ISOLATION OF HIGH QUALITY DNA FOR AFLP AMPLIFICATION IN *CASUARINA EQUISETIFOLIA* L.

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Summary. High quality DNA was isolated from *Casuarina equisetifolia* which is widely planted in tropics and subtropics for its multiple utilities. Two commonly used methods, CTAB based and the commercially supplied DNeasy^{TM} Plant Mini Kit (QIAGEN GmbH, Germany) were tested for their efficiency in isolating high quantity and quality DNA to be utilized for AFLP analysis. The CTAB based protocol was modified by introducing additional steps and manufacturer's protocol was followed for the commercial kit. DNA yield and purity were monitored by gel electrophoresis and by determining absorbance under UV (A260/A280 and A260/A230). Both ratios were between 1.9 and 2.1, indicating the absence of contaminating metabolites. The average DNA yield obtained from 100 mg needle tissue using the CTAB based protocol and the commercial kit was 17 mg and 6 mg, respectively. Further, the purity of DNA was assessed by enzymatic digestion with *Eco*RI and *Hind*III and the DNA was also tested for suitability by AFLP-PCR analysis. A unique AFLP pattern was achieved for the five ortets tested showing the utility of DNA for fingerprint studies. Based on the quantity of isolated DNA, the CTAB based method is recommended for *C. equisetifolia* DNA extraction and it can be utilized for other *Casuarina* species with high content of fiber and phenolic compounds.

Keywords: *C. equisetifolia*, DNA isolation, restriction digestion, AFLP, CTAB method

***Abbreviations*:** AFLP - amplified fragment length polymorphism; CTAB – cetyltrimethyl ammonium bromide; EDTA – ethylenediamine tetraacetic

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acid; TE buffer – 10 mM Tris-HCl, pH 7.5, 1mM EDTA, pH 8.0; TBE buffer- 90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.2.

INTRODUCTION

Casuarina equisetifolia is a multipurpose plantation species, best suited in the agrarian ecosystems in South and South East Asia. A short rotation period, ability to improve soil fertility and ready marketability are the major attractions for this species. Clonal plantations of this species are common in India, China, Vietnam and Egypt that are raised from the out performing phenotypes selected from provenance trials. International provenance trials were established in 20 tropical and subtropical countries for evaluating variations existing within the species (Pinyopusarerk et al., 2004). Breeding programs were established through selection of best provenances and establishment breeding populations (Nicodemus et al, 2001). DNA markers are regarded as the best tools to accelerate breeding in tree species and marker-assisted breeding have been reported for many tropical trees (Ho et al., 2002). Among the dominant markers, amplified fragment length polymorphism (AFLP) is regarded as the best system for monitoring breeding programs in forest trees, construction of high-density genetic maps and quantitative trait loci maps because of its high throughput nature and better genetic resolution.

In *C. equisetifolia* and *C. junghuhniana* provenances variations were assessed using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers (Ho et al., 2002; Ho et al, 2004). RAPD variations were used to identify introgressive hybridization in *C.equisetifolia* (Ho et al, 2002). Genetic analysis of *Casuarina* species (and *Allocasuarina*) and clonal fingerprinting of *C. equisetifolia* were performed with ISSR and Fluorescent-ISSR markers (Yasodha et al., 2004). No reports about the use of AFLP for members of the family *Casuarinaceae* are available.

AFLP technique requires isolation of DNA with high purity for restriction digestion. Isolation of DNA free of metabolites particularly, polysaccharides and phenolic compounds is most essential because these compounds can irreversibly bind to nucleic acids during extraction steps (Varadarajan and Prakash, 1991), may interfere with DNA isolation, cloning, characterization (Bousquet et al., 1990) and inhibit the activity of DNA modifying enzymes (Pandey et al., 1996; Abdulova et al., 2002). Recent studies have indicated that extraction of DNA is not always simple or routine and the published protocols are not necessarily reproducible for all species (Doulis et al., 2000; Lin et al., 2001).

Higher amounts of polyphenols particularly tannins, fibrous nature and xerophytic adaptations of *Casuarina* needles make the DNA isolation difficult in this species. Needle tissues are known to be difficult for DNA extraction and were classi-

fied as "recalcitrant" or "hard" tissues (Csaikl et al., 1998). Therefore, in the present study, two different methods, which are routinely used for many plant species, were tested to isolate DNA from needles of *Casuarina*. DNA was further subjected to restriction digestion analysis and AFLP-PCR amplifications.

MATERIALS AND METHODS

Plant material

Needle samples from five ortets of 5-year-old hedged *C.equisetifolia* were used for DNA isolation. The growing tips of the needles were collected and the fresh sample was washed in double distilled water, dried on a paper towel for 1-2 min and stored at –20º C.

DNA isolation

DNA was isolated using two routinely used extraction protocols (Doyle and Doyle, 1990 and Qiagen DNeasy Plant Mini kit). In both methods, 100 mg needle tissue was ground to a fine powder with liquid nitrogen in a pre-chilled mortar.

Method 1

The first attempt to isolate DNA utilized a modified method of Doyle and Doyle (1990). The tissue powder was transferred immediately into pre-warmed (65 ºC) extraction buffer (2 % w/v CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 100 mM Tris-HCl, pH 8.0, 0.2% b-mercaptoethanol) and incubated for 40 min at 65 ºC in a water bath with gentle mixing per every 10 min. Chloroform: Isoamyl alcohol (24:1) extraction was carried out twice and the DNA was precipitated by addition of 8.0 ml of ice-cold 2-propanol and placed at –20 ºC for one hour. DNA was processed to remove RNAs as described earlier (Doyle and Doyle, 1990) and the pellet was dissolved in 100 ml of double distilled water. Further purification of DNA was carried out twice by extracting with TE buffered phenol/chloroform/isoamyl alcohol (25:24:1). Thirty µl of 3.0 M sodium acetate was added, vortexed for 2 s followed by the addition of 650 ml of ice-cold ethanol and vortexed again for 2 s. The DNA sample was placed at –20 ºC for 30 min. DNA was washed with 70% ethanol, air-dried and resuspended in 25 ml TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM Na_2EDTA) and stored at – 20 ºC until analysis.

Method 2

The second method utilized a commercial kit to extract DNA, the DNeasy Plant Mini Kit (Qiagen, Germany, Cat. No.69103). Isolation procedures were followed accord-

ing to the manufacturer's instructions. In the final step DNA was eluted with 75 ml TE buffer and stored at – 20ºC.

Evaluation of nucleic acids

Each DNA solution was assayed for UV absorption spectrum using a UV/VIS spectrophotometer (NanoDrop Technologies, USA). The absorbance was recorded at 230, 260 and 280 nm to estimate yield and quality. Further, *C. equisetifolia* genomic DNA (140.0 ng) was digested with 5 units each of *Eco*RI and *Hind*III (MBI, Fermentas, Lithuania) in the recommended buffer at 37 ºC for 4 h. DNA digestion was assayed by visual inspection after agarose gel (0.8%) electrophoresis.

AFLP Analysis

AFLP products were generated with 200 ng genomic DNA, following the protocol of Vos et al. (1995) using the AFLP Analysis System I (Gibco- BRL, Life Technologies, USA) kit. Genomic DNA (5.0 ml) was double restriction digested for 6 h at 37 ºC with 1.25 units each of *Eco*RI and *Mse*I in a 25 ml volume. After heat inactivation for 15 min at 70 ºC, restriction site derived adapters were ligated for 2 h at 20 ºC with 24 ml of Adapter-ligation solution and 1 unit of T_4 Ligase. The ligation mix was diluted with an equal volume of TE buffer and pre-amplified with adapter-derived primers having an additional (+1) nucleotide at the 3′ end and amplified using 20 cycles of 30 s denaturation at 94 ºC, 60 s annealing at 56 ºC and 60 s extension at 72 ºC. The reaction products were diluted 30-fold with TE buffer. The second amplification was performed with a combination of *Eco*RI and *Mse*I primers that had three selective nucleotides each. Primer combinations used included: E-ACG / M-CAG; E-ACG / M-CAC and E-ACG / M-CAA. The second amplification was performed in a 20 ml final volume with 10 cycles of 94 ºC for 60 s, 65 ºC for 60 s with a decrease of -1.0 ºC per cycle, and 72 ºC for 90 s followed by 30 cycles of 94 ºC for 30 s, 56 ºC for 60 s and 72 ºC for 60 s. AFLP reaction products were separated in 6% (w/v) denaturing polyacrylamide in 1X TBE buffer and visualized by silver staining using the method described by Briard et al. (2000).

RESULTS AND DISCUSSION

The source tissue from which DNA is extracted plays a critical role in determining DNA quantity and quality (Weising et al., 1994; Abdulova et al., 2002). Mature *Casuarina* needles consist of extensive lignified and fibrous tissue and high tannin content interfering with DNA isolation. Rapidly growing young needles which must maximize photosynthetic efficiency and growth may not invest in lignified structural tissue and therefore, they serve as an ideal starting material for DNA isolation.

The total yield of DNA isolated by method 1 and method 2 was 17.0 mg/100mg fresh weight tissue and 6.0 mg/100mg fresh weight tissue, respectively (Table 1). The purity of isolated DNA was assessed by the UV absorbance ratios. The A260nm/A230 nm ratio was 2.09 and 2.18 while the A260 nm/A280 nm ratio was 2.10 and 1.93 for DNA isolated by method 1 and 2, respectively, indicating the absence of major contaminants. DNA obtained using the two methods appeared on the agarose gel as a single band of average size approximately 23 kb and with no trailing of denatured DNA (Figure 1). Further, the complete digestion of DNA using *Eco*RI and *Hind*III (an enzyme particularly inhibited by the presence of polysaccharides) showed that the isolated DNA was free of polysaccharides and other contaminating inhibitory substances (Figure 1). Similarly, pure and high DNA yield from needle tissues was reported by Doulis et al (2000) in *Cupressus sempervirens* and Stange et al (1998) in *Pinus radiata* with modified CTAB and Qiagen commercial kit. CTAB-based extraction methods have proved to yield good quality DNA and are frequently used for DNA isolation from needles of many forest tree species like *Pinus radiata* (Stange et al., 1998), *Taxus wallichiana* (Khanuja et al., 1999), *Ulmus glabra, Abies alba, Pinus sylvestris* (Csaikl et al., 1998) and *Picea abies* (Scheepers et al., 1997).

Table 1. DNA yield obtained by two extraction methods

Method	Quantity (mg) /100mg fresh tissue ± SD	A260/A280 ± SD	A260/A230 ± SD
Method 1	17.0 ± 3.9	2.10 ± 0.904	2.09 ± 0.569
Method 2	6.0 ± 2.5	1.93 ± 0.573	2.18 ± 0.513

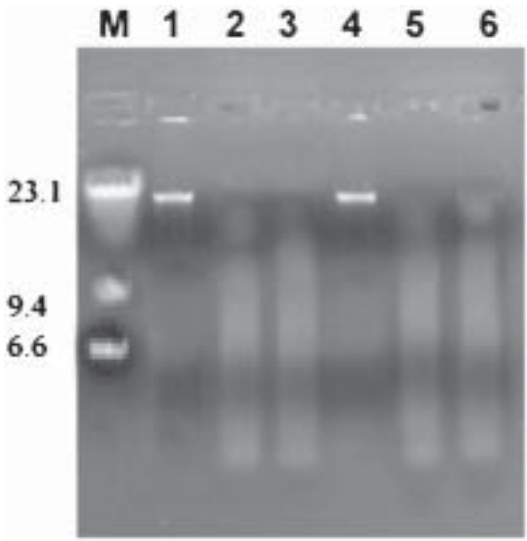
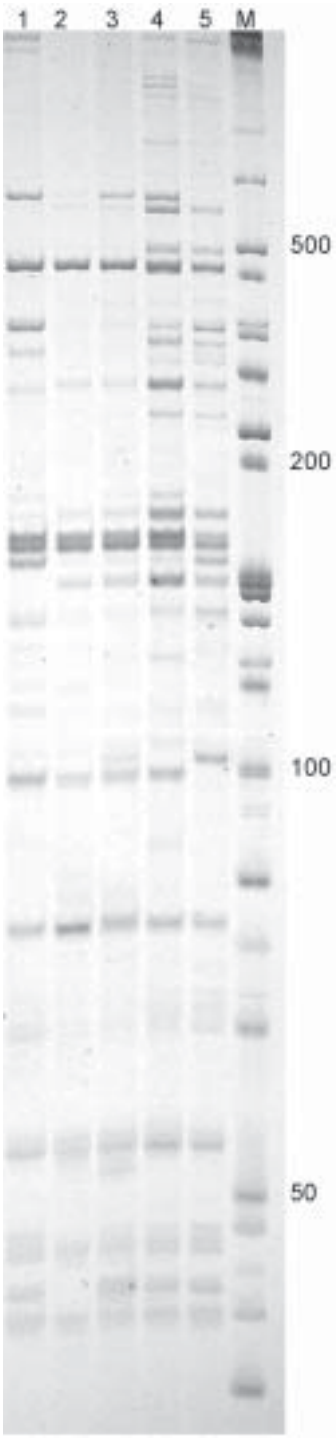


Figure1. DNA isolated from *C.equiserifolia.* Lanes: M-Lambda-HindIII digest, I-DNA isolated following method 1, 2&3-Method 1 DNA digested with *Eco* RI and *Hind* III, 4- DNA isolated following method 2, 5&6-Method 2 DNA digested with *Eco* RI and *Hind* III



AFLP procedure is influenced by the quantity and quality of the DNA preparation requiring more complex DNA purification methods (Henry, 2001). Hence, DNA isolated from *C. equisetifolia* was subjected to AFLP analysis to evaluate the suitability of DNA for fingerprinting studies. The procedure described for the amplification of AFLP markers in *C. equisetifolia* was tested with five different individuals and unique AFLP fingerprint patterns for each individual were obtained (Figure 2). Silver staining of the polyacrylamide gels was found to be advantageous for AFLP in carrot (Briard et al., 2000). In *C. equisetifolia* about 20-30 bands were observed with few faint bands which were excluded from the data analysis.

In conclusion, both methods used in the present study produced pure DNA suitable for AFLP analysis. Method 1, a CTAB based modified protocol of Doyle and Doyle (1990) yielded high-quantity DNA and can be recommended for *C. equisetifolia* DNA extraction. Casuarinas have needles with high content of fiber and phenolic compounds. Therefore, this protocol can be adapted for other *Casuarina* species.

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Figure 2. AFLP-PCR in *C. equisetifolia* using DNA isolated by method 1. Lanes 1-5: Five ortets, M - DNA ladder

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