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RESEARCH ARTICLE

High-molecular-weight DNA extraction from six *Quercus* species of Kumaun Himalaya, India

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Abstract

Quercus is an economically and ecologically important globally distributed genus of family fagaceae comprises of trees and shrubs. For genetic diversity, phylogenetic and evolutionary study of the species of this genus along their distribution, molecular tools can be beneficial. For them isolation of good quality DNA is prerequisite. High phenolic content of leaf tissue of *Quercus* makes it difficult to isolate good quality DNA free from phenol, lignin and pigments by conventional CTAB method. A reliable protocol for DNA isolation from frozen leaves of six *Quercus* species of Kumaun Himalaya has been standardized. The isolated DNA was found suitable for molecular studies and can be used to assess genetic diversity, phylogeny, and other molecular marker based studies.

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INTRODUCTION

The isolation of pure, high-molecular-mass genomic DNA is essential for many molecular biology applications (Nagori and Purohit, 2012). There are a number of protocols available for the DNA extraction from plant material, but often it is difficult in many plants due to presence of metabolites that interfere with DNA isolation procedures and subsequent applications (Arbi et al., 2009). Extraction of DNA is generally hindered by polyphenols and their quinone oxidation products and by carbohydrate polymers, isolation of DNA using the CTAB complex gives a product of inadequate purity (Murray and Thompson, 1980; Manning, 1981).

Quercus is an economically and ecologically important genus of family fagaceae comprises of trees and shrubs. In India the oak flora is distributed in the sub Himalayan region from Jammu and Kashmir in the Northwest, Garhwal and Kumaun hills of Uttarakhand and Himanchal Pradesh in the Central zone, to Manipur and Arunanchal Pradesh in the Northeast (Pandey and Tamta, 2014) and molecular tools may be beneficial for genetic diversity, phylogenetic and evolutionary study of its species along their distribution. The target six *Quercus* species includes 5 evergreen species, namely *Q. glauca* (phaniyat oak), *Q. leucotrichophora* (banj oak), *Q. floribunda* (tilonj oak), *Q. lanuginosa* (rianj oak) and *Q. semecarpifolia* (brown oak) in the Central Himalaya between 1000 to 3600 m asl (Champion and Seth, 1968) and one deciduous species *i.e. Q. serrata* (Manipuri oak) which is planted in an altitude of 1370 m asl (Pandey and Tamta, 2012).

For molecular studies isolation of good quality DNA is prerequisite. However due to high phenolic content, tannins and polysaccharides in leaf tissues of *Quercus* (Beldeanu, 2008), it is very difficult to isolate good quality DNA free from phenol, lignin and pigment by conventional methods. These metabolites cannot be eliminated during DNA isolation and can inhibit the DNA polymerase activity during polymerase chain reaction (PCR) (Pandey, 1996). A good quality of DNA corresponds to a high specificity of PCR amplified products. Therefore, in the present study attempts were made and a simple, reliable and reproducible protocol for isolation of DNA from frozen leaves of six *Quercus* species of Kumaun Himalaya, India has been standardized. There is limited/no reports are

available regarding the molecular study in genus *Quercus* from the country and present attempt will work as a molecular blueprint for genus *Quercus* as well as other tree species.

Materials and Methods

Plant material. Young leaves were randomly collected from mature elite trees growing in different identified wild populations of Kumaun hills. Plucked leaves were kept in -20°C mini-chiller and brought to the laboratory and stored at -20^{0}C till use.

Solutions. Extraction buffer: 2% (w/v) CTAB, 100 mMTris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA (pH 8), 0.5% 2-mercaptoethanol (v/v), 1% PVP (w/v); chloroform-isoamylalcohol- 24:1 (v/v); TE buffer- 10 mMTris-HCl (pH 8); 1 mM EDTA; Isopropanol (Stored at -20°C); 70% ethanol; 5M NaCl; 1xTAE- Tris base, Glacial acetic acid, 0.5M EDTA (pH 8.0) were prepared.

DNA extraction. Frozen leaf tissues of *Quercus* species kept at -20^{0}C for five days were surface sterilized with 70% ethanol and leaf-midrib was removed by using a sterile blade. Pre-chilled mortar and pestle were used to grind the sample in the presence of liquid nitrogen and PVP (1:1; ratio of tissue: PVP). The frozen powdered sample was transferred to 5ml of extraction CTAB buffer containing 0.5% β-mercaptoethanol (added freshly) in 50-ml centrifuge tube. It was mixed vigorously by vortexing and incubated at 60°C for 60 min in water bath followed by treatment with equal volume of chloroform: isoamyl alcohol (24:1) and mixed for 2 min. The upper aqueous phase obtained by centrifugation at 15000 rpm for 15 min at room temperature, was transferred to a fresh autoclaved centrifuge tube by using wide-bore pipette, chloroform extraction was repeated if extract still contain color. DNA was precipitated by gently mixing with equal volume of chilled isopropanol to obtain fibrous DNA and incubated for 30 min at 4^{0}C for complete precipitation. DNA fibers were spooled out using bent capillary tube and transferred to new autoclaved centrifuge tube having 70% ethanol for 30 min for washing, this step was repeated if there is any visible adhering contaminant remaining. DNA pellet was air dried and suspended in 50-200 µl of 1xTris EDTA (TE) buffer, if necessary brief heating in water bath at 65°C was provided to dissolve pellet in TE and stored at -20°C till use.

DNA Qualitation and Quantification. The quality of DNA was checked by agarose (Promega) gel electrophoresis (GeNei). Samples were prepared by taking 5.0 µl of DNA and 0.5µl of 6x bromophenol blue dye (0.25% Bromophenol Blue and 50% glycerol) mixed well with the help of a micropipette. Samples were electrophoresed in 1xTBE buffer for 1h, initially at 60 V until DNA comes out from the well and then increased up to 80 V in 0.8% agarose gel matrix having 10mg/ml ethidium bromide (EtBr). Gel was photographed under UV light using Gel Documentation System (Bio Red). Quantity and purity of DNA was estimated using UV–vis Spectrophotometer (Thermo Scientific, UV1) by measuring the absorbance at 260 and 280 nm. DNA quantity was assessed using the formula:

$$\text{DNA quantity } (\mu g/\mu l) = \frac{OD_{260}\text{X Dilution factorX50}}{1000}$$

OD: optical density

The absorbance ratio (A_{260}/A_{280}) between 1.6-1.9 was considered as pure and used for PCR amplification.

PCR reactions were carried out in 0.2 ml polypropylene PCR tubes (Bangalore GeNei, India) using Thermal Cycler (Master Cycler Personal, Eppendorf). The original DNA samples were then diluted to 50 ng/µl for reaction mixture. Each 50 µl reaction mixture contained 1x Taq buffer A (10mMTris-Cl, 50 mM KCl), 200µM dNTPs, 0.2 µM oligonucleotide primers, 1U Taq DNA polymerase (Bangalore GeNei, India) and 50ng template DNA, nuclease-free water was used to makeup final volume up to 50µl. The PCR programming for the amplification of cpDNA fragments was as follows: initial denaturation at 95ºC for 8 minutes followed by 34 cycles of denaturation at 95ºC for 1min, primer annealing step at 58 and/or 60ºC for 1min, elongation step at 72ºC for 2 min and a final extension step at 72ºC for 10 min. The amplified PCR products were run on 1.5% agarose (w/v) gel and amplified products were compared using 1kb DNA ladder (Bangalore GeNei, India). The band intensities were visualized and photographed under gel documentation system. Primer pairs which were able to produce clear bands in every sample were selected and considered the best primers for amplification of targeted regions (mat k and trnL-trnF intergenic spacer).

Results

Leaves of *Quercus* species in plant DNA extraction creates problems due to the presence of polysaccharides and certain secondary metabolites, which have been observed to interfere with DNA isolation procedure and inhibit the

activity of a wide range of DNA-modifying enzymes, such as restriction enzymes, polymerases, and ligases. Two CTAB based protocols of genomic DNA extraction, one CTAB based method described by Doyle and Doyle (1990) and another CTAB based method with modifications were tested in the present study for yield, quality and suitability of DNA for molecular biology. The CTAB based protocol without any modification yielded creamish color DNA with high viscosity which could not be dissolved in TE buffer and was not suitable for electrophoresis because it trapped in to the wells during electrophoretic separation (Plate-1c, Lane 1-4). The modified CTAB method not only yielded high quality DNA in each sample but also showed purity (Plate-1bLane 1-6). The principle modification in this method included use of 2.0% addition of PVP, repetition of chloroform: isoamylalcohol precipitation, finally the elution of DNA fibers using bent capillary tube without centrifugation. Higher PVP concentration prevented the browning of DNA (since its ability to form complexes with polyphenols through hydrogen bonding) generally caused by the presence of polyphenols. Long term chloroform:isoamyl alcohol treatment eluted out chlorophyll and other pigments and dyes. In CTAB based protocol 'centrifugation' resulted in compact DNA pellets with polyphenols and other contaminants, which was hard to solubilize in ethanol and TE also. Therefore, to overcome the formation of contaminated pellets, the protocol was modified by substituting the step with directly spooling off DNA fibers. The modification has increased solubility, because only fragile DNA threads were taken off without centrifugation and transferred directly in 70% ethanol for washing. Increased solubility in ethanol facilitated the removal of polysaccharides from DNA, and purity was increased due to this. Agarose gel electrophoresis verified no evidence of RNA contamination and confirmed that the isolated DNA was of good quality (1.65-2.10). The yield range was varied according to species. *Quercus glauca* yielded maximum 13.17 µg followed by *Q. lanuginosa* 5.97 µg, *Q. floribunda* 2.94 µg, *Q. semecarpifolia* 2.80 µg, *Q. leucotrichophora* 1.89 µg and *Q. serrata* 1.68 µg DNA/gram of leaf tissue. The isolated DNA was found suitable for molecular biology experiments and the amplified products of all six *Quercus* species with primer pair (forward-AAAGGCCCGTTTGATTCCCCA and Reverse-ACCAGCTGAGCTATCCCGACCA) is depicted in Plate-1d.

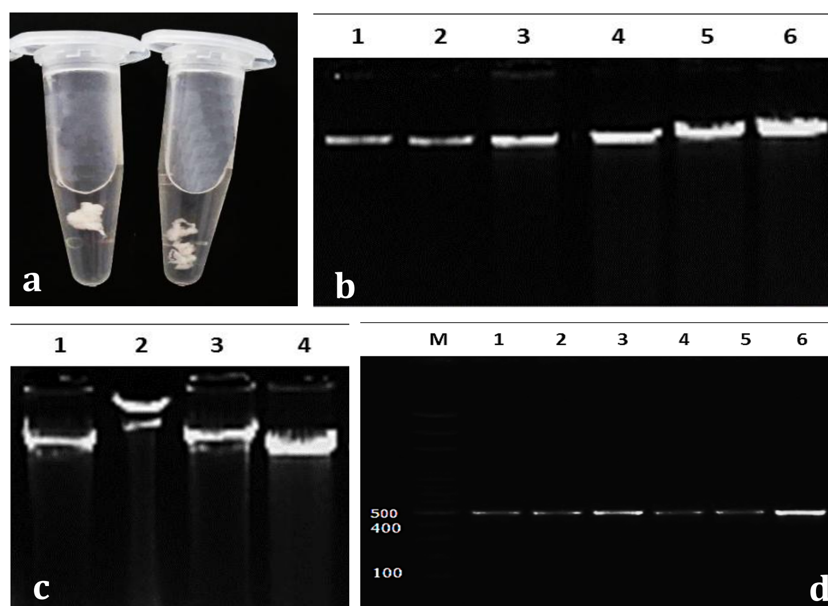

Plate-1: DNA extraction and amplification of target cpDNA regions of six *Quercus* species of Kumaun Himalaya.

a. Spooled extracted DNA suspended in 70 % ethanol **b**. lane1-6 DNA extracted through modified CTAB method (1 *Q. leucotrichophora* 2 *Q. glauca* 3 *Q. floribunda* 4 *Q. semecarpifolia* 5 *Q. serrata* 6 *Q. lanuginosa*) **c.** lane1-4 DNA extracted through CTAB method without modification (1 *Q. glauca* 2 *Q. serrata* 3 *Q. semecarpifolia* 4 *Q. lanuginosa* **d.** Amplification of 500bp DNA of six *Quercus* species; M marker (1kb, 100

lane ladder) 1 *Q. semecarpifolia* 2 *Q. leucotrichophora* 3 *Q. serrata* 4 *Q. floribunda* 5 *Q. glauca* 6 *Q. lanuginosa*

Discussion

To obtain molecular biology study grade genomic DNA several modifications in the original CTAB procedures have been reported from plants with high polysaccharides and polyphenols compounds (Porebski and Bailey, 1997; Michiels et al., 2003; Haquen et al., 2008; Nagori and Purohit, 2012). In the present study addition of increased amount of PVP, repeatition of chloroform: isoamylalchol precipitation step followed by spooling of DNA threads directly, provide good quality DNA from leaves using CTAB based DNA extraction. In normal CTAB method high viscosity of extracted DNA may be due to high endogenous levels of polysaccharides, phenolics and other organic constituents (Sarwat et al., 2006). PVP forms complex hydrogen bonds with phenolic compounds and coprecipitates with cell debris upon lysis (Ghaffariyan et al., 2012). All phenolic compounds as well as polysaccharides, bind firmly to nucleic acids during DNA extraction and interfere with subsequent reactions (Angeles et al., 2005; Hanania et al., 2004; Puchooa and Khoyratty, 2004). In addition to phenolic compounds in the leaves, polysaccharides also interfere with biological enzymes such as polymerases, restriction endonucleases and ligases (Michiels et al., 2003), which results unsuccessful amplification during PCR, PVP has been used to extract genomic DNA from other polyphenol-rich plants such as cotton, sugarcane, lettuce and strawberry (Aljanabi et al., 1999) and several conifers (Kim, 1997). Since CTAB binds to fructans and other polysaccharides and forms complexes that removed during subsequent chloroform extraction (Michiels et al., 2003; Jitu and Kr, 2008) so the repetition of chloroform: isoamyl step produce polysaccharide free DNA.

Conclusions

The method described here is (i) swift- one step (centrifugation) was reduced and pellet solubility time was also shortened, (ii) modest- concentration of PVP was increased and pellet was directly eluted and (iii) reliable- it has been successfully tested in six evergreen and deciduous *Quercus* species. The DNA obtained from described method was free of polysaccharides and secondary metabolites, as shown by the absence of viscous or brown substances. Therefore this method can be used effectively in molecular and evolutionary research, and can be applied for the processing of number of samples of *Quercus* as well as other polyphenols and polysaccharide rich samples.

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