

Full Length Research Paper

## Optimization of DNA extraction and amplified fragment length polymorphism (AFLP) analysis of pomegranate (*Punica granatum* L.)

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Pomegranate (*Punica granatum* L.) is a plant rich in polysaccharides, polyphenols and secondary metabolites, which makes it difficult to obtain high quality DNA. The present study reports a quick, simple and inexpensive method to isolate genomic DNA suitable for amplified fragment length polymorphism (AFLP) analysis and other PCR-based applications. This method is a modification of a protocol described by Doyle and Doyle (1990). It is a cetyl trimethyl ammonium bromide (CTAB)-based protocol modified by the use of potassium acetate (KoAc) and polyvinylpyrrolidone (PVP) to remove polyphenols and polysaccharides and a high concentration of  $\beta$ -mercaptoethanol to reduce oxidation. Moreover, the final optimized protocol was then compared with three different methods, which are routinely used for many plant species. The results show that our modified CTAB protocol produced a high yield (>500 ng/ $\mu$ l) of good-quality DNA ( $A_{260}/A_{280} >1.8$ ) compared to the other three methods. The DNA purity was further confirmed by complete digestion with *Eco*RI and *Mse*I enzymes. The modified CTAB protocol used in this study could be a useful protocol for extraction of high quality DNA not only for pomegranate but also for other plants rich in polysaccharides, polyphenolics and secondary metabolites. Using this method, DNA was extracted from 67 accessions of pomegranate. The DNA was then used for AFLP analysis. To optimize the AFLP protocol, the effects of  $MgCl_2$  concentration during selective amplification, the dilution level of pre-amplified DNA and the cycle number used in the pre-amplification were studied. After optimization of the reaction conditions, AFLP was used to study genetic diversity among Iranian pomegranate accessions.

**Key words:** Pomegranate, DNA extraction, amplified fragment length polymorphism (AFLP), secondary metabolites.

### INTRODUCTION

Pomegranate (*Punica granatum* L.), a species in the *Punicaceae* family, is a fruit-bearing deciduous shrub or small tree growing to between 5 and 8 m tall. It is native to Iran and perhaps some surrounding areas (Stover and Mercure, 2007), and that is spread from Iran to other areas (Levin, 1994). Iran is center of pomegranate diversity. About 800 accessions of pomegranate originating

from different provinces of Iran have been gathered in Yazd pomegranate collection. Germplasm collections can only be exploited in breeding programs after they are properly analyzed. Molecular marker techniques based on PCR, such as random amplified polymorphic DNA (RAPD) (Williams et al., 1990), inter simple sequence repeat (ISSR) (Zietkiewicz et al., 1994) and amplified

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**Table 1.** List of pomegranate accessions used in this study.

Accession name	Population	Main characteristic		
		Taste	Peel color	Aril color
Tabestani	Markazi	Sour	Pink	Red
Alak Shirin	Markazi	Sweet	Red	Red
Torsh Zoodras	Isfahan	Sour	Red	Pink
Malas Shahvar	Isfahan	Sweet-Sour	Red	Pink
Anbari Riz	Isfahan	Sour	White	Pink
Bazri Marvast	Yazd	Sweet	Red	Red
Daneh Ghermez Harati	Yazd	Sour	Red	Red
Jari Poost Sefid	Kerman	Sour	White	Pink
Shahi Daneh Ghermez	Kerman	Sweet	Red	Red
Torsh Mahani	Kerman	Sour	Red	Red
Shirin Hasteh Riz	Kerman	Sweet	Red	Red
Poost Siyah Saveh	Saveh	Sweet	Black	White
Torsh Poost Sefid Saveh	Saveh	Sour	White	Pink
Sandani Saveh Torsh	Saveh	Sour	Red	Red
Mesri Torsh kazon	Fars	Sour	Black	White

fragment length polymorphism (AFLP) (Vos et al., 1995) are powerful tools for plant breeding and genetic analysis. Among different marker systems available at present, AFLP is a DNA fingerprinting method with a high reproducibility; covering a great number of genome loci in one analysis. Due to its reproducibility and its powerfulness, this tool has been widely used for the characterization and genetic analysis of many plants (Vos et al., 1995). AFLP technique requires isolation of DNA with high purity for restriction digestion. Isolation of DNA free of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and tannins is most essential because these compounds can irreversibly bind to nucleic acids during extraction steps (Varadarajan and Prakash, 1991; Mishra et al., 2008).

Pomegranate contains exceptionally high amounts of polysaccharides, secondary metabolites and polyphenols including mainly ellagitannin, gallotannin, and anthocyanin which interfere with DNA isolation procedure. In their oxidized forms, polyphenols covalently bind to DNA, giving it a brown color and making it useless for most research applications (Aljanabi et al., 1999). There are several DNA extraction protocols for high polyphenol and polysaccharide containing plant species (Ghaffari et al., 2011; Abd-Elsalam et al., 2011; Wang, 2010; Sanchez-Hernandez and Gaytan-Oyarzun, 2006), but none of these are applicable to all plants. Since plants contain high amounts of many different substances, it is unlikely that just one DNA extraction protocol is suitable for all plants (Loomis, 1974).

In pomegranate, several DNA extraction methods have been successfully used (Pirseyyedi et al., 2010; Hasnaoui et al., 2010; Sarkhosh et al., 2006). However, these methods are not useful for AFLP analysis. Therefore, in

this study, we reported a rapid, simple and inexpensive protocol for isolation of high-quality DNA from pomegranate that can be used for AFLP analysis and other PCR-based applications. Furthermore, the final optimized protocol was then compared with three different methods which are routinely used for many plant species. This is the first report focusing on optimization of DNA extraction for AFLP analysis of pomegranate.

## MATERIALS AND METHODS

Plant material consisted of young leaves of adult trees sampled from different geographic populations. These were collected from Yazd pomegranate collection of National Plant Gene, Bank of Iran. In this collection, about 800 accessions of pomegranate originating from the different populations of Iran were gathered. In the present study, the number of plants tested per population varied from one to four (Table 1). Leaves were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for future use.

### DNA extraction

DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) procedure described by Doyle and Doyle (1990) with some modifications. Briefly, Young leaves (100 mg) were ground to a fine powder in liquid nitrogen and mixed with 500  $\mu\text{l}$  of extraction buffer (3% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.3 M NaCl; 4% polyvinylpyrrolidone; 2%  $\beta$ -mercaptoethanol). Samples were incubated at  $65^{\circ}\text{C}$  for 20 min, subsequently, washed with an equal volume of chloroform/isoamylalcohol (24:1), and centrifuged at 12 000 rpm for 10 min. The supernatant was mixed with 200  $\mu\text{l}$  potassium acetate (koAc) and 500  $\mu\text{l}$  of cold isopropanol carefully and cooled for 15 min and the DNA was pelleted by centrifugation at 12 000 rpm for 15 min. Pellets of DNA were then washed with 70% ethanol twice, dried and dissolved in 200  $\mu\text{l}$  of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and extracted DNA was stored at  $-20^{\circ}\text{C}$  for later use.

### Comparison analysis with other DNA extraction protocols

The DNA extracted with modified CTAB protocol was compared with three different methods. In Method I, genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions; this system uses advanced silica-gel membrane technology for isolation of total DNA without phenol, chloroform or ethanol precipitation. The buffer system allows selective binding of DNA to a membrane. In Method II, DNA was extracted using the Sodium dodecyl sulphate (SDS) mini preparation method of Dellaporta et al. (1983). In Method III, DNA was extracted by the method described by Lodhi et al. (1994).

### DNA quantification

The yield of the DNA samples was determined by measuring the absorbance at 260 and 280 nm in a UV Spectrophotometer and the purity of the DNA was then determined by calculating the ratio of absorbance at 260 nm to that of 280 nm ( $A_{260}/A_{280}$ ). DNA concentration and purity was also determined by running the extracted genomic DNA samples on 1% agarose gel using 0.5× TAE buffer (10 mM Tris-HCl and 1 mM EDTA, pH. 8.0). A DNA molecular weight size marker was used on each gel. The gels were stained with ethidium bromide and visualized under UV fluorescence.

### Optimization of AFLP reaction

For optimization of AFLP reaction, factors like  $MgCl_2$  concentration during selective amplification, the dilution level of pre-amplified DNA and the cycle number used in the pre-amplification were tested. Genomic DNA (50 to 100 ng) was digested with 5U each of *EcoRI* and *Tru9I* (isoschizomer of *MseI*) (Fermentas) restriction enzymes in a 40  $\mu$ l volume and this reaction was incubated at 37°C for 10 h, followed by incubation at 65°C for 15 min for denature of the restriction enzymes. Digested DNA fragments were then ligated with 5 pmol of *EcoRI* adapter, 50 pmol of *MseI* adapter and 1U T4 DNA ligase (Fermentas) at room temperature for 4 h in a final volume of 28  $\mu$ l. The mixture was then diluted 10-fold with sterile distilled water. Pre-amplification PCR reaction was performed using primers carrying no selective nucleotide, pre-amplification reaction containing: 4  $\mu$ l of 1:10 diluted restriction-ligation mixture, 0.6  $\mu$ M of each pre-amplification primer, 1× PCR reaction buffer, 5 mM  $MgCl_2$ , 0.2 mM dNTPs and 1U *Taq* DNA polymerase in a volume of 28  $\mu$ l. The mixture was amplified in a Bio-Rad thermocycler using the following profiles: 94°C for 30 s, 56°C for 60 s and 72°C for 60 s with varying cycle numbers (15, 20 and 25 cycles). Pre-amplified DNA was checked for the presence of smear of fragment by 1.5% agarose gel electrophoresis, the pre-amplification products were then diluted to various concentrations (10, 20, and 50- fold) to optimize the selective amplification. The selective amplification was performed using the *EcoRI* and *MseI* primers with three selective nucleotides at 3' ends. Selective amplification reaction included 4  $\mu$ l of diluted pre-amplified DNA, 0.75  $\mu$ M of each primer combination, 1× PCR reaction buffer, different  $MgCl_2$  concentrations (2, 3.2 and 4 mM), 0.3 mM dNTPs and 1U *Taq* DNA polymerase in a volume of 20  $\mu$ l. The reaction was performed following the profile: 1 cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, then 15 cycles of 94°C for 30 s, 65°C for 30 s (with decreasing ramp of 0.7°C each cycle) and 72°C for 60 s followed by 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s and a final extension of 72°C for 10 min.

### Gel electrophoresis and silver staining

The final PCR products were mixed with an equal volume of loading buffer composed of 99% formamide, 10 mM EDTA pH 8, 0.1%

xylene cyanole and 0.1% bromophenol blue; the mixture was denatured at 95°C for 5 min and cooled immediately on ice. 3  $\mu$ l of each sample was loaded on a pre-warmed 6% polyacrylamide gel running with 1× TBE buffer, electrophoresis was carried out at 80 W and 50°C for 1 h until the forward-running dye reached the end of the gel, followed by silver staining using the procedure below: after electrophoresis, the gel was fixed in 1% acetic acid for 15 min. After rinsing in distilled water, the gel was stained with a 0.2% silver nitrate solution for 20 min. The stained gel was rinsed twice with distilled water and was placed in a developing solution composed of 3% sodium carbonate and 0.06% formaldehyde until solution became dark. It was placed again in the developing solution until the bands became visualized; the development was stopped with 1% acetic acid and after rinsing in distilled water, the gel was dried and was scanned.

## RESULTS AND DISCUSSION

### Evaluation of quantity and quality of DNA

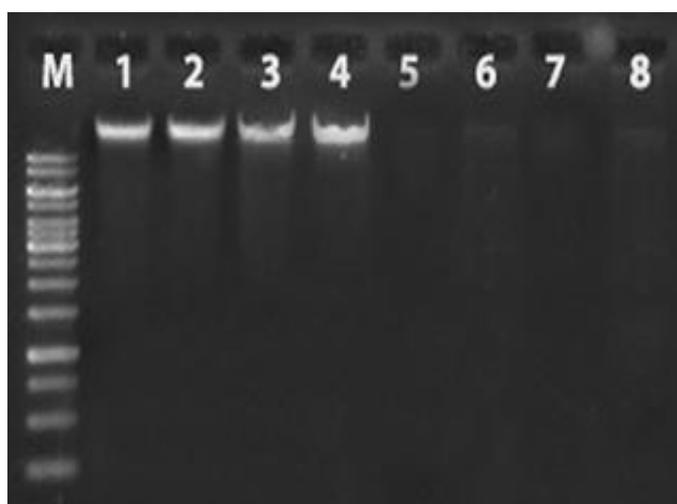
In pomegranate, several molecular markers have been used for genetic studies (Melgarejo et al., 2009; Sarkhosh et al., 2006; Pirseyedi et al., 2010; Jbir et al., 2008; Yuan et al., 2007a). Since pomegranate is a plant rich in polyphenols and polysaccharides, and these inhibitors interfere with *Taq* DNA polymerase and restriction enzymes, their removal can have a great impact on successful molecular-based studies and PCR reaction. CTAB based extraction methods are frequently used for DNA isolation from plant tissues. The extraction technique of Doyle and Doyle has been applied successfully in horticulture and fruit trees, such as, apricot (Yuan et al., 2007b), plum (Ilgin et al., 2009), and grape (Lodhi et al., 1994).

In the present study, significant changes to the method reported by Doyle and Doyle (1990) consisted of higher CTAB and  $\beta$ -mercaptoethanol concentrations, use of potassium acetate (KoAc) and polyvinylpyrrolidone (PVP). Detergents like CTAB and PVP remove polyphenols and polysaccharides, while the  $\beta$ -mercaptoethanol reduces oxidation (De la Cruz et al., 1997). PVP has been used to remove polyphenols from leaf tissues in several protocols (Lodhi et al., 1994; Doyle and Doyle, 1987; Aljanabi et al., 1999; Kim et al., 1997). PVP or PVPP (polyvinylpoly-pyrrolidone) efficiently forms complex hydrogen bonds with polyphenolic compounds and alkaloids, which can be separated from DNA by centrifugation (Maliyakal, 1992). However, in some cases, brown color of DNA in our study was not lost with PVP. Only when potassium acetate has been applied, removing of phenolic compounds was possible.

In this study, the modified CTAB protocol resulted in the greatest yield of DNA. A lesser amount of DNA was extractable from the Qiagen commercial kit. The methods of Lodhi et al. (1994) and Dellaporta et al. (1983) produced extremely little DNA. The results of the quantification are displayed in Table 2. The  $A_{260}/A_{280}$  absorbance ratios varied between 1.13 and 2.03 for DNA extracted with Dellaporta et al. (1983) and modified CTAB protocol respectively; indicating the highest and lowest protein contamination among the four methods, respectively.

**Table 2.** Yield and purity of DNA obtained by four extraction methods.

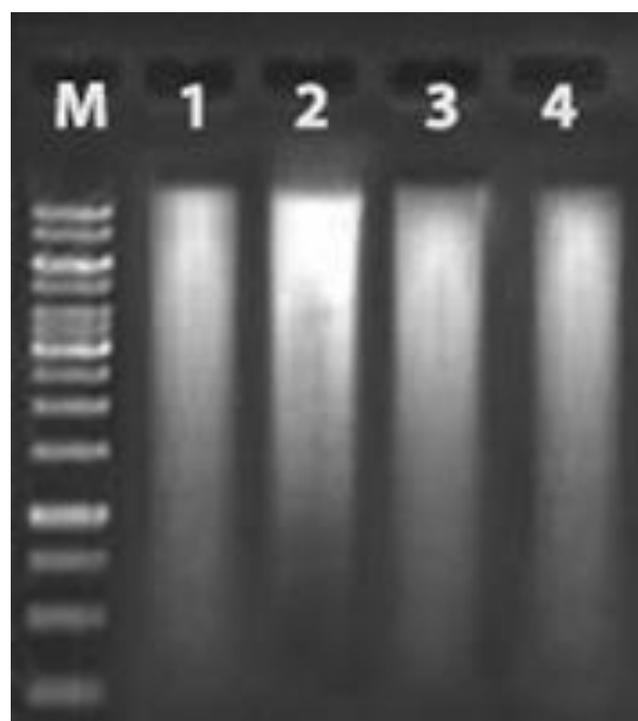
Extraction method	$A_{260}/A_{280}$ ratio	DNA yield (ng/ $\mu$ l)
Modified CTAB	2.03	532.47
Qiagen DNeasy	1.49	295.84
Lodhi et al. (1994)	1.14	3.38
Dellaporta et al. (1983)	1.13	2.03



**Figure 1.** Genomic DNA extracted by the following four different protocols: Lanes 1 and 2, the modified CTAB; lane 3 and 4, Qiagen DNeasy Plant Mini Kit; lanes 5 and 6, Lodhi et al., (1994) and lanes 7 and 8, Dellaporta et al., (1983). Lane M represents the Molecular weight marker—GeneRuler™ 1 kb DNA ladder.

When DNA is extracted, protein frequently remains in the DNA solution. Protein is tightly bound to DNA, and complete removal of protein is not always possible. Generally, the peak of UV absorption is at 260 nm for DNA and at 280 nm for protein. Thus, when a solution contains both DNA and protein, absorbance at 260 nm is mainly due to the DNA present, and absorbance at 280 nm is due to protein. The expected ratios for extracted DNA samples should range from 1.7 to 2.0 (Miller et al., 2009). DNA obtained using the modified CTAB protocol and isolation kit appeared on the agarose gel as a single band of high molecular weight (greater than 10,000 base pairs) and there is little or no evidence of degradation, shearing, or RNA contamination. Lodhi et al. (1994) and Dellaporta et al. (1983) methods failed to produce a visible band of genomic DNA (Figure 1).

Products of digestion with *EcoRI* and *MseI* restriction enzymes showed that DNA obtained by the modified CTAB protocol was completely digested (Figure 2). So these results confirmed that the extracted DNA by the modified CTAB protocol has a better quality and quantity compare with the other three methods. Using our modified CTAB protocol, high quality DNA samples from different populations of pomegranate consisting of 67

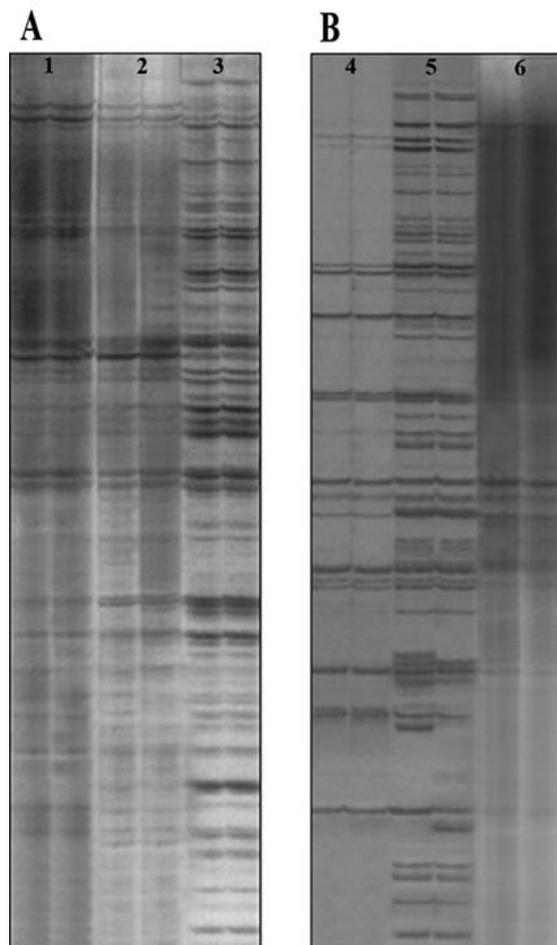


**Figure 2.** Restriction digestion of DNA isolated by the modified CTAB protocol. Lanes 1 to 4 show genomic DNA from four accessions of pomegranate digested with *EcoRI*/*MseI* enzymes. Lane M shows 1 kb DNA ladder.

accessions were extracted by a single person in less than one day. The obtained DNA was suitable for applications involving restriction enzyme digestion or PCR-based applications.

### Optimization of AFLP reaction

The extracted DNA with our modified protocol was subjected to AFLP analysis. Optimization of AFLP reaction is essential for band generation and reproducibility of the results. Therefore, we examined the effects of different  $MgCl_2$  concentrations during selective amplification, the dilution level of pre-amplified DNA and the cycle number used in the pre-amplification on the quality of AFLP profiles. Figure 3 shows the effects of different  $MgCl_2$  concentrations (2, 3.2 and 4 mM) and the dilution

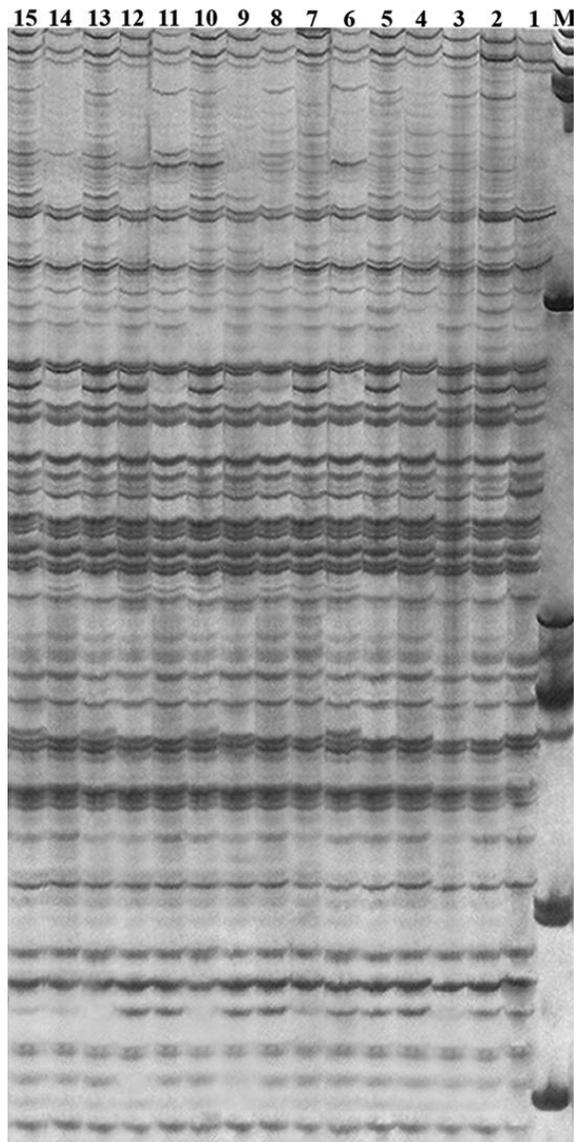


**Figure 3.** Effects of dilution level of pre-amplified DNA (A) and  $MgCl_2$  concentration (B) on the AFLP fingerprint. Lanes 1-3 represent different dilutions of DNA (10-, 20- and 50-fold, respectively) and lanes 4-6 represent different  $MgCl_2$  concentrations (2, 3.2 and 4 mM respectively).

level of pre-amplified DNA (10, 20, and 50-fold) on the quality of AFLP profiles. In higher DNA concentrations, there was a high background smear due to competitive inhibition between fragments during PCR that completely disappears in low concentrations so that the bands were scorable. This background smear was also present in higher  $MgCl_2$  concentrations. Furthermore, decrease in pre-amplification cycle numbers resulted in poor amplification. The use of 50-fold dilution, 3.2 mM  $MgCl_2$  and 25 cycles of pre-amplification resulted in the best resolution and clarity of profiles (Figure 4). After optimization of the reaction conditions, polymorphism among Iranian pomegranate accessions was studied using different primer pairs.

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**Figure 4.** AFLP profiles of pomegranate on 6% polyacrilamide gel using DNA isolated by the modified CTAB protocol. The selective primer combination used was E-AAC/M-CAC. Lanes 1-15 represents accessions as described in Table 1, M is the 50 bp DNA ladder.

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