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Full Length Research Paper

Genomic DNA extraction from sapwood of *Pinus roxburghii* for polymerase chain reaction studies

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A method for extraction of genomic DNA from sapwood tissues of mature tall trees of *Pinus roxburghii,* where collection of needle tissues is extremely difficult has been standardized. The extracted DNA was comparable to that obtained from the needle tissue in terms of yield and purity. The yield of extracted DNA ranged from 6.98 to 19.668 µg / 100 mg tissue and A_{260} / A_{280} ratio ranged from 1.70 to 1.87. The polymerase chain reaction (PCR) amplification of the DNA extracted from sapwood tissue using random amplification of polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers was similar to that of DNA extracted from the needle tissues.

Key words: *Pinus roxburghii,* DNA extraction, sapwood, random amplification of polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), simple sequence repeat (SSR).

INTRODUCTION

Pinus roxburghii Sargent (Chir pine or long needle pine), with a long rotation of 120 years is a precious timber-resin resource, native to the outer range and principal valleys of the Himalayas from Afghanistan to Arunachal Pradesh in northeastern India between 450 and 2300 m altitude. It covers very large areas as pure forests and also with other species, particularly at its upper and lower limits. The species provides a variety of goods for the society and the resin industry of the country. Tree improvement and conservation programs have been initiated with the use of DNA marker techniques to study the population genetic structure and genetic diversity in this species. However, extraction of genomic DNA is the first essential step for molecular diversity/similarity analysis. Generally, the DNA is extracted from the young tissues in their active growth for obtaining high quality and yield. Young leaves are the most preferred starting material. DNA from young needles can be extracted following a combination of the protocols given by Doyle and Doyle (1990) and Stange et al. (1998). However, in case of *P. roxburghii* where the trees are very tall (~30 m) and situated in steep hilly terrain, obtaining foliage tissues is highly laborious and time consuming. Therefore, an alternative method of extraction was developed using sapwood peeled from the trunk of trees at approachable height.

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MATERIALS AND METHODS

The cetyl-trimethylammonium bromide (CTAB) extraction buffer consisted of 100 mM Tris-HCl (pH-8.0), 20 mM ethylenediaminetetraacetic acid (EDTA) (pH-8.0), 1.4 mM NaCl, 3% CTAB, 1% polyvinylpyrollidone (PVP) and 0.2% β-mercaptoethanol. The bark of the tree was obtained using bark shaver and transported to the laboratory wrapped in zip pouch inside ice box. The sapwood (developing xylem near cambium and bark) was separated and cut into fine pieces. One gram fine-shredded tissue was ground in liquid nitrogen and incubated in 5 ml extraction buffer for 2 h at 65°C in water bath. The mixture was extracted with equal volume of chloroform : isoamyl alcohol (24:1). Two phases were separated by centrifuging at 5000 rpm for 20 min using refrigerated centrifuge (Hermile model: Z233 MK2) at 4°C. An equal volume of cold isopropanol was added to the supernatant, mixed well and incubated overnight at -20°C. DNA was pelleted by centrifuging at 5000 rpm for 20 min at 4°C. The pellet was washed with 1 ml of 76% ethanol for 45 min by gentle inverting and then pelleted by centrifuging at 5000 rpm for 5 min. The pellet was washed again

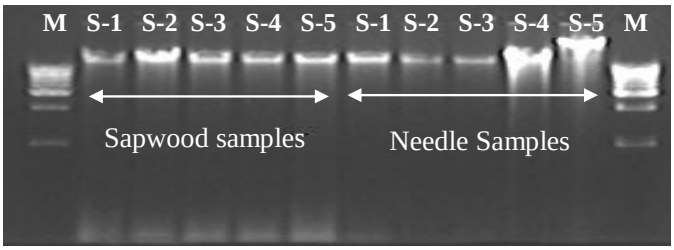

Figure 1. Genomic DNA extracted from sapwood and needles of five individuals of *P. roxburghii* on 1% agarose gel. M: 1 kb DNA ladder.

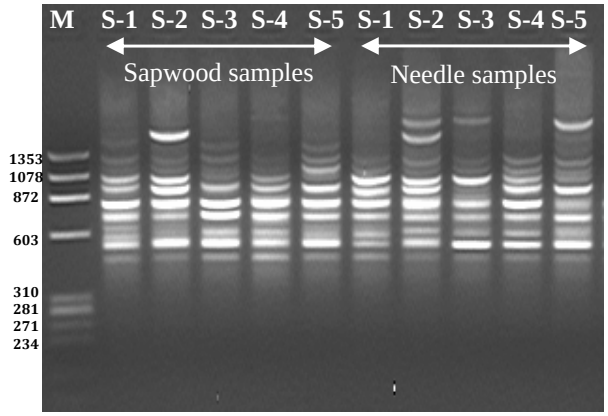

Figure 2. RAPD profile of sapwood and needle samples of five genotypes of *P. roxburghii* generated by the primer M-182. Sequence: 5'GTT CTC GTG T 3', M: ϕ X 174 DNA/ *Hae* III Digest.

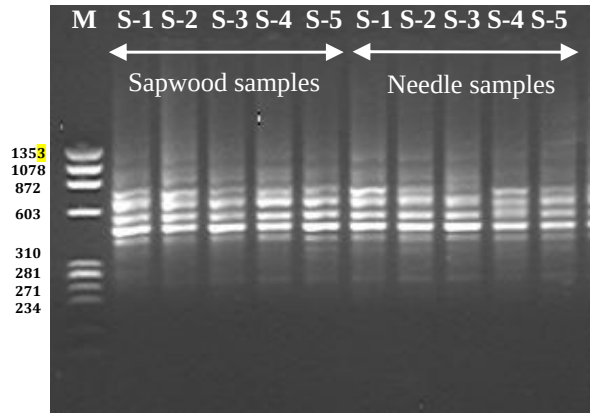

Figure 3. ISSR profile of sapwood and needle samples of 5 genotypes of *P. roxburghii* generated by the primer UBC-809. Sequence: 5'AGAGAGAGAGAGAGAGG-3', M: ϕ X 174 DNA/ *Hae* III Digest.

with 500 µl of 70% alcohol by centrifuging at 5000 rpm for 15 min. After vacuum drying for 10 min, the pellet was re-dissolved in 50 µl of Tris-EDTA (TE) buffer. The quantitative analysis was done based on A_{260}/A_{280} spectral absorbance ratio using Biophotometer (Eppendorf). The size, purity and integrity of the DNA was also confirmed through electrophoresis on 1% agarose gel (Figure 1) containing ethidium bromide (0.5 µg/ml) at 80 V for 1 h. Finally, the extracted DNA was subjected to PCR amplification using random amplification of polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) primers and compared with DNA extracted from the young needle tissues.

RAPD analysis

The extracted DNA was subjected to RAPD analysis using primer M-182, 5'GTT CTC GTG T 3' (Mosseler et al., 1992). PCR for RAPD marker amplification was performed using 10 ng genomic DNA as a template. PCR was performed on a thermal cycler (Biorad, Mycycler) incorporating 10 ng genomic DNA to a 25 µl reaction mix containing 1X *Taq* buffer, 3 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.4 µM primer and 1 unit of *Taq* polymerase (Applied Biosystems). The PCR amplification parameters consisted of: one cycle at 94°C for 2 min; 41 cycles each at 94°C for 45 s; 37°C for 1 min and 72°C for 1 min followed by a final elongation at 72°C for 10 min. Amplification products were visualized on 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) run at 100 V for 3 h (Figure 2).

ISSR analysis

ISSR analysis was carried out in a 20 µl volume of reaction mixture containing 1X *Taq* polymerase buffer, 1.75 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.4 µM primer, 1 unit of *Taq* polymerase (Applied Biosystems) and 10 ng template DNA. ISSR primer (University of British Columbia, UBC-809 Sequence: 5'AGAGAGAGAGAGAGAGG-3' was used for the amplification (Hong et al., 2007). The PCR amplification was programmed for: one cycle at 94°C for 5 min; 40 cycles each at 94°C for 30 s, 57°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 10 min. Amplification products were visualized on 2.0% agarose gel (Figure 3).

SSR analysis

For SSR analysis, primer Pt 71936 was used (forward sequence: 5' TTCATTGGAAATACACTAGCCC 3' and reverse sequence: 5'AAAACCGTACATGATTCCC 3') (Vendramin et al., 1996). PCR amplification was carried out in a 15 µl reaction mixture containing 1X *Taq* polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.2 µM of each primer, 1 unit of *Taq* polymerase (Applied Biosystems) and 15 ng template DNA. The PCR amplification program consisted of: one cycle at 95°C for 5 min; 35 cycles each at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min followed by a final extension at 72°C for 8 min. Amplification products were visualized on 3% metaphor-agarose gel (Figure 4).

RESULTS AND DISCUSSION

The developed protocol yielded DNA that was comparable to the quality of DNA extracted from the needle tissues as evident from the A_{260}/A_{280} ratios and the yield of the DNA from needle and sapwood tissues (Table 1). The value of 1.8 indicates a high purity of the extracted DNA while values below and above 1.8

Table 1. Comparison of the yield and quality of DNA extracted from sapwood and needle samples of *P. roxburghii*.

S/N	Sample	A_{260}/A_{280} (ratio)		DNA yield (µg/100 mg tissue)	
		Sapwood	Needle	Sapwood	Needle
1	S-1	1.87	1.86	6.98	22.632
2	S-2	1.71	1.72	18.108	12.792
3	S-3	1.70	1.72	12.398	14.016
4	S-4	1.82	1.72	18.108	46.232
5	S-5	1.74	1.68	19.668	31.204

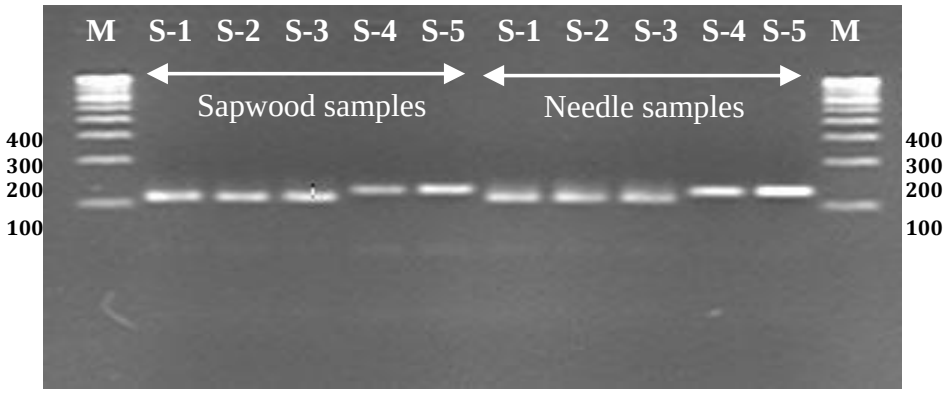

Figure 4. SSR profile of sapwood and needle samples of 5 genotypes of *P. roxburghii* using SSR primer Pt71936. Forward: 5' TTCATTGGAAATACACTAGCCC 3'; Reverse: 5' AAAACCGTACATGAGATTCCC 3'. M: 100 bp DNA ladder.

denotes contaminations of proteins and RNAs, respectively. Samples S1 and S4 however yielded lesser quantity of DNA than that expected, as the extraction is difficult from the sclerenchymatous tissues. The quality of the DNA was also evident from the agarose gel (Figure 1). The results of RAPD (Figure 2), ISSR (Figure 3) and SSR (Figure 4) marker analyses were almost similar for DNA extracted from the sapwood and needles of the same genotype. There are other published reports of DNA isolation from wood of *Cyclobalanopsis* species (Ohyama et al., 2001), Oak (Deguilloux et al., 2002) and species of Dipterocarpaceae (Rachmayanti et al., 2006, 2009). The differences in genomic DNA extracted from bark and wood of different zones in *Robinia* trees were studied using RAPD-PCR (De Filippis and Magel, 1998). In sapwood and heartwood, the random amplification of polymorphic DNA and reverse transcription polymerase chain reaction of RNA was studied (Magel et al., 2002). DNA from processed wood and herbarium samples of *Gonystylus bancanus* was reported (Asif and Cannon, 2005) for identification of endangered tropical timber species. The protocol reported for DNA isolation from *G. bancanus* (Asif and Cannon, 2005) with modifications worked well for *P. roxburghii* in our study. The DNA extracted from sapwood was equally good like that obtained from green needle tissue in terms of both purity and yield. The quality of DNA was also found adequate for RAPD, ISSR and SSR analysis. The results indicate the usefulness of the protocol for the molecular marker investigations of *P. roxburghii*. Also, the method of DNA extraction from sapwood of *P. roxburghii* is extremely useful for molecular studies involving mature tall trees, where collection of foliage tissue is difficult and labour intensive and often professional tree climber is hired for such work in the field.

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