INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 3, p. 223-231, 2017

RESEARCH PAPER OPEN ACCESS

Extraction of purified plants gDNA free of Secondary metabolites

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Key words: gDNA, Molecular Techniques, Peanut, Polyphenols, Polysaccharides

http://dx.doi.org/10.12692/ijb/10.3.223-231 Article published on March 17, 2017

Abstract

Extraction of high-quality gDNA from plants is a basic step in molecular biology research. In some plant species like peanut getting the genomic DNA in its purified form is a very challenging job as compared to other crops. The reason behind which is the coexistence of different secondary metabolite impurities i.e., polyphenol and polysaccharides with genomic DNA. In this paper, we have optimized a protocol for extracting high-quality gDNA which is amenable to various improvement studies like PCR amplification and other molecular research. The OD 260/280 ratio for the extracted DNA was found within the range of 1.8 to 1.99 while OD 260/230 ratio was between 2.0 to 2.20, using Nano Drop ND-2000/2000C Spectrophotometer. Similarly, the concentration of the extracted DNA for all the extracted samples was greater than 300 ng/ul. The obtained results through our current method indicate the fitness of the extracted DNA for using it in PCR amplification and other molecular research. The novelty in our protocol is the use of Buffer A before CTAB treatment of the samples.

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Introduction

Peanut, also known as groundnut (*Arachish ypogaea* L.), is one of the prominent legumes crops throughout the globe. The main fact that this crop has worldwide growing adaptability is its lower needs of inputs as compared to other crops and more importantly is its adaptation of semi-arid growing conditions. It provides a good share in human nutrition in terms of containing about 25% of protein and 50% of oil in mature seeds. The cultivated peanut is originated from the hybridization of two diploid species i.e., *Arachis duranensis* (A-genome) and *Arachis ipaensis* (B-genome) followed by a rare spontaneous duplication of chromosomes making its genome allotetraploid having 2n = 4x = 40 (Seijo *et al*., 2004-2007; Favero *et al*., 2006; Milla *et al*., 2005).

DNA extraction is pivotal to molecular biology and especially to biotechnology. It is the preliminary step for imminent molecular biology research. To further improve the yield, quality and stress tolerance of peanut varieties various techniques are applied i.e., Marker-assisted breeding, Quantitative trait locus (QTL) analysis, gene cloning and other different types of genetic and genomic tools. Getting good results through these techniques a supreme quality of genomic DNA is needed to be isolated from the available genetic resources of peanut. But many plants crops like peanut containing secondary metabolites which affect not only the extraction of high-class genomic DNA (gDNA) but also hamper the succeeding reactions (Kotchoni and Gachomo, 2009; Kotchoni *et al*., 2011). In these crop plants isolating gDNA in its purified form is a very challenging job due to highly contamination by various impurities including polysaccharides, terpenes and other polyphenols compounds. Among these contaminator compounds, the polyphenols are the most serious ones making the extracted DNA sample unsuitable to be used in various upcoming research applications (Shepherd *et al*., 2002; Li *et al*., 2002). Therefore it always remains a thirst to have a good and consistent DNA isolating protocol. One of the promising steps to minimize the contamination level of these compounds is to avoid the selection of old and mature leaves for DNA extraction because mature leaves have the phenol and polysaccharides in comparatively high amount. Due to the need of best quality consistent DNA isolation in the past various efforts were made in a diverse range of plant species i.e., *Arachis hypogaea, Pinus radiate*, *Mucuna pruriens*, *Parkia timoriana* and *chickpea*etc (Sharma *et al*., 2000; Claudia *et al*., 1998; Padmesh *et al*., 2006; Dipankar *et al*., 2006; Robert *et al*., 2003).

The thirst for exploration of a method of extracting gDNA having both higher yield and quality has led to the optimization of a wide range of DNA extraction protocols. It is essential that the extracted DNA must be cleansed from cellular substantial in a manner that avoids degradation. In the current paper, we have optimized a very simple protocol that gives a comparatively good quality of gDNA, which can be used for various molecular research applications like PCR amplification etc.

Materials and methods

Biological material and study location

Plant samples for DNA extraction were taken and the current protocol was optimized at State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, College of Crop Science, Fujian Agriculture and Forestry University Fuzhou, Fujian, China.

Reagents and chemicals required

Reagents and chemicals required for the current DNA extraction protocol include Buffer A (Table 1), Buffer B (Table 2), Chloroform: isoamyl alcohol (24:1), ice-chilled 2-propanol, RNase A, 95% and 75% Ethanol.

DNA extraction protocol

Preliminary steps

To start grinding of samples ones must ensure the sterilization of equipment used for grinding, which in most cases consists of mortar and pestle. In the case of using grinder machine for grinding of the samples its spike part which comes in contact with the samples should be sterilized after grinding each sample to avoid the risk of contamination among the samples.

The 37°Cand 65°C water baths should be pre-heated. Another important preliminary step is to preheat the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (in our case buffer B) in the 65°C water bath. The distilled water must also be autoclaved to make it double distilled. Label the centrifuge tubes with permanent marker and made sure the availability of required micro-pipettes.

Grinding and tissue disruption

Take the leaf and thoroughly wash it with 75% ethanol to make it surface sterilized. Then put about 1 gram of that sample in a mortar (if you are using mortar and pestle) or in 2 ml centrifuge tube (if you are using the grinding machine) and after adding liquid nitrogen grind it completely until to the fine powder.

Treatment with Buffer A

After making the sample turn into fine powder then add 1000 ul of buffer A (Table 1) and put the samples for 10 minutes on ice. This step is the most important one to eliminate the contaminators like polysaccharides and other polyphenol compounds. Buffer A contain β-mercaptoethanol which denature the protein and polyphenol compound. Along with these two, buffer A, also containing Ethylene diaminetetra acetic acid (EDTA) which is a good chelating agent, which neutralizes the negative charge on DNA and bring the molecules closer.

The other constituents of buffer A are Tris and salt (NaCl) which provide buffer condition and precipitation of DNA, respectively. After 10 minutes of ice treatment centrifuge the samples on 5000 rpm for 5 minutes. For best results, it is better to repeat the step. Then discard the supernatant and add 1000 ul of buffer B to tubes having samples.

Application of Buffer B

After adding buffer B (Table 2) put the samples in 65°C water bath for 15 to 30 minutes. Invert the sample thoroughly to mixed it properly after each 5 minutes till the completion of the incubation period. The additional ingredient in Buffer B is CTAB which is a detergent for the disruption of the membrane and separates the DNA and the histone of the chromosome. Centrifuge the samples on 12000 rpm/10 minutes after 30 minutes of 65°C incubation. Then, transfer the supernatant to another new sterilized centrifuge tube and discard the lower phase.

Adding chloroform: Isoamyl alcohol (24: 1)

In this step 750ul chloroform: isoamyl alcohol (24: 1) is added to the samples and gently inversion is carried out until to turn the sample milky. Then centrifugation is carried out at 12000 rpm for 10 minutes. After centrifugation, the upper portion (supernatant) is transferred to another tube and the lower phase is discarded. During pipetting out the supernatant extreme care should be taken to avoid touching or pipetting out the interphase. Because in this marginal layer which separates the two phases is the region where the denatured contaminants are aggregated. Care should be taken during this step to avoid the vigorous shaking of samples because it can shear the DNA.

Purification of DNA

To ensure the complete removal of RNA from the samples add 1 ul of RNase A (10 mg/ml) to the DNA samples. After adding the RNase A, incubate the samples for 40 minutes in 37°C water bath. After incubation add the same amount of chloroform: isoamyl alcohol (24: 1) and centrifuge the samples at 12000 rpm for 10 minutes. Repeat the chloroform: isoamyl alcohol (24: 1) addition and centrifugation until the middle layer completely disappears.

Precipitation

To precipitate the extracted DNA transfer the supernatant to 1.5 centrifuge tube and add 450 ul of Ice chilled 2-propanol.

Then put the samples for 15 minutes in -80°C freezer. Ice chilled 2-propanol precipitate the DNA in the solution. After 15 minutes incubation, centrifuge the samples on 12000 rpm for 10 minutes to make the pellet of the precipitated DNA.

Washing

Washing of pellet is carried out through 75% ethanol by centrifugation at 12000 rpm for 5 minutes 2 to 3 times. This step is important to remove the remaining alcohol and salts from the extracted DNA samples.

Drying

Discard the ethanol completely with care and place the open centrifuge tubes in the dryer for 10 minutes or at room temperature till the pellet become completely dry. Putting the samples at room temperature will require little longer time as compared to the drier.

Re suspending DNA

The extracted DNA pellet can be resuspended in TE buffer (10mM Tris: 1mM EDTA) and double distilled water. In our case, we used 100ul double distilled water but for long storage, TE buffer is preferred.

Quality assessment of the extracted DNA

To assess the quality of extracted DNA the samples were checked through Nano Drop ND-2000/2000CSpectrophotometer (Thermo Fisher Scientific Inc, USA), agarose gel electrophoresis, different storage temperatures, and PCR amplification.

Results

Quality analysis of extracted DNA through NanoDrop ND-2000/2000c Spectrophotometer

We extracted good quality of gDNA through the current extraction protocol. The OD260/280 ratio for the extracted DNA was found in the range of 1.8 to 1.99 while the OD260/230 ratio was between 2.0 to 2.20, using Nano Drop ND-2000/2000C Spectrophotometer (Fig. 1). These ratios indicate the fitness of the extracted DNA for using it in sequencing and PCR amplification. Similarly, the concentration of the extracted DNA for all the samples was above 300 ng/ul.

Table 1. Buffer A composition.

Reagent	Volume	Final concentration
1.0 M Tris-HCl	20ml	0.2 M
0.5M EDTA-Na	10ml	0.05M
NaCl	1.5g (0.25 M)	0.25M
β-mercaptoethanol	1% (V/V)	1%（V/V）
PVP-40	2% (W/V)	2%（W/V）
ddH2O	69ml	
Total	100ml	

Analysis of extracted DNA quality by gel electrophoresis

The extracted gDNA was quantified on 1% agarose gel to further check its quality.

The gel pictures (Fig. 2A) shows various samples extracted from peanut and (Fig.2B) i.e., DNA extracted from different plants are very clear from protein and polysaccharides contaminators. These pictures are a clear indication of our protocol is suitability not only for peanut but also for other plants like tobacco, rice, green chilli, and beans.

Analysis of storage stability of the extracted DNA

The extracted gDNA through our current protocol was also subjected to quality test upon various storage conditions. Same samples were stored at different temperature i.e., -20°C (for 30 days) and -80°C, 4°C, 37°C and at room temperature (for 7 days).

Then 1 ul of each sample was run on 1% agarose gel on 150V for 20minutes (Fig.3) to know about its quality after its treatment with different storage conditions.

Comparison of DNA extracted with and without Buffer A, application

To check the importance of buffer A, we extracted five samples treated with buffer A and without buffer A treatment. The samples treated with buffer A were very clear from the secondary metabolites (Fig.4A) while other five, not treated with buffer A, were extremely contaminated by secondary metabolites and instead of clear bands share bands were found for these samples (Fig.4B). These results are the clear justification for the superiority of our developed protocol for extracting gDNA from plants having highly coexistence of secondary metabolites with genomic DNA.

Table 2. Buffer B composition.

Reagent	Volume	Final concentration
1.0 M Tris-HCl	10 ml	0.1 M
0.5M EDTA-Na	4 ml	0.02M
CTAB	2grams	2% (W/V)
NaCl	8.2grams	1.4 M
β-mercaptoethanol	2mL	2%
PVP-40	3grams	3% (W/V)
ddH2O	84ml	
Total	100ml	

Analysis of extracted DNA quality through PCR amplification

To further assess the quality of the extracted DNA, Polymerase Chain Reaction was carried out. Samples were amplified using AhqBW1gene primer through an initial temperature of denaturation 94°C for 5 minutes followed by 94°C for 15 seconds.

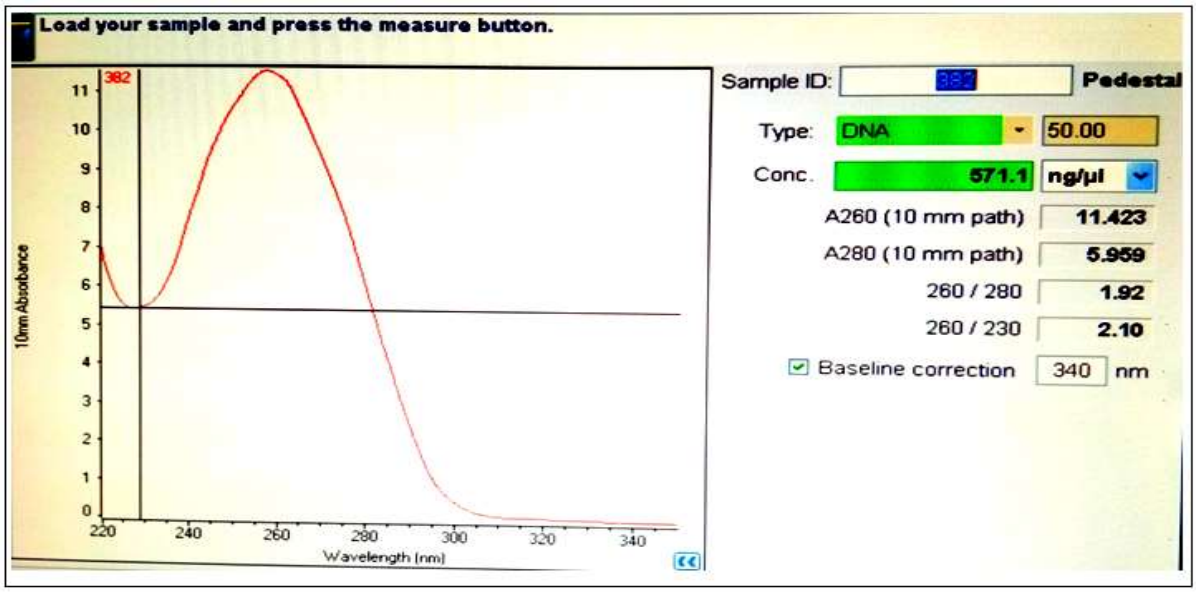

Fig. 1. Assessment of extracted DNA quality through Nano Drop Spectrophotometer.

The annealing temperature was kept 55°C for 15 seconds followed by initial extension of 2 minutes and 30 seconds at 72°C and the final extension of 72°C for 6 minutes. Polymerase Chain Reaction was carried out in 20 µl volumes including 10ul Prime STAR® Max DNA Polymerase, 0.5ul of each forward and reverse primer, 8ul ddH_2O and 1ul of DNA template. After PCR amplification the PCR product was separated through 1% agarose gel using 2 kb DNA marker (Fig. 5).

Discussion

The purpose of optimizing the current protocol was to get good quality of the gDNA from peanut,

which is significantly challenged by secondary metabolite contamination as compared to other crop species.

Using CTAB method for DNA extraction is the most preferred method for plants. The fact behind its preference is that it is a great cationic surfactant.

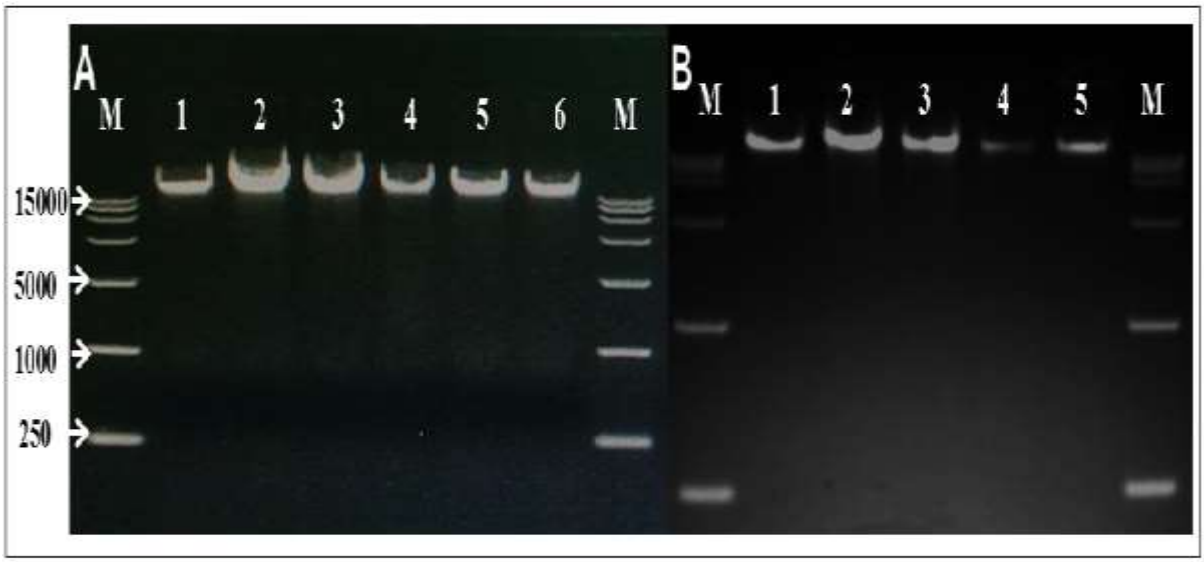

Fig. 2. AM= 15000kb DNA marker, 1,2,3,4,5,6= Peanut samples. (B): M= 15000kb DNA marker, 1= Peanut, 2= Tobacco , 3= Green Chilly, 4= Rice , 5= Bean.

Plants like peanut having a high amount of secondary metabolites contaminators which badly affect the quality of gDNA during extraction for various purposes. But when CTAB is used in combination with some other chemicals like Polyvinylpyrrolidone (PVP) and NaCl it gives a very good quality of gDNA and this is why CTAB extraction method is the superior and preferred one among all other methods. The first goal in gDNA extraction is its concentration and through the current method, it was above 300ng/ul, which is good to be used for different purposes like PCR amplification and sequencing. Our results are supported by Cavallari *et al.*, (2014).

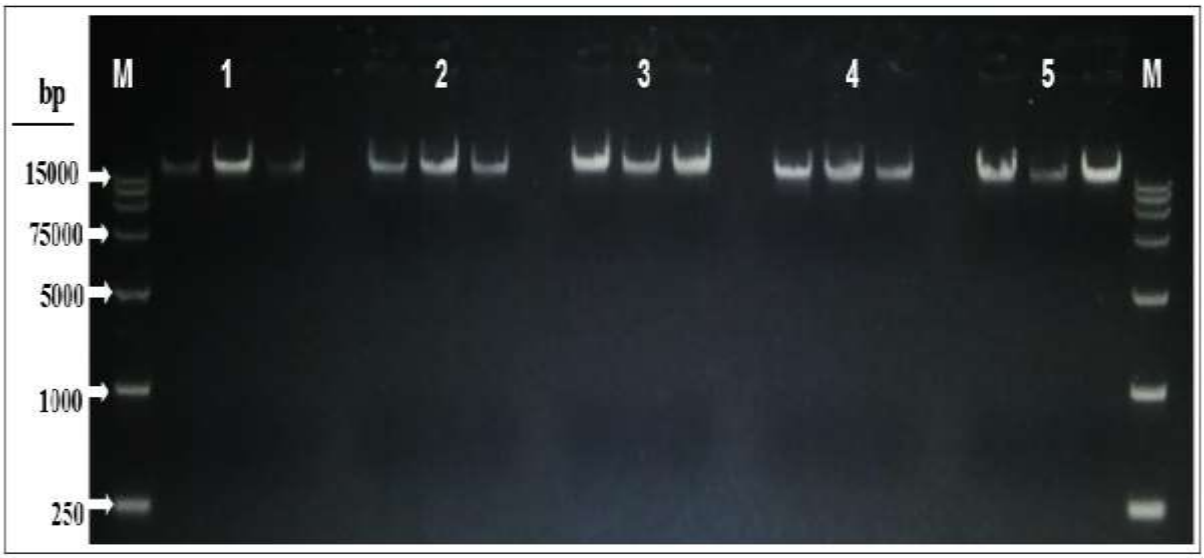

Fig. 3. Samples stored on different temperature i.e., 1 = -80°C (for 7 days), 2 = -20°C (for 30 days), 3 = 4°C (for 7 days), 4 = 37°C (for 7 days), 5 = room temperature (for 7 days).

Our method is further supported by Sherma *et al.*, (2000), they also used a CTAB method with some modification to isolate gDNA from plants having secondary metabolite contaminators. We used good concentration of CTAB and sodium chloride (2% w/v and 1.4 M, respectively) followed by chloroform: isoamyl alcohol washing to remove the polysaccharides and proteins.

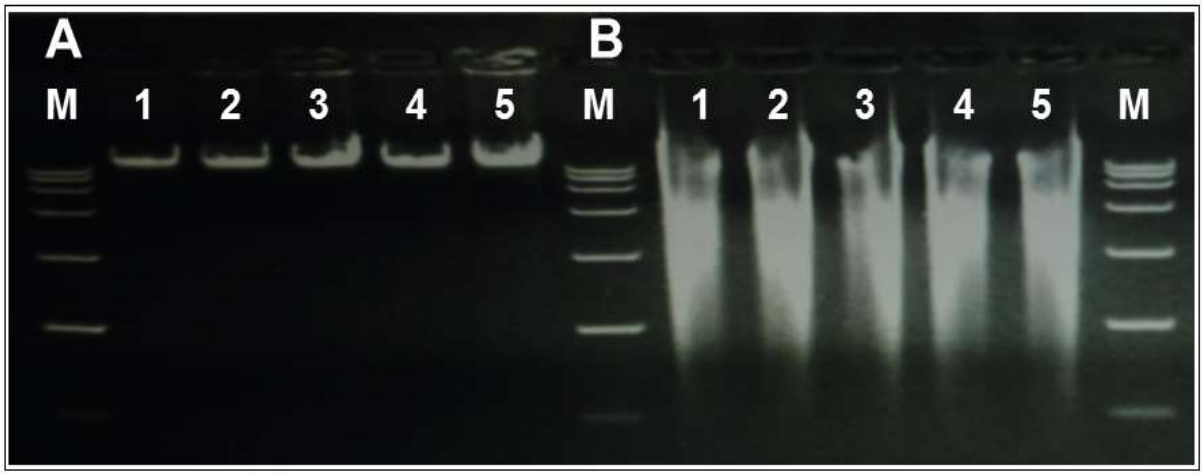

Fig. 4. (A) Samples extracted with Buffer A (B) Same samples extracted without Buffer A.

Some other scientists also applied high concentrations of CTAB and NaCl followed by multiple washing through chloroform: isoamyl alcohol for removing the polysaccharides and proteins (Dipankar *et al.*, 2006; Paterson *et al.*, 1993; Suman *et al.*, 1999; Murray and Thompson, 1980). Along with concentration for gDNA, it is must to have high quality.

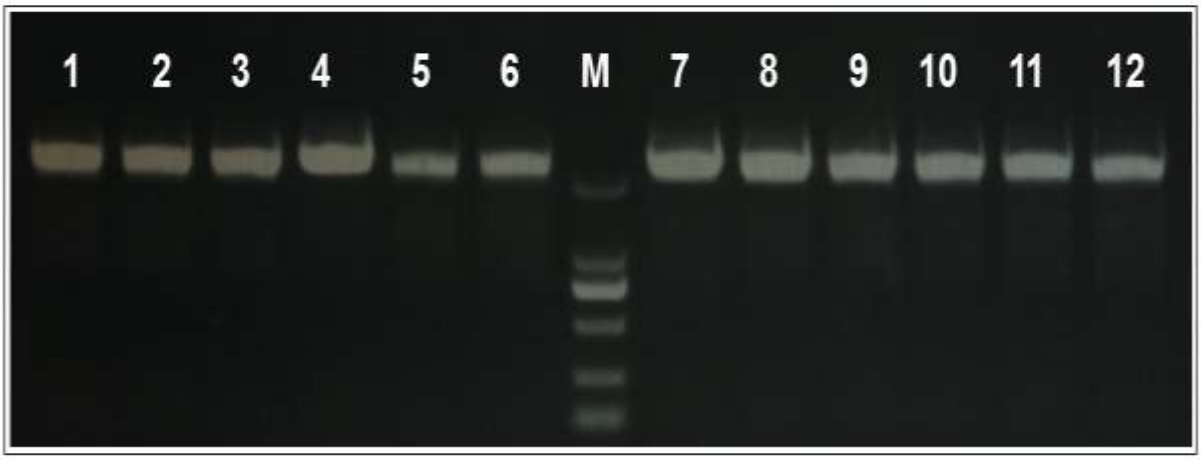

Fig. 5. PCR amplification of the extracted gDNA.

The quality of extracted gDNA through our current protocol was OD260/280 from 1.8 to 1.99 while the OD260/230 ratio was between 2.0 to 2.20, which is good quality. The good quality is considered to have the Nano-Drop ratios of OD 260/280 and 260/230 from 1.80 to 2.0 and 2.0 to 2.20, respectively (Kasem *et al.*, 2008).

Conclusion

We have established a protocol for obtaining good quality gDNA from plants, having the high level of secondary metabolites. The pronounced method is a clear improvement over previous methods due to the application of buffer A. Obtained gDNA through this protocol is suitable to be used in various molecular research applications and can be applied for extracting the gDNA from different oilseed crops.

Acknowledgment

The work was supported by National Science Foundation of China (NSF 31601337). We are thankful to the College of Crop Sciences, Fujian Agriculture and Forestry University for providing the laboratory and facilities.

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