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GENETIC RELATIONSHIP BETWEEN QUALITY AND NON QUALITY WOOD OF PTEROCARPUS SANTALINUS.L., (RED SANDERS) AN ENDEMIC TREE SPECIES BY USING MOLECULAR MARKERS

R.USHA,* S.JHANSI RANI, T.GEETHA PRASUNA
Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam, (Women's University)
Tirupati, Andhra Pradesh, India.
***Corresponding author:E.Mail: ushatirupathi@hotmail.com, Phone Number: 09704704646.**

ABSTRACT

Pterocarpus santalinus. L., popularly known as 'Red Sanders' or 'Red Sandal Wood' is a member of the family Fabaceae. It is an endangered and endemic taxon in the Eastern Ghats of India. The genetic variability among individuals of *P.santalinus* from nursery grown and Forest grown plants, was examined by using Random Amplified Polymorphic DNA (RAPD). Seeds were collected from different regions of Chittoor district. The genomic DNA was extracted by CTAB method. Out of 20 primers, 7 primers were used for PCR amplification, which was found to give reproducible and scorable bands with high percentage of polymorphism. The final numerical analysis included 46 total numbers of bands with 63.04% polymorphism. Maximum polymorphism was showed in PCR reaction with OPA 8. These primer showed 85 percent polymorphism as all these bands obtained were polymorphic with size ranging from 1000 bp to 1.5kb. Dendogram clearly shows that, among all the isolates, there is relationship between the plants growing in forest areas and in nurseries. There is close relationship between two forest growing regions i.e. Nagalapuram and Mamandur region and in between nursery growing plants.

Keywords: Dendogram, Polymorphism, PCR, RAPD, Red Sanders.

1. INTRODUCTION

P.santalinus.L., popularly known as 'Red sanders' or 'Red Sandal wood' is a member of the family Fabaceae, a timber yielding tree. The species is medium sized tree, which grows up to 10.00-11.00m height. Naturally occurring in the dry deciduos forests of south India. The timber of *P.santalinus* is highly priced. Wood is also exported to Japan for manufacture of musical instruments called 'Shamisen'. Timber with wavy or rippled grain fetches fancy price. The importance of this timber is obvious as the quality wood fetches the foreign currency worth of Rs 75000/- per tone approximately.

Commercially there are two types of red sander trees: quality trees and non quality trees. Quality trees have wavy grain structure in the wood where as non-quality trees have no wavy grain structure in their wood. Until a tree attains an age of about 25 years it is not possible to know whether the tree belongs to quality or non-quality type.

The demand for medicinal plants is increasing in both developing and developed countries because of products being non-narcotic, having no side effects, easily available at affordable prices and some time the only source of health care available to the poor. With increasing demand for medicinal plants their commercial cultivation is very essential to reduce the pressure on forest and retain the safe population in natural conditions. *P.santalinus* is one of such species, which it is being exploited recklessly and which is already included in the endangered category of IUCN red list. Apart from this, heartwood has medicinal property. The heartwood is rubbed with water, honey, ghee and oil, applied as collyrium to alleviate defects of vision. Also used for treating skin diseases, bone fracture, leprosy, ulcers and mental aberration. Wood paste applied on skin eruptions, inflammation and on forehead to relieve headache. Wood powder is used to control haemorrhage, bleeding piles and inflammation. An infusion of the wood is used in controlling diabetes. Apart from this durability of timber makes the use of it for making toys, ornamental house posts, agriculture implements and picture frames.etc. Because of presence of wavy grain, deep-red colored heartwood, having medicinal value and durability of timber makes the high demand for timber of cultivation of the species. Since, *P.santalinus* is extremely slow growing species due to wavy and straight grained nature wood.

Molecular markers are most popularly used for the determination of related polymorphisms and mating system parameter, genotype characterization and marker assisted selection. RAPDs are currently the fore runners among PCR based techniques for estimation of genetic diversity of forest species mainly because of their rapidity, reliability and ability to handle large sample sizes.

Knowledge of genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources and breeding. Without determining the diversity reliably, it would not be possible to identify molecular markers or qualitative trait associations. Moreover the viability and purity of rootstocks can be analyzed through the utilization of fingerprints based on molecular markers. To this end, DNA markers are being widely used in studying polymorphism between species or in populations. The application largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of

polymorphism and reproducibility of products (Virk, 2001; Fernandez, 2002). Techniques using Random Amplified Polymorphic DNA (RAPD) markers are simple, fast and sensitive. They require no prior knowledge of the DNA sequence and can amplify a large number of DNA fragments for reaction. The introduction of DNA markers based on the polymerase chain reaction (PCR) technology has led to the development of several novel genetic assays that can be used for many purposes in plant genetic analysis such as cultivar identification and gene mapping. RAPD markers that result from the PCR amplification of genomic DNA fragments using short oligonucleotide (usually 10-mers) of arbitary sequence as primers (William, 1990) provide a fast and easy approach for taxonomic classification and cultivar-typing of fruit trees. This assay has the advantage of being readily employed, requiring very small amounts of genomic DNA, and eliminating the need for blotting and radio-active detection (Cipriani, 1996). This technique, regardless of its sensitivity to reaction conditions, problems with repeatability and amplifying of non-homologous sequences, has been successfully used for the assessment of genetic diversity in plants (Maria, 2008) Factors such as speed, efficiency and amenability to automation make it one of the most suitable methods for germplasm management with respect to estimating diversity, monitoring genetic erosion and removing duplicates from germplasm collections (Khadari, 2003).

In the present study, the genetic variability by visiting different nursery plantations and forests areas of *Pterocarpus* plants and observed the morphological characters of both plants.

2. MATERIALS AND METHODS

Sample collection: *P. santalinus* seeds from seven different regions of Chittoor district were collected and used for present study and compared the results between naturally grown forest plants and plants grown in the nurseries.

Extraction of DNA by CTAB method: DNA extraction was done by procedure given by Murray and Thompson (1980) with slight modifications. *Pterocarpus* seeds were weighed (1g) and ground in pestle and mortar, grinded mixture was transferred into centrifuge tubes carrying 25 ml of preheated (65 ºC) 2 per cent CTAB extraction buffer to make a slurry. The tube were incubated at 65ºC for an hour and stirred occasionally with the help of the sterile glass rod. Centrifuged at 14,000 rpm for 10 min. Equal volume of chloroform: Isoamylalcohol (24:1) was added to each tube and mixed gently. Samples were centrifuged at 10,000 rpm for10 min at room temperature. Then upper aqueous phase was precipitated with 0.6 volume of ice cold isopropanol and 0.1 vol of 3M sodium acetate (pH 5.2) and spinned at 15,000 rpm for 15 min at room temperature. Then, kept for overnight incubation. Supernatant discarded and 70% ethanol was added to pellet and centrifuged at 10,000 rpm for 10 min. Ethanol was poured out carefully and kept for drying at room temperature. Nucleic acid obtained was dissolved in sterile distilled water and stored at -20ºC in small aliquots.

Optimization of polymerase chain reaction: Composition of PCR (Palm cycler) reaction was optimized by varying the concentration of template DNA (25, 50, 75 and 100 ng), *Taq* DNA polymerase (0.5, 1.0 and 1.5 units) and MgCl2 concentration (3.5 and 7.5 mM). The standardized amplification assay was as follows: Template DNA 25ng, *Taq* DNA polymerase (Genei)- 0.5 units; MgCl2 -5mM; dNTP (Genei)100µl each; primer (Operon Technology)-1µm, Buffer (Genei)-1x in a reaction volume of 25µl. Different PCR protocols given by Pascual et al. (2000) and Lee were tested for obtaining best amplification of nucleic acid of the isolation under investigation. The polymerase chain reaction was performed by using Thermo Hybaid cycler, with following temperature profile. The initial denaturizing at 94ºC for 2 min followed by denaturizing at 92ºC for 1min; extension at 37ºC for 2 min with final elongation of 72ºC for 5 min.

Primer screening was carried out using 20 primers from OPA series (Operon technology) for molecular variation analysis. The primer that gave reproducible and recordable amplification was used in the analysis of variability of the genotypes.

Agarose gel electrophoresis: To the 25µl amplification product obtained after the PCR reaction, 2µl loading dye (Bromophenol blue) was added and loaded into individual wells of 1.2% agarose in 1X TBE buffer. Electrophoresis (Genei) was carried out at 60 V for 3 hours and the gel was stained with ethidium bromide (1µg/ml). The observation was made on a transilluminator under UV light. 1 kb ladder (Bangalore Genei) loaded in one lane as a marker.

3. RESULTS AND DISCUSSION

Scoring of bands and data analysis: Each amplification product was considered as RAPD marker and recorded across all samples. Data was entered using a matrix in which all observed bands or characters were listed. The RAPD pattern of each isolate was evaluated; assigning character state '1' to all the bands that could be reproducible and detected in the gel and '0'for the absence of band.

Quantification of the DNA was done after RNase treatment. The purified and quantified DNA was stored at -20^{o}C in small aliquots in sterile distilled water. The DNA of each isolate was subjected to polymerase chain reaction using 10 mer random primers (Operon Technology Inc. USA). The PCR conditions were optimized in terms of concentration of template DNA, *Taq* DNA polymerase and $MgCl_2$ concentration. Varying concentrations of template DNA from 25 ng to 100 ng in a reaction volume of 25 µl, 25 ng DNA gave maximum number of reproducible bands and thus was considered ideal and used subsequently in all analysis. A titration of different concentration of *Taq* DNA polymerase and $MgCl_2$ showed that 0.5 Units of *Taq* DNA polymerase and 5 mM $MgCl_2$ in the final reaction mixture gave optimum, reproducible and well resolved results. A higher or lower concentration resulted in either sub optimal or lack of complete amplifications. The final amplification assay contained 25 ng genomic DNA, 0.5 Units of *Taq* DNA polymerase, 100 mM each of dNTP's, 5 mM $MgCl_2$, 0.6 µM primer and 1X*Taq* buffer in a PCR reaction volume of 25 µl.

Primer selection and survey: Primer survey was carried out by using 20 primers from OPA series of Operon technology Inc., USA. Out of 20 primers used for amplifications of *Pterocarpus* isolates, seventeen resulted in either sub-optimal or non-distinct amplification products. Therefore, these were discarded and remaining 7 were used for PCR amplification which were found to give reproducible and scorable bands with high percentage of polymorphism (Table No.1). Hence, the final numerical analysis included 46 total number of bands with 63.04% polymorphism was resulted from 7-primers amplification. PCR amplification with these primers was done twice before scoring for presence and absence of bands. Numbers of amplification products obtained were specific to each primer and ranged from 2 to 11.

Seven selected primers gave total of 46 amplifications products, out of which 29 were polymorphic. Among the 20 primers of OPA series, 7 primers produced scorable and reproducible amplifications in all the isolates. Maximum polymorphism was showed in PCR reaction with OPA 8. These primers showed 85 percent polymorphism as all these bands obtained were polymorphic with size ranging from 1000 bp to 1.5 kb. They were closely followed by OPA 10 with 81 percent polymorphism, respectively.

The banding pattern of 4 and 5 was found to be identical with primers OPA 2 and OPA 6. In case of primers OPA 1 majority of the bands were found to be common among all isolates. In case of primer OPA 8 total number of bands was 7 out of which 6 were polymorphic. The size of amplified products varied from 500 bp to 3kb. Jaccards similarity co-efficients between the isolates were calculated. Banding profiles obtained with 7 random primers for 7 isolates of *Pterocarpus santalinus* were analysed on the basis of presence and absence of the bands.

Similarity matrix thus produced indicated that 6 and 1 were genetically distinct as they showed only 44.7% similarity followed by 6 and 4 and (53.8%). While the isolate 5 and 4 were found to be genetically similar as 83.3% similarity was observed between the isolates followed by 81.1% similarity between 4 and 1 isolates.

Among all the isolates, there is relationship between the plants growing in forests and in nurseries. There is close relationship between two forest growing plants i.e., Nagalapuram and Mamandur plants and also in nursery growing plants.

The characterization of *Pterocarpus santalinus* isolates by RAPD has proved useful in separating all the isolates from each other. It has also provided us with primer markers that can be used to separate and distinguish each isolate. This possibility of distinguishing different isolates by a simpler technique of genomic fingerprinting based on PCR-RAPD could be of great importance for use in patent protection of plant strains of biotechnological use, where easily detectable markers are not available.

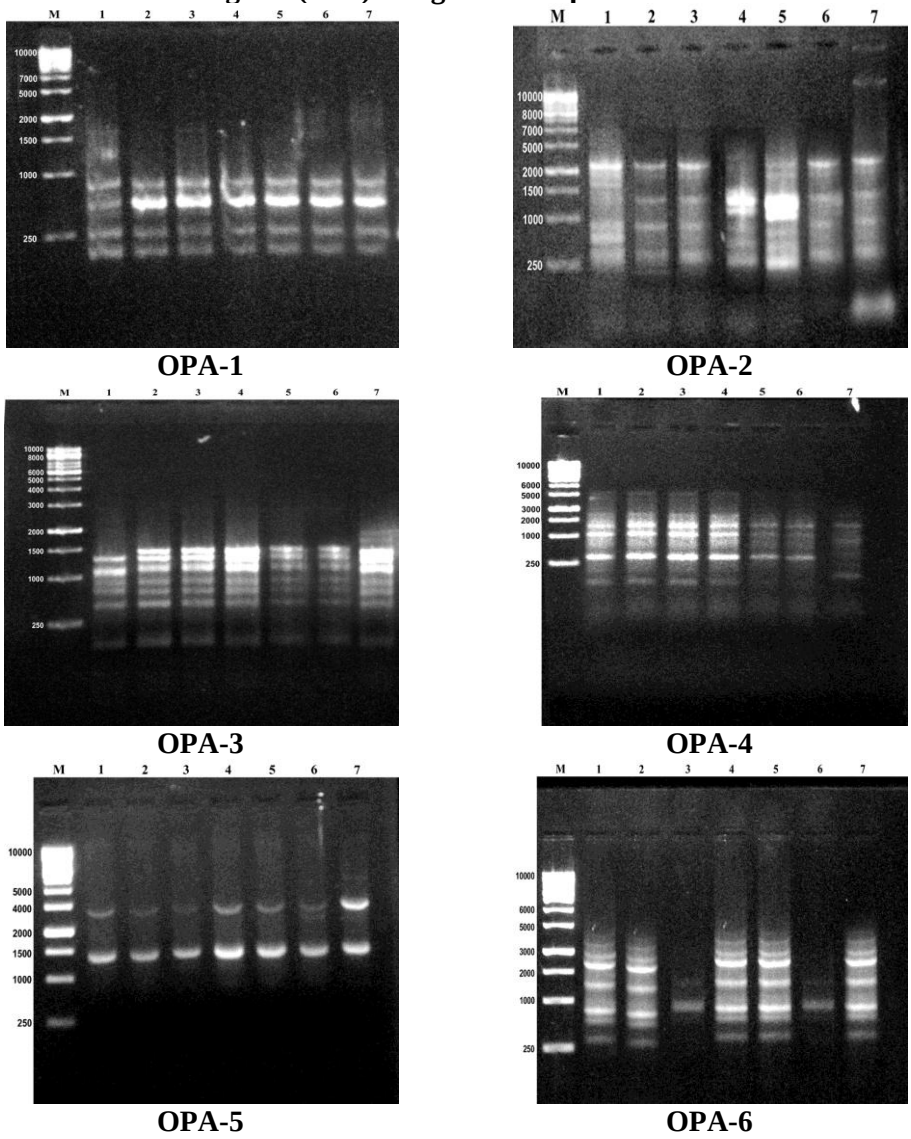
The purification, cloning and sequencing of selected amplifications which are being performed will allow the development of *Pterocarpus* spp. specific primers to be used in a fast and reliable screening of efficient isolates by PCR.

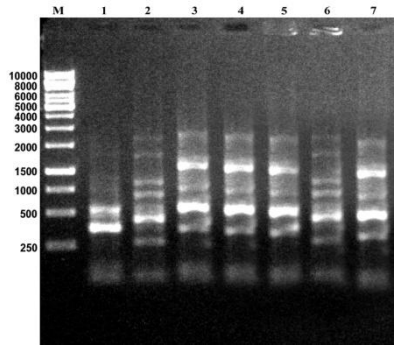
Table No: 1 Banding pattern of *Pterocarpus santalinus*

Name of the primer and sequence	Total Number of bands	Polymorphic bands
OPA-01 5^1-CAGGCCCTTC-3^1	4	0
OPA-02 5^1-TGCCGAGCTG -3^1	8	4
OPA-03 5^1-AGTCAGCCAC -3^1	8	5
OPA-04 5^1-AATCGGGCTG -3^1	6	4
OPA-05 5^1-AGGGGTCTTG -3^1	0	0
OPA-06 5^1-GGTCCCTGAC -3^1	2	1
OPA-07 5^1-GAAACGGGTG -3^1	0	0
OPA-08 5^1-GTGACGTAGG -3^1	7	6
OPA-09 5^1-GGGTAACGCC -3^1	0	0
OPA-10 5^1-GTGATCGCAG -3^1	11	9

Total Number of bands – 46
Polymorphic bands – 29
Total percentage of polymorphism – 63.4%

Figure.1. Banding patterns of RAPD fragments of Pterocarpus santalinus from different regions (1 - 7) using different primers





OPA-8

M Marker 4. Nagalapuram
1. Karvetnagaram 5. Mamandur
2. Chelluru 6. Talakona
3. Kapilatheertham 7. Satyavedu

By using dendogram, one major cluster was formed, these cluster divided into two sub clusters. First sub cluster having 4, 5 and 7; second sub cluster having 1 and 2. Remaining genotypes i.e., 3 and 6 did not grouped with these clusters, they formed separate branch.

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Figure.2. Hierarchical cluster analysis

* * * * * * H I E R A R C H I C A L C L U S T E R A N A L Y S I S * * * * * *

Dendrogram using Average Linkage (Between Groups)

Rescaled Distance Cluster Combine

CASE Label	Num	0	5	10	15	20	25
VAR00004	4						
VAR00005	5						
VAR00007	7						
VAR00001	1						
VAR00002	2						
VAR00003	3						
VAR00006	6						

Proximity Matrix

	Matrix File Input						
Case	VAR00001	VAR00002	VAR00003	VAR00004	VAR00005	VAR00006	VAR00007
VAR00001	1.000						
VAR00002	.744	1.000					
VAR00003	.639	.625	1.000				
VAR00004	.811	.780	.778	1.000			
VAR00005	.649	.634	.611	.833	1.000		
VAR00006	.447	.605	.625	.538	.636	1.000	
VAR00007	.610	.674	.575	.732	.711	.553	1.000

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