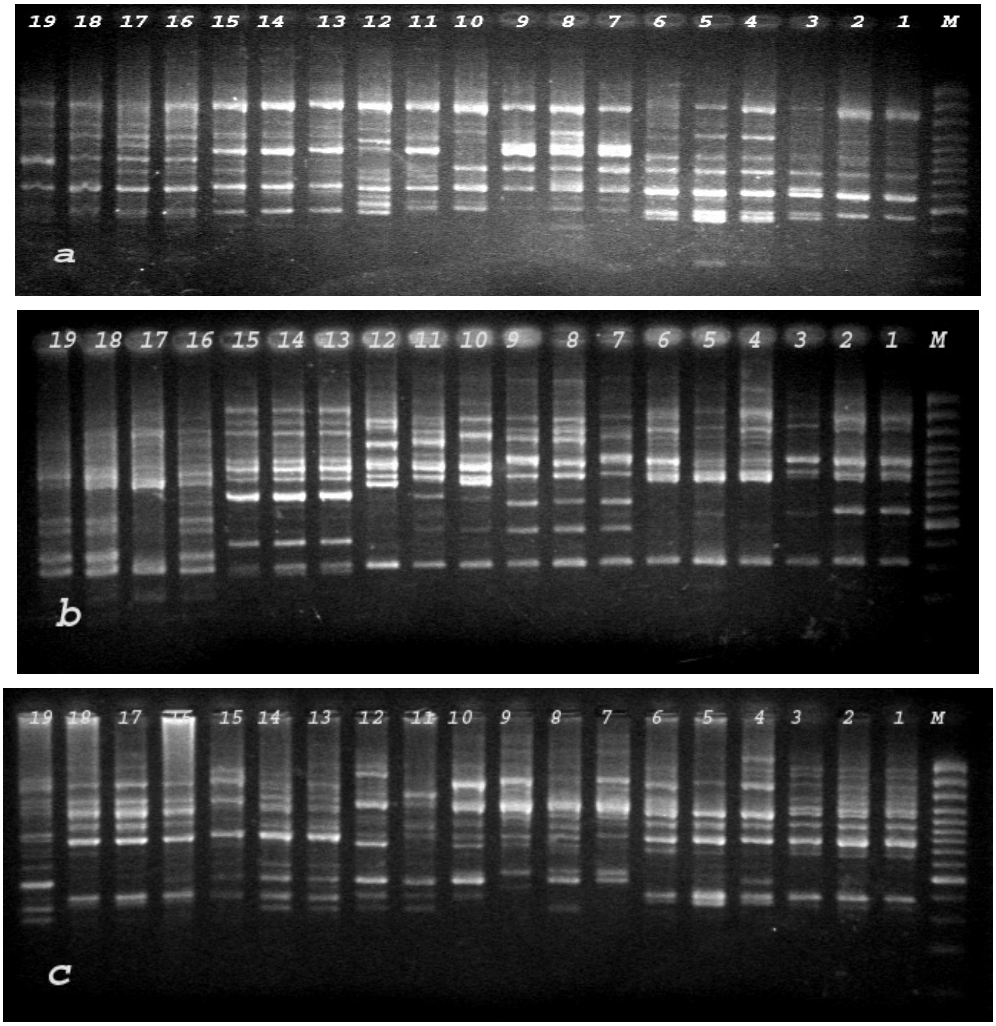


**Figure 2:** PCR amplification of nineteen different Cicer genotypes with primer OPZ10 (a) , OPB10 (b) and OPAC09 (c). M: weight marker.

considerable number of plant species, DNA preparation tends to be brown colored due to the oxidation of polyphenols to quinonic compounds. These are powerful oxidizing agents that damage DNA and protein [16]. As a consequence, yield of high molecular weight DNA from plants is often very poor. Even in the presence of soluble-polyvinylpyrrolidone (e.g. PVP-40) and polyphenol absorbents such as bovine serum albumin (BSA) in the isolation buffer at concentrations of 1–4 %, the phenolic compounds adhere to DNA in solution, forming a colored matrix around the DNA. The result indicated that the quantity of the genomic DNA was about 50-150 ng $\mu l^{-1}$ (Fig. 1).

There was neither RNA contamination nor any sign of degraded DNA in all samples. It seems that RNA is degraded during the extraction process. A 260/280 ratio of our protocol was 1.53-1.88 indicating very low levels of contaminating proteins. DNA isolated by this method yielded strong and reliable amplification products showing its compatibility for RAPD-PCR using random decamer primers (Figures 2a,b and c). DNA was well digested with three different enzyme (*EcoRI, MseI* and *HindIII* ) (Fig. 3), indicating the absence of large amounts of impurities and inhibitors in extracted DNA. Complete digestion with restriction endonucleases and amplification in PCR indicate the absence of polysaccharides. Almost all the tested parameters for RAPDs like the concentration

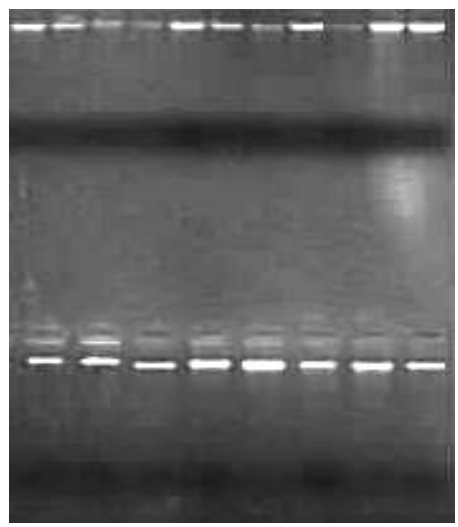

**Figure 1:** Genomic DNA isolated from genotypes including seven different *Cicer* species.

of template DNA, primer, magnesium chloride, *Taq* polymerase, dNTPs and temperature and time intervals during denaturation, annealing and elongation were also optimized which also had an effect on amplification, banding patterns and reproducibility.

There was no interference with PCR amplification reactions. Large amounts of RNA in the sample can cheated Mg2+ and reduce the yield of the PCR. A incubating the DNA in 65◦C for 30 min in the end of extraction procedure, degraded RNA into small ribonucleosides that do not contaminate the DNA preparation, and yielded RNA-free pure DNA. The present optimized protocol for DNA isolation and RAPD technique may serve as an efficient tool for further molecular studies. Our protocol involves two step use of chloroform initially because of precipitation of chlorophyll and proteins. The addition of 5M NaCl and ethanol help in precipitation of DNA. Previous studies indicated that the addition of high concentration of NaCl increased the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of the polysaccharides and DNA [17] and same results observed in *Vitis* species [18].

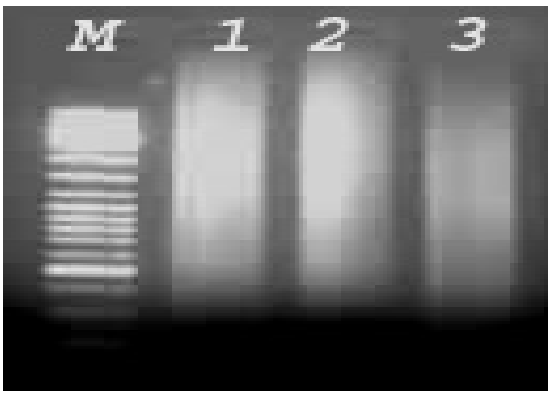


**Figure 3:** Restriction endonuclease digestion of DNA isolated with *EcoRI* (lane 1), *HindIII* (lane 2) and *MseI* (lane 3), M: Weight marker.

The conditions described in the present work, modified for use in RAPD analysis, consistently amplified DNA fragments of plants belonging to different species. The present optimized protocol for DNA isolation and RAPD technique may serve as an efficient tool for further molecular studies. Cost is always an important consideration when conducting DNA analyses of any type. Normally the need of purity, yield and the abundance of initial plant material would be the factors most influencing the choice of extraction method. In performing population studies using RAPD, the time consuming step is often isolating DNA from numerous samples. For researchers using amplification techniques on hundreds of plant DNA samples, large yields are likely to be less important than speed and cost of sample preparation. This procedure, with minor modifications in equipment, may useful for DNA extractions in the field, and it can be directly applied to many different plants. Our method is comparable to conventional plant DNA isolation methods in terms of the speed of isolation, requiring 3-4 h from the fresh tissue up to the final DNA resuspension. In performing population studies using RAPD, the time consuming step is often isolating DNA from numerous samples. For researchers using amplification techniques on hundreds of plant DNA samples, large yields are likely to be less important than speed and cost of sample preparation. This procedure, with minor modifications in equipment, may useful for DNA extractions in the field, and it can be directly applied to many different plants. We have found that this method generates DNA that yields more reproducible results in the RAPD system than does DNA generated by other isolation methods. This allows breeding programs to utilize marker-assisted selection more efficiently and cost effectively than previous methods.

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