

A New and Simple Method for Isolating Genomic Dna from *Julandaceae* for Genetic Diversity Analysis

Zhang Fan and Wan Xueqin

College of Forestry, Sichuan Agricultural University, Ya'an, Sichuan 625014, China

Abstract: Isolation of high-quality DNA from long stored *Julandaceae* leaves or dried *Julandaceae* leaves is particularly difficult because of their high levels of polyphenols, lipin, tannin, pigment, terpene and other compounds. The yields and quality of genomic DNA are considerably affected when the common protocol for DNA isolation is applied to the long stored or dried *Julandaceae* leaves. A simple, rapid and efficient protocol for the extraction of DNA from the *Julandaceae* leaves is described. The modified hexadecyltrimethylammonium bromide (CTAB) procedure, which uses high concentrations of polyvinylpyrrolidone (6% [w/v]) and 5% bentonite in the high-salt-concentration extraction buffer to remove polyphenols and polysaccharides. This protocol results in high yields of DNA. The average yield of DNA ranged from 986-1824 µg/g of fresh weight of leaves. Downstream results indicate that DNA quality is sufficient for random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analyses. Extractions are scaled down to suit 1.5-mL Eppendorf tubes, allowing easier handling and enhanced sterility.

Key words: *Juglans* % *Carya* % Genomic DNA % Molecular markers

Abbreviations: AFLP, amplified fragment length polymorphism;
RAPD, random amplification of polymorphic DNA; CTAB, hexadecyltrimethylammonium bromide;
EDTA, ethylene-diaminetetraacetic acid; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone.

INTRODUCTION

Many studies require high quality genomic DNA from plants. Many other ingredients or different treatments in plants could influence its DNA quality. Polysaccharides are common in *Julandaceae* leaves with silica gel treatment or long frozen prior to DNA extraction, which makes it rather difficult to obtain high quality genomic DNA from their tissues [1-3]. Many molecular enzymes, such as restriction endonucleases, DNA ligase and DNA polymerases, are inhibited by polysaccharides and other secondary metabolites [4-7]. In addition, polysaccharides can concentrate the sample, resulting in an extremely viscous DNA pellet [8]. At present, a major bottleneck is how to isolate purified DNA from *Julandaceae* with silica gel treatment or long frozen.

Existing extraction methods were used to fresh young *Julandaceae* leaves [9-12]. While in the *Julandaceae* leaves with silica gel treatment or long frozen, we did a dozen of experiments as previously reported and found the extracted DNA with dirty yellow and highly viscous, so it was not suitable for the followed manipulation and

analysis. In this context, we concluded that the previously protocols were not suitable for long frozen or long silica gel treatment materials. The aim of this study was to establish a new protocol for DNA isolation from *Julandaceae* leaves with silica gel treatment or long frozen to get high quality DNA that is suitable for generation of molecular markers, such as RAPD and AFLP.

MATERIALS AND METHODS

Material Collection: Seeds of one wild *Juglans regia* population, one wild *Juglan sigillata* population and one wild *Carya cathayensis* population were collected from forestry and horticultural college of Sichuan Agricultural University in China. *J. regia* trees that located at Qingba Mountain, Daduhe valley and southern Ganzhi district in Sichuan province, *J. sigillata* trees at plateau of Yunnan province and *Carya cathayensis* trees at Xinjiang. All the trees were 80 years old at the time of seed collection in 2005. Seeds were germinated and planted in the greenhouse. Leaves from one year old trees

were categorized into two groups: one has been stored frozen at -20°C for seven months and the other was stored in silica gel at room temperature prior to DNA extraction at the same time.

DNA Isolation: The successful procedure of DNA isolation along with the modifications was carried out as followed:

Equipment and reagents

Mortar and pestle

Water bath

Beckman centrifuge (Avanti J-20xp)

UV-1601 spectrophotometer (Shimadzu, Japan)

Liquid nitrogen

Extraction buffer I: 0.25 M NaCl, 1 M Tris-Hcl pH8.0, 0.5 M EDTA pH8.0.

Extraction buffer II: 1.4 M NaCl, 1M Tris-Hcl pH8.0, 0.5M EDTA pH8.0, 2% (w/v)CTAB.

\$-mercaptoethanol

Polyvinylpyrrolidone

Phenol

Bentonite

Isopropanol

Chloroform

70% and 96% (v/v) ethanol

RNase

Protocol

- C Approximately 100 mg of leaf tissue were cut into pieces to a pre-chilled mortar and grinded with 6 mg PVP-40 in liquid nitrogen to obtain a fine powder.
- C The powder was quickly transferred to a 1.5 mL sterile Eppendorf tube and then 500 µL of extraction buffer I and 5 µL \$-mercaptoethanol were added and mixed by inversion.
- C The mixture was held at 0 °C for 20 min and was transitorily centrifuged at 10, 000 g at room temperature for 5 min. The flow-through liquid was discarded and the deposit was reserved in the same tube.
- C 500 µL Buffer II (pre-heated at 65°C) was added to remaining deposit. The tube was placed in a 65 °C water bath for 30 min and was shaken every 10 min.
- C 500µL 1:1 chloroform-Phenol (v/v) and 5mg bentonite were added into the tubes and mixed gently by inversion for 10 min.
- C The mixture was centrifuged at 13, 000 g for 5 min and the supernatant liquid was transferred to a fresh tube.
- C 500µL chloroform-octanol (24:1; v/v) was added and mixed.

C Repeat Step 6.

C 500 µL cold isopropanol (0 °C) was added and mixed. Then the tubes were incubated at -20°C for 30 min to precipitate DNA.

C The mixture was centrifuged at 10, 000 g for 5 min and the supernatant was poured off carefully to avoid losing the DNA pellet. The tubes were inverted to remove the residual water with filter paper.

C The pellet was rinsed with 1 ml 70% and 96% (v/v) ethanol and was dried by vacuum.

C DNA was dissolved in 50 µL of TE buffer.

C 1.5 microliter of RNase (1mg/ml) was added to DNA solution and was incubated at 37°C for 3 hour. And then DNA solution was held at 4°C.

C To test effectiveness and efficiency of this protocol, we restricted the DNA with commonly used restriction enzymes and also tried generation of RAPD and AFLP markers.

UV Spectrophotometric Analysis: Dilute the genomic DNA with TE buffer and use Eppendorf UV Biophotometer to measure the ratio A_{260} / A_{280} and DNA concentration. The ratio A_{260} / A_{280} was determined in order to assess the purity of the sample.

Restriction Digestion: Two hundred and fifty ng of genomic DNA were digested with 3 units of restriction enzymes under optimal temperature and the buffer was added for 3 hours. The digested DNA was electrophoresed on 0.8% agarose at 5 V/cm along with undigested DNA as a control.

RAPD Analysis: PCR amplifications were carried out according to the parameters established by Wang *et al.* [10]. PCR reactions were made as follows: 10×PCR buffer 2 µl, 2.5mmol/l dNTPs 1.8µl, MgCl₂ 2.5Mm, Taq DNA polymerase 0.4 U, decamer primer 12.5 pmol and DNA template 40 ng. The PCR reactions system was 20µl. 10-mer Primer (5'GGAAGCTTGG 3') was used for DNA amplification. PCR reaction procedure used in this experiment was listed as follow: pre-denaturalised for 5 min at 94°C, then 40 cycles of 1 min at 94°C for template denaturation, 1 min at 36? for primer annealing, 2 min at 72°C for primer extension, followed by a final extension of 10 min at 72°C followed be cooling to 4°C. RAPD primers were synthesized by Shanghai Sangon Inc. The amplified products were size fractionated on 1.2% agarose gel and visualized under UV light after ethidium bromide staining.

AFLP Analysis: AFLP experiments were performed according to the protocol described by Fan *et al.* [13]. Genomic DNA (300-400 ng) was double-digested with the Eco I and Mse I enzymes at 37°C for 2 hrs. A small aliquot of the digested DNA was run on a 1.5% (w/v) agarose gel to check if the DNA digestion was complete. The digested samples were incubated at 70°C for 15 min to inactivate the restriction endonucleases. EcoRI and Mse I adapters were ligated to the digested DNA samples to generate template DNA for amplification. Pre-amplification was carried out with primers each carrying one selective nucleotide (EcoR I + A, Mse I + C) in a thermocycler for 20 cycles set at 94°C denaturation (30 sec), 56°C annealing (60 sec) and 72°C extension (60 sec). The initial denaturation was done at 94°C for 30 sec and the final extension at 72°C for 8 min. The amplification products were diluted 20-fold in TE buffer and stored at -20°C. Selective AFLP amplification was carried out with EcoR I + 3 primers and Mse I + 3 primers and 5 µL of the diluted PCR products from the pre-amplification. The PCR amplifications were carried out as follows: one cycle at 94°C for 30 sec, 65°C for 30 sec and 72°C for 60 sec; followed by 12 cycles of touchdown PCR in which the annealing temperature was decreased by 0.7°C every cycle until a touchdown annealing temperature of 56°C was reached. Once reached, another 20 cycles were conducted as described above for pre-amplification. The reaction product (2 µL) was mixed with an equal volume of formamide loading buffer (98% [v/v] formamide, 10 mM EDTA, 0.005% [v/v] of each of xylene cyanol and bromophenol blue) denatured by incubating at 90°C for 5 min and quickly cooled on ice. The products were analyzed on 5% (w/v) denaturing polyacrylamide gels. The gel was run at constant power (50-55 W) until the xylene cyanol was about two-thirds down the length of the gel. AFLP bands were detected by silver staining as the procedures described by Bassam *et al.* [14].

RESULTS AND DISCUSSION

Compared with other methods, our procedure was optimized by making changes. First, collection the deposit by centrifugation at low speed before cell nucleus lysis facilitated removal of polysaccharides and polyphenol. Second, a high salt concentration, together with the high amounts of PVP and bentonite prevented oxidization of the secondary metabolites. Bentonite was also successfully applied in the RNA isolation of *Jatropha curcas* containing high levels of polysaccharides and polyphenols [15].

By using this method, we successfully isolated good-quality and high-yield DNA from the long-stored or dried *Julandaceae* leaves. The average yield of DNA ranged from 986-1824 µg/g of fresh weight of leaves. Spectrophotometric analysis at A_{260}/A_{280} revealed that the ratio ranged from 1.802 to 1.894 (Table 1), indicating little contamination by proteins and macromolecules. To further evaluate DNA quality, we subjected the obtained DNA to restriction digestion (Figure 1), RAPD (Figure 2) and AFLP analysis (Figure 3). With the use of 180 random primers in RAPD analysis, each primer generated 6-11 bands, with an average of 7 bands. Most of the bands ranged in size from 250bp to 2.0 kbp. Figure 2 showed the profiles amplified with random primer of RA-15. With the use of 48 pairs of AFLP primers, each combination of primers generated 48-60 bands, with an average of 52 bands. Most of the bands ranged in size from 100bp to 2.0 kbp. Figure 3 showed the profiles amplified with the combination of E35/M61 primers. The amplified bands showed high resolution and polymorphism among *Julandaceae*.

Secondary Metabolites in plant are common problems when extracting DNA from *Julandaceae* samples, especially those stored extensively at -20°C for a long time. Polysaccharides contamination could cause

Table 1: The purity and yield of DNA isolated following modified protocol. Results were expressed as the mean of 3 samples (standard error)

No.	Population	A_{260}/A_{280}	Yield, µg/g of Fresh Weight
1	<i>J. regia</i>	1.802 (0.023)	1648 (153)
2	<i>J. regia</i>	1.815 (0.126)	1124 (96)
3	<i>J. sigillata</i>	1.867 (0.201)	1576 (129)
4	<i>J. sigillata</i>	1.894 (0.216)	986 (46)
5	<i>Carya cathayensis</i>	1.828 (0.013)	1824 (150)
6	<i>Carya cathayensis</i>	1.847 (0.056)	1063 (62)

Note: No 1, 3 and 5 signed the DNA extracted from leaf materials stored frozen at -20°C for seven months; No 2, 4 and 6 signed the DNA extracted from leaf materials stored in silica gel at room temperature for seven months

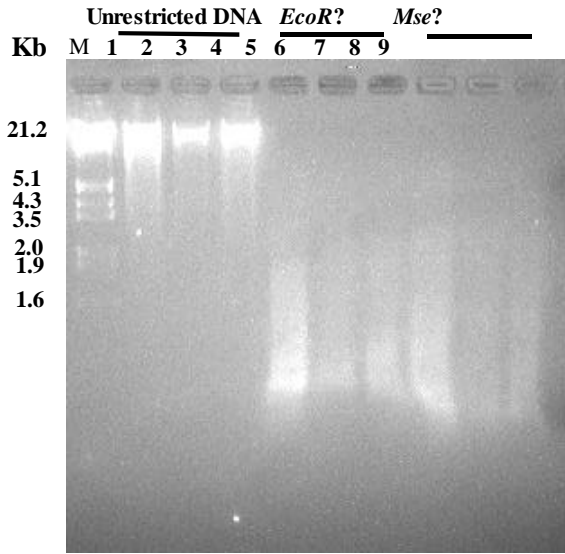


Fig. 1: Restriction pattern of DNA isolated following modified protocol. Two hundred and fifty ng of genomic DNA from *Juglans regia*, *Juglan sigillata* and *Carya cathayensis* were loaded either undigested (lanes 1, 2, 3, respectively) or after *EcoR I* (lanes 4, 5, 6, respectively) and *Mse I* (lanes 7, 8, 9, respectively) digestion. 8 DNA digested with *EcoR I* + *Hind III* was loaded as marker (lane M).

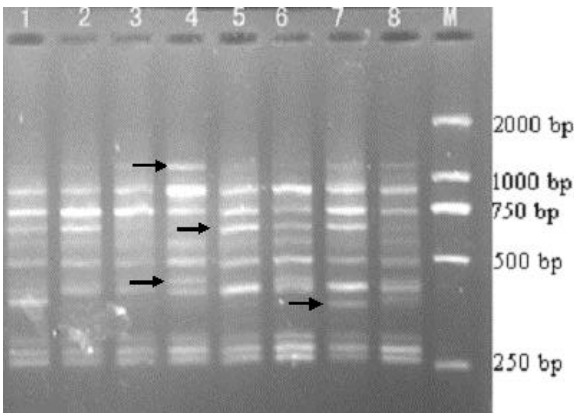


Fig. 2: RAPD fingerprinting patterns of *Julandaceae* using the primer RA-15(5'GGAAGCTTGG 3'). Use 8-*Hind III* as a molecular weight marker. Note: Lanes 1, 7 and 8 are individuals of *Carya cathayensis*; Lanes 3, 4 and 6 are *J. regia*; the rest are *J. sigillata* detected by RAPD. The blank arrows present part of polymorphism loci.

viscous DNA pellets due to its DNA concentration. At present, many researchers usually store leaves in silica gel prior to DNA extraction, they should also consider

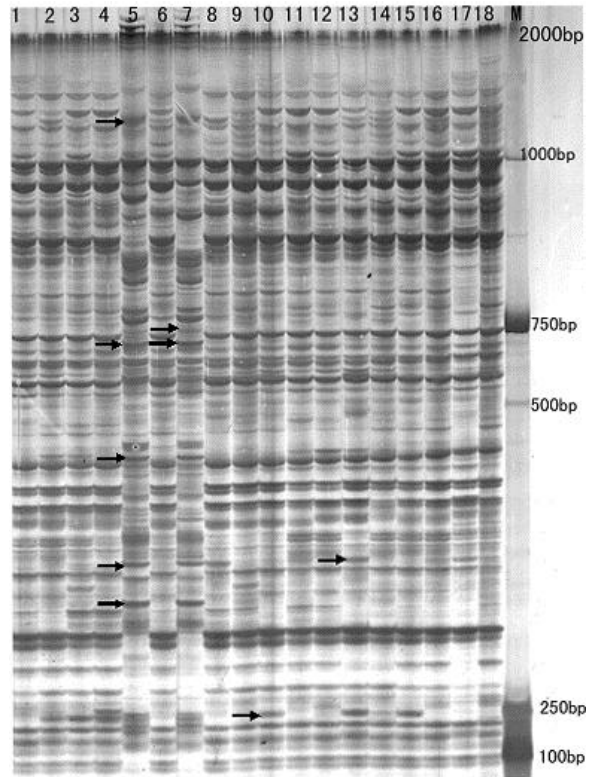


Fig. 3: AFLP fingerprinting patterns of *Julandaceae* using the primer combination E35/M61 (5' GACTGCGTACCAATTCACA 3' / 5'GATGAGTCTGAGTAACTG 3'). Use 8-*Hind III* as a molecular weight marker.

Note: Lanes 4 and 7 are individuals of *Carya cathayensis*; Lanes 3, 5, 7 and 15 are *J. regia*; the rest are *J. sigillata* detected by AFLP. The blank arrows present part of polymorphism loci.

evaluating the effectiveness of their method on such material. In this study, we used PVP-40 and 5% bentonite during extraction and obtained good integrity and purity DNA from *Julandaceae* which demonstrated that the method described was an excellent method for extracted DNA from *Julandaceae* leaves stored frozen for a long time or stored in silica gel. Bentonite is clay generated frequently from the alteration of volcanic ash, consisting predominantly of smectite minerals, usually montmorillonite. As an absorbent, bentonite can effectively eradicate protein and secondary metabolites such as polyphenols, lipin, tannin, pigment and terpene [16-18]. Up to now, bentonite was only carried out in RNA extraction [15], whereas little information is available in DNA extraction.

In the paper, *Juglans regia* and *Juglans sigillata* were belonged to *Juglans* genus, *Carya cathayensis* was classified into *Carya* genus, while all belonged to *Julandaceae*. Thus, isolating DNA of these materials was significance.

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