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An efficient protocol for DNA isolation from the genus *Pyrus*

Mohammad Islam*, Habib Ahmad, Imtiaz Ahmad Khan

Department of Genetics, Hazara University, Mansehra, KP, Pakistan

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Abstract

This paper communicates an economical, handy, quicker and reliable protocol of DNA isolation from different parts e.g. herbarium specimens, bark, wood, leaves etc. of pears. The protocol is recommended under the normal lab conditions without using any sophisticated equipment and costly kits/chemicals. Best quality of DNA was extracted from any part of the plant. The PCR product was amplified for 18S rRNA genes of various *Pyrus* genotypes. The genes were cleaned with gel elution Kit (K0513, Fermentas) and sequenced accordingly. The sequence data obtained was BLAST against the entire available nucleotide database using online tool at NCBI access and the land races were successfully discriminated. The protocol presented here is recommended for reliable extraction of best quality and high yield of DNA from woody plants.

* **Corresponding Author:** Mohammad Islam ✉ mislamsw2@gmail.com

Introduction

Pears belonging to the genus *Pyrus* of family Rosaceae (Peng *et al*., 2000) are cultivated in Europe and Asia for more than three thousand years and currently cultivated in temperate regions in more than 50 countries (Bell 1990; Bell *et al*., 1996; Teng *et al*., 2004) including Pakistan (Islam *et al*., 2012; Hussain *et al*., 2006). Nearly one and a half dozon of pears are available as land races in the traditional farms of the northern Pakistan, which has never been explored with reference to their species and genetic diversity. Before starting the detailed biosystematic endeavor of pears, it was imperative to optimize protocol for its DNA isolation of plant parts available in different parts of the year, which can be used for genetic characterization of pears, which have not been identified due to low morphological diversity and overlapping traits (Jang *et al*., 1991; Chevereau *et al*., 1997; Frankhan *et al*., 2004). The recent application of molecular biology have revolutionized the use of DNA for better understanding of genome structure, evolution and identification of species, which needs high quality genomic DNA (Kim *et al*., 2000; Kimura *et al*., 2003). Various protocols have been report for isolation for yielding high quality DNA (Saghai-Maroof *et al*., 1984; Doyl, 1991; Yamamoto, 2003) each having their own limitations and scope. The longer processing time, need of expensive equipments, costly kits/chemicals and low quality yield generally hampers their application in fruit trees (John, 1992; Kim *et al*., 1997; Shepherd *et al*., 2002; Aganga *et al*., 2003; Do *et al*., 1991; Shepherd *et al*. 2002; Verma *et al*. 2007). The low quality yield is due to variety of chemical constituents in trees (Scott *et al*. 1996; Shepherd et al. 2011), which needs modification in the protocol accordingly (Barzegari *et al*. 2010; Li *et al*., 2010; Smyth *et al*. 2010).

The land races of pear and its allied species, for centuries, remained a source of food and marginal earning for traditional communities of Western Himalayas including Pakistan.These resources are still available in the subsistence farms of the area. Nothing is known about its species diversity and genetic potential of the widespread landraces of the area, which is imperative for commercial utilization of these resources. We were successful in optimizing cost effective, easy to use and widely applicable protocol for the extraction of good quality genomic DNA for genetic fingerprinting of pears.

Materials and methods

Plant materials

Plant materials such as shoots, fallen dry leaves bark, wood and herbarium specimens were collected from different areas of Northern Pakistan and Azad Kashmir and were dried at room temperature.

Methodology

The materials were ground to fine powder through pestle and mortar, at room temperature. The ground samples were packed and labeled properly. For DNA Isolation, 0.12g of the powdered material was put into 15µl centrifuge tube, which was added with 7.00 ml of 3% heated (60°C) CTAB solution. The constitution of CTAB solution was kept as; 3%CTAB, 100mM Tris-HCl, 2.5 M NaCl, and 20mM EDTA (both the Tris and EDTA's pH was kept standard to 8.0). The powdered samples were mixed well in the CTAB solution, with the help of micropipette tip. Lysis Buffer (10% SDS, 0.1 M Tris HCl, 20 mM EDTA pH. 8.0, 0.1 M Tris-HCl pH. 8.0) 1.00 ml, β. merceptoethanol 200µl and PVP 0.1g were added to each sample. The samples were water heated at 60°C for 1.00 hrs and the tube was inverted after 15.00 min. 500 µl of Chloroform Isoamyl Alcohol in the ratio of 24:1, were added to each sample and centrifuged at 10,000 rpm for 15min. The supernatant was shifted to another eppendorp tube and 500 µl Chloroform Isoamyl Alcohol were again add to each samples. It were again centrifuged at 10,000 rpm for 15min. The supernatant was shifted to eppendorp tube and 500 µl of Isopropenol and 30 µl Sodium acetate was again added to each samples. The samples were gently inverted and kept at -20°C for 1 hour and centrifuged at 12,000 rpm for 15min for getting the DNA pellet. The pellet was kept in 70 % ethanol for

10 minutes and slowly inverted few times. The ethanol was discarded and the pellet was dried at 35°C for 30 minutes. Each pellet was added with 30µl Tris EDTA (TE) buffer and water heated at 60°C for 5 minutes. Only 5.00µl of the extracted genomic DNA was run and checked on 1.00% agarose gel. The gel was visualized in gel documentation system, its report is provided in Fig. 1.

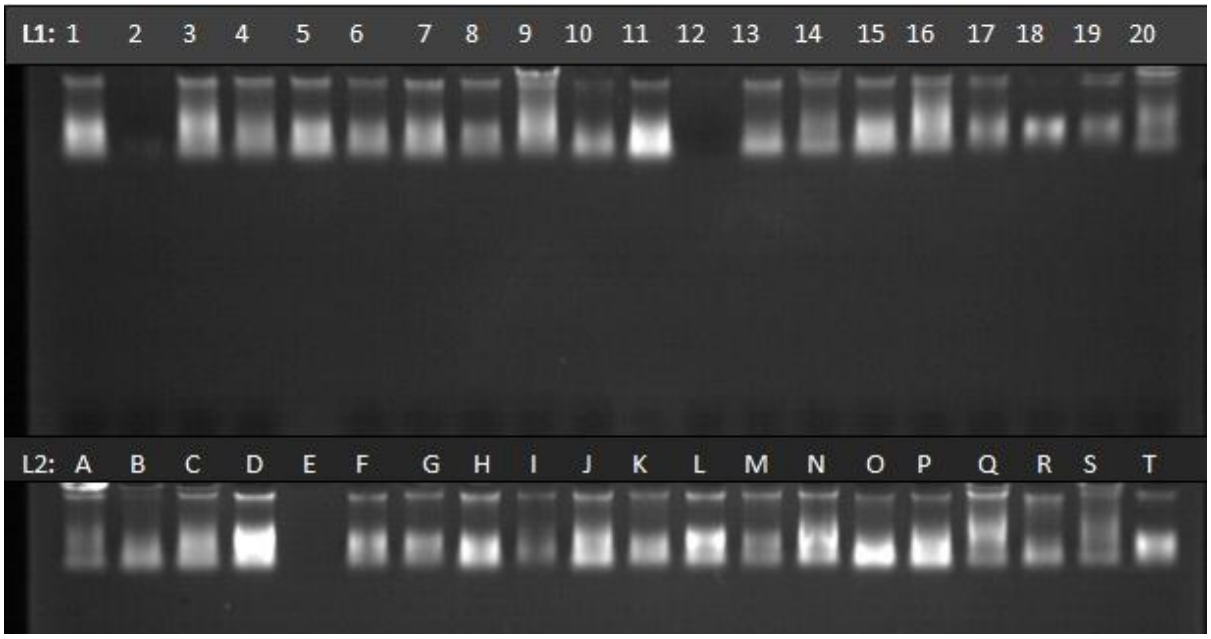

Fig. 1. Gel documentation image of genomic DNA extracted from Pyrus samples, from bark (L1) and from wood (L2).

Polymerase Chain Reaction (PCR)

PCR was performed for the amplification of 18S rRNA genes of different *Pyrus* genotypes. A typical PCR mix contained 200µM of dNTPs, 25mM of $MgCl_2$, 10x Taq Buffer, 20 pmol of forward and reverse primers each, 50ng of template DNA, 0.5 µl (2.5U) of Taq DNA Polymerase; made the final volume of 25µl dH_2O. The conditions for amplification of the said gene in thermal cycler (Applied Biosystems-2720) were adjusted at 94°C for 4 minutes as pre PCR denaturation step, 35 repeated cycles of denaturation for 40 seconds, annealing was 54°C for one minute, extension at 72°C for 1.5 minutes. A final extension step was carried out at 72°C for 5 minutes. The PCR products of all the samples were resolved on 1.5 % agarose gel in gel electrophoresis (Fig. 2).

Elution of gel

The PCR amplified fragments were eluted from the gel. The gel along the amplified fragment was excised with scalpel and kept in eppendorp tube and maked with allotted number. Gel elution Kit (K0513, Fermentas) was used for purification of the excised fragments. 700µl of binding solution was added to each sample and incubated at 56°C for 5-10 minutes and gently shacked every 3 minutes to mix well. A 5µl of Salica beads were added to each samples, incubated at 56°C for 5 minutes and centrifuge at 7000 rpm for 1.00 min. The supernatant was discarded and 500µl wash buffer was added to the pellet and centrifuged again at 10000 rpm for 1.00 min. The pellet was air dried after discarding the supernatant. The pellet was dissolved in 50 µl of TAE buffer and centrifuged at 10000rpm for 2 minutes. The upper liquid layer containing eluted DNA was carefully shifted to another eppendorf tube. 5µl DNA was loaded into 1.5% agarose gel for the conformation of DNA and the gel was visualized in gel documentation system (Fig. 3).

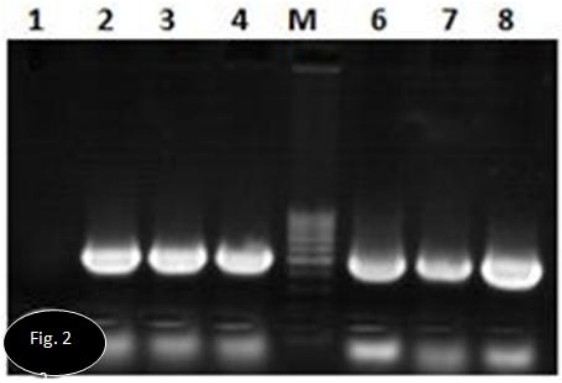

Fig. 2. PCR amplification of a 500 bp fragment of 18s rRNA of *Pyrus* genotypes. Number 1 shows negative control, 2, 3, 4 are th nuclear DNA bands from *P. sinkiangensis*, 6 is from the land race Shaker, 7 from *P. communis*, 8 from *P. hopeiensis*, M. showing the marker.

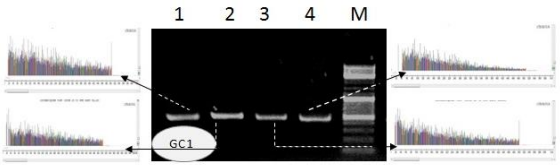

Fig. 3. This Fig. shows the presence of the cleaned genes. Number 1 shows the amplified DNA segment from *P. sinkiangensis*, 2 shows Shaker Batang, 3 shos Gultar Tango, 4 shows *P. pyrifolia*, M shows the marker DNA and arrowheads showing their respective sequence.

Results

Our experience of DNA extraction from bark, wood, branches, fallen dry leaves of pears, herbarium specimens and some other plants i.e *Brassica* and *Rosa* spp including mushrooms (not incorporated in this paper) through using the modified protocol of a number of scientists (Saghai-Maroof *et al.*, 1984; Doyle 1991; Kim *et al.*, 1997; Kumar *et al.*, 2003; Yamamoto, 2003) and its PCR product as given in Fig 2 shows this protocol is excellent for quality DNA extraction from *Pyrus* species throughout the year. No degradation of DNA was observed in the samples. It is evident from the figure that all the samples had approximately similar amount of best quality DNA. In this protocol no sophisticated equipments, no high grade chemicals, no RNase treatment even during grinding no liquid nitrogen or ovan were used. For incubation, common gas heater and steel pot was used because of less availability of electricity, the plant materials were not stored in -20°C but they are grinded and store at room temperature as stock materials. By using the above protocol, we were able to extract high quality genomic DNA (Fig. 1) suitable for PCR reaction and downstream applications. Furthermore to counter check the quality of the extracted DNA, the PCR amplified products were eluted and sequenced. The sequence data showed that the amplified fragments were indeed 18S rRNA of pears. The sequence data obtained was BLAST against all the available nucleotide database using online tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), The protocol developed in this study can act as a useful tools for molecular biology of woody trees, herbarium specimens and mushrooms. It is economical, less time consuming and handy in application.

Discussion

Through this protocol, DNA is extracted throughout the year, from any part of plant, no sophisticated equipments, no high grade chemicals, even no liquid nitrogen, no RNAs were used. The extracted DNA are intact, have high quantity, quality and highly suitable for many molecular biological applications such as PCR; nucleotides sequencing, RAPD and SSR etc. DNA isolation from mature trees of high altitude is difficult job due to the presence of large number of phenolic compounds, polysaccharids (Gupta *et al.*, 2011) co-precipitation of impurities such as terpenes, polyphenolics (Aganga et al. 2003) highly viscous polysaccharides (Do *et al.*, 1991; Shepherd *et al.*, 2002) rigid polysaccharide cell wall, tannin, secondary metabolites and pigments (Verma *et al.*, 2007). However, isolation of genomic DNA from mature tree species is an important issue in plant molecular biology (Barzegari *et al.*, 2010; Li *et al.*, 2010; Smyth *et al.*, 2010). To overcome on the above hindrance and resolve this issue, the protocol developed in this study can act as a useful tools for molecular biologists working on woody trees and herbarium specimens. The DNA extracted using our

protocol is suitable for PCR and other downstream applications in molecular biology.

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