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# DEVELOPMENT OF RAPD POLYMORPHIC DNA BASED SCAR MARKER FOR IDENTIFICATION OF *THESPESIA POPULNEA* – A BARK DRUG USED IN AYURVEDA

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## ABSTRACT

**Introduction**: *Thespecia populnea (*L.)Sol.ex Corr, one among *Panchavalkala*, the bark of which is used medicinally in various diseases. Because of similar appearance of bark drugs, quality standard parameters are essential requisite. Molecular markers are like DNA SCAR markers are most reliable methods of quality parameters. Hence an attempt made to generate DNA SCAR markers of bark drug *T. populnea*. **Materials and Methods:** Authenticated bark samples of *T. poppulnea* were collected, along with samples of *Panchavalakala.* DNA extraction carried out through CTAB method and purified. Templates of all five species of *Panchavalkala* were used for RAPD. Based on RAPD results, unique fragments were selected for SCAR marker development. **Results:** Unique band of ~500bp was observed for *Thespesia populnea* which was not seen in other species. This fragment was gel purified, amplified and used for cloning into T vector. Clones were confirmed by amplification using vector primers and was sequenced. Sequence obtained was analysed and primers were designed to give ~450bp product for this region and reconfirmed.

**KEYWORDS:** *Thespecia populnea (*L.), *Panchavalakala.*

## INTRODUCTION

*Thespesia populnea (*L.)Sol.ex Corr. a compact quick growing evergreen medium sized tree found commonly at coastal regions of India and the Andamans.[1] Bark of this tree is grey to brown, fissured, often knobby, fibrous, young parts covered with peltate scales. Leaves are

simple, 14x11 cm, heart shaped, ovate, dark green. Flowers solitary, yellow with purple base and capsules brown, globose.[2]

Astringent bark of this tree is used popularly used in Indian system of medicine, for the treatment of skin diseases, gynecological problems, infertility, hemorrhoids, chronic dysentery etc.[3] Dextrorotatory gossypol is the main active principle of the bark.[4] In Madagascar the sap juice is said to be used in herpes; whereas oil prepared out of bark and capsule is used in gonorrhea.[5] It is also claimed that the pregnant women who consumes the bark powder along with cumin seeds delivers a male child.[6] It forms an ingredient of important five bark drugs *Panchavalkala* which are *Nyagrodha(Ficus benghalensis*), *Udumbara(Ficus racemosa*), *Ashwatha(Ficus religiosa), Plaksha(Ficus lacor), Parisha(Thespesia populnea)* used in Indian system of medicine.[7] Bark drugs of herbal origin, always found mixed with similar other barks of same species or subspecies. Hence quality standards of drug used will be uncertain.

Growing worldwide interest in medicinal plants, simultaneous fast expanding global market for herbal drugs necessitated quality ensured herbal products. Authenticity is the first and foremost part of standardization.[8] It includes standard macroscopic features, microscopic illustrations with cellular structures, powder characters. Pharmacopeas, Monographs, Materia medica published on herbal drugs have given sufficient information about these.[9]

DNA analysis has been proved as an important tool in herbal drug standardization especially for the identification of pharmcognostically indistinguishable genuine drug from admixture. DNA fingerprints are a bar-code like patterns generated by amplification of chromosomal DNA, which is unique for every living thing.[10]

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from polymerase chain reaction amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence.[11] The limitation of RAPD is that it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. To overcome these problems SCAR(sequence characterized amplified region) marker is developed by cloning and sequencing RAPD marker(unique band) and further designing specific primers for the sequence obtained.[12] These SCAR primers are used to identify and differentiate the strain of interest from others.

With respect to all above facts discussed, an attempt has been made to develop SCAR marker of popularly used bark drug *T. populnea* an ingredient of *Panchavalkala.*

## MATERIALS AND METHODS

**Plant material:** Fresh 10 bark samples of *T. poppulnea* tree were collected from various parts of India, along with samples of *Panchavalakala*. Authenticated bark samples were frozen at -70°C till further use.

**Preparation of bark powder:** Bark samples were crushed into small pieces using autoclaved pestle and mortar and powdered thoroughly. Individual pestle and mortar were used for each sample and collected the powder in a fresh container separately, till further use.

**DNA isolation**: DNA isolation of these bark pieces was done using different methods including urea method, DNAzol method, CTAB method and modified CTAB method. CTAB method DNA isolation was found better for RAPD PCR, from the point of yield and quality. The crushed powder was treated with 70% ethanol before DNA extraction. Water wash to the powder was given to remove soluble pigments and compounds that may interfere in the experiment.[13]

**CTAB method DNA extraction:** Ethanol and water washed bark powder was homogenized with CTAB buffer thoroughly. To this Beta-Mercapto ethanol was added and incubated for 90 minutes at $60^0$C in a water bath. Later it was centrifuged at 10,000 rpm for 15 minutes and supernatant was decanted into fresh tubes. For this equal volume of 100% chloroform was added and mixed well for 10 minutes by inverting tube. Again it was centrifuged at 10,000 rpm for five minutes. Aqueous layer was separated and relocated in to fresh tube and to this added equal volumes of iso-propyl alcohol. Another time, it is centrifuged at 10,000 rpm for 15 minutes. Supernatant was discarded and DNA pellets were washed with 0.5 ml of 70% ethanol and centrifuged at 10,000 rpm for 5 minutes. DNA was suspended in 1X TE buffer. This plant DNA was further purified using silica membrane based column and quantitated on agarose gel.[14]

**RAPD PCR[15]:** Templates of all the 5 species of *Panchavalkala* were used for RAPD**.** PCR was arranged using master mix consisting of 100µM each of dNTPs, 100mM KCl, 1.5mM MgCl2, 0.1% each of tween 20 and Nonidet P40, 15 p moles of random primer and 1u of taq polymerase in 40 µl volume. 100ng of template was used. PCR Cycle conditions were set as,

initial denaturation at 94˚C for 5 minutes, denaturation for 30 seconds, annealing for 1 minute at 45˚C, extension for 90 seconds at 72˚C and final extension for another 7 minutes.

After amplification, the samples were run in 2% agarose gel using DNA marker with 0.1, 0.2, 0.3, 0.6, 1.0, 1.5, 2, 2.5, 3 and 3.5 Kb fragments.

Unique band of ~500bp was observed for *Thespesia populnea* which was not seen in other species. This fragment was gel purified, amplified and used for cloning into T vector. Clones were confirmed by amplification using vector primers and was sequenced. Sequence obtained was analysed and primers were designed to give ~450bp product for this region. Randomly selected *Panchvalkala* samples were used for checking the efficacy of the primers designed.

**RESULTS**

DNA extraction of plant samples were carried out through CTAB method and purified. Templates of all five species of Panchavalakala were used for RAPD. RAPD treid with different primers like OPC-06, OPB-10, OPA-02.(Figure1-4). Based on RAPD results, unique fragments were selected for SCAR marker development. The bands that were found only in *T. polunea* species and not in others were selected for further processing. The unique band thus obtained was further purified from the gel and used for cloning into T vector. The white colonies obtained were inoculated in LB media with antibiotic and plasmid isolation was done. The plasmids with retarded mobility were selected for confirmation with PCR using M13 primers. Positive clone was further purified and sequenced. The sequence obtained was used for designing SCAR primers. (Fig 5-7). Highlighted sequence and primer designed was shown in Fig.8 and Table 2.

The primer selectively amplifies only *T. populnea* samples. It was observed that some samples gave very faint amplification(Fig 6 & 7). Thus SCAR marker for *T. populnea* is successfully developed and can be used to identify and differentiate the same from other admixture wherever it is used.

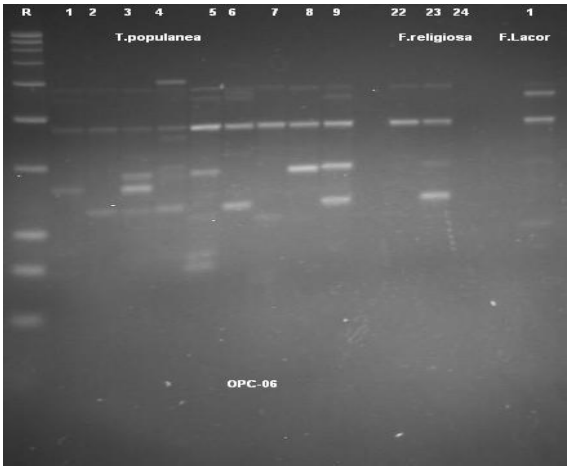


**Fig. 1: RAPD pattern obtained using OPC-06 primer.**

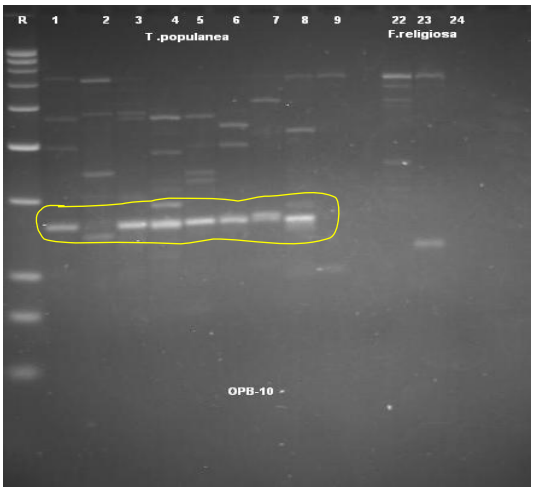


**Fig. 2: RAPD pattern obtained using OPB-10 primer.**

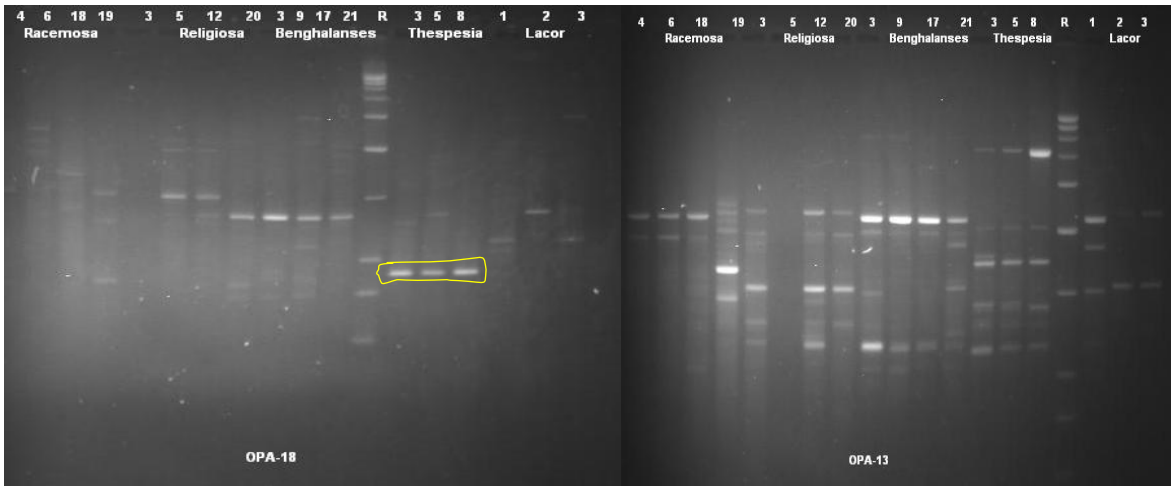


**Figure 3: RAPD with selected samples of 5 Ficus species using OPA-18 and OPA-13 primers.**

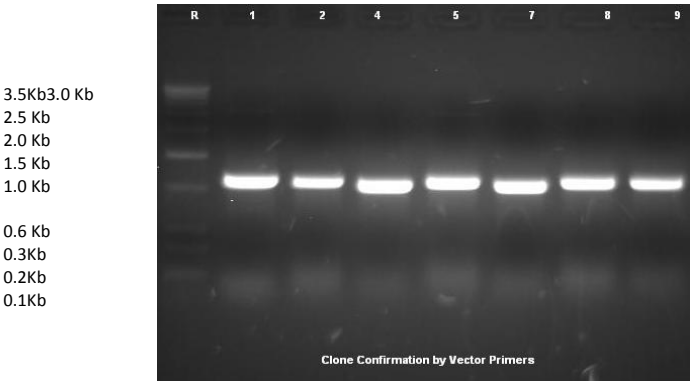


**Figure 4: Clone confirmation: 550 bp unique fragment(with vector primers the size is ~760bp).**

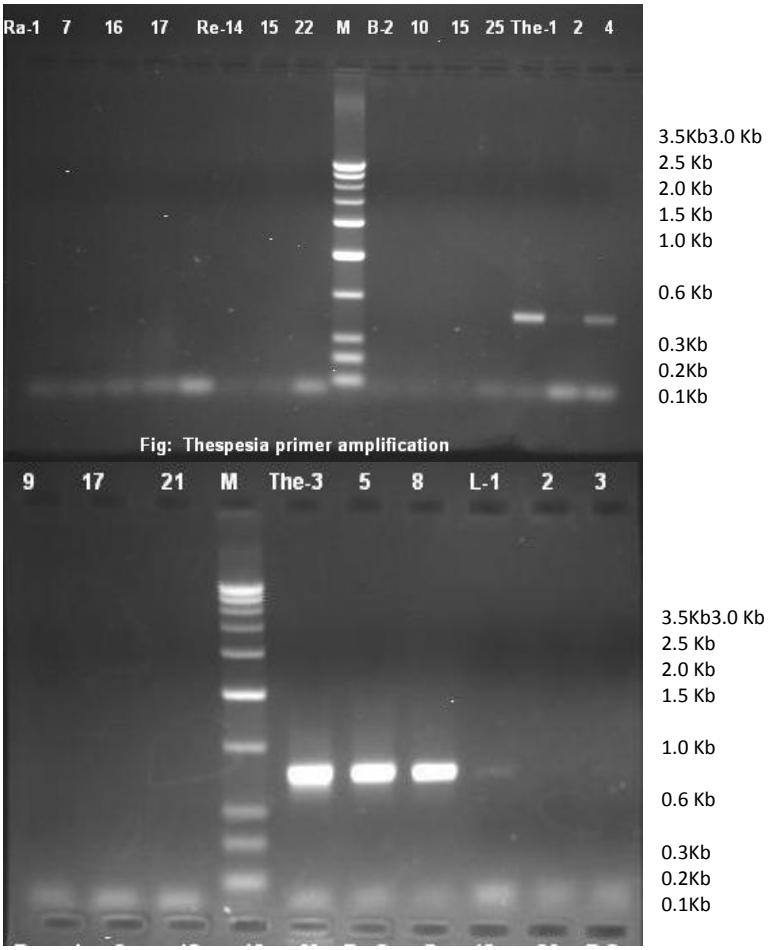


**Figure 5: Lane1-3, *Ficus religiosa*, lane4-marker with 3.5,3.0,2.5,2.0,1.5,1.0,0.6,0.3,0.2 aand 0.1kb fragments, lane5-7 *T.populnea*, lane 8-10 *Ficus lacor* samples.**

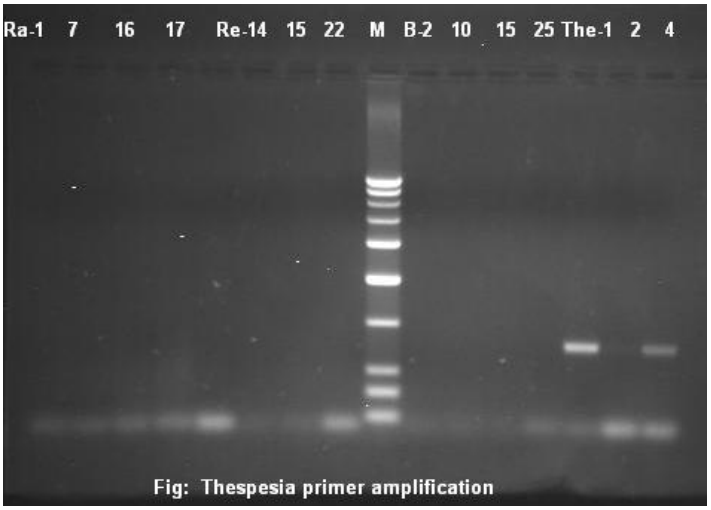


**Figure 6: Lane1-4 *F.racemosa*, lane5-8 *F.religiosa*, lane 9 marker with 3.5,3.0,2.5,2.0,1.5,1.0,0.6,0.3,0.2 and 0.1kb fragments, lane10-13 *F. benghalensis*, lane 14-16 *T.populnea.***

GGAGAATCATCATTTTAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCC
ATGGTTCTGCTGGGACACCTCTCAGTGTTTTGTCTCGAACTCGGGCTACAAGACT
CTATTTTAGGTGTACTTTATATTAAAGAAAAGGGAATTTGAAGCTTTATTCAAAT
ACTTGAACCCCAATACCAAAGTACATCAGGAAGAAATTAATATTTGGAGTGCAA
GTCACGAGTTTTACATTTTTGGACCGCATCCATGCATTTTACTCCCAGGATCATTA
CTCCTAAACTACTTGGCCTTTGAAGCTGAAATTTTTTTTGGAGATTCTTAGCTCAC
AAATCTATCAGAAAGTCTTTTTCCCAAATTAATTTGAAGTTGTTATCATGTCCAGA
AAAATCATGCAAACCAGATGACAATTGGGCACTATTATTGAATCCACTTGTGCTT
CAATTCAAAAATGGTGGCTAAGCTTATCTTGAATGTACTATGCATTGTCATTTTCT
TCTTCCTAAATCTCTATAAATAGGCTTTAGTCCCAGCAGATCCATGGCCGCGGGA
TATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTT
GGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATG
GTCATAGCTGTTTCCTGTGTGAAATTGT

**Figure 8: Highlighted green letters-flanking Nco I sites, blue letters-vector sequence, red letters-primer sequences, purple highlight-random primer sequence.**

**Table 1: Primer sequence**

| | |
|---|---|
| ThpFp | 5’ctgctgggacacctctcag’3 |
| Thp Rp | 5’gcctatttatagagatttaggaa'3 |

(Note: PCR product- 462 bp).

## DISCUSSION AND CONCLUSION

Drug authenticity is a major question in herbal drug research, due to increased demand and simultaneous non-availability.[16] Quality control parameter majors such as macro-microscopy, physicochemical standards, and other analytical techniques have limitations in deciding the authenticity due to geographical variation, secondary metabolite qualitative, quantitative variation etc.[17] DNA based markers such as RAPD, SCAR, AFLP, SSR are unique in identifying the reliability of species and are accurate.[18]

Developing a SCAR marker requires use of two specific primers designed from nucleotide sequence developed in cloned RAPD fragment linked to a trait of interest. A polymorphic band of interest should be identified and eluted from agarose gel, and nucleotide sequence of eluted DNA fragment can be determined. Based on sequence uniqueness, the nucleotide sequence of the polymorphic DNA band is used to synthesize SCAR primers.[19]

*Thespesia populnea (*L.)Sol.ex Corr.*(Parisha*) a component of *Panchavalkala*, the bark of which is used in Ayurveda, in different formulations. Because of similar look with other bark samples, limited availability made this sample admixed with other bark pieces. Hence as an authentication parameter RAPD polymorphic DNA based SCAR marker was tried. 10 samples of T. populnea bark pieces along with other samples of Panchavalkala collected from different areas of India were collected under the guidelines of botanist. DNA extraction of each sample was done using CTAB method, purified using silica membrane based column and the concentration was determined. PCR was set for RAPD of these DNA samples. After amplification, the samples were run in 2% agarose gel using DNA marker with 0.1, 0.2, 0.3, 0.6, 1.0, 1.5, 2, 2.5, 3 and 3.5 Kb fragments.

Unique band of ~500bp was observed for *T. populnea* which was not seen in other species and it was gel purified, amplified and used for cloning into T vector. Clones were confirmed by amplification using vector primers and were sequenced. This sequence obtained was again analysed and primers were designed to give ~462bp product for this region, which is unique for *T. populnea.*

The SCAR primers designed were synthesized and used for amplification on selected Ficus species. Amplification was seen only with *T. populnea* samples.

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