

RAPD studies on genomic DNA isolated from antimalarial plants: *Eucalyptus globulus* Labill. (Eucalyptus) and *Artemisia annua* L. (Artemisia)

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Abstract

This research paper reports a comparison of the genomic DNA constitution between two selected antimalarial plants, *i.e.*, *Eucalyptus globulus* Labill. and *Artemisia annua* L. The Random Amplification of Polymorphic DNA (RAPD) analysis was performed to detect the polymorphism between them. Genetic analysis of these plants relies on high yields of pure DNA samples. Optimized conditions for DNA isolation and Polymerase Chain Reaction (PCR) polymerization were provided. The method involves a modified Cetyltrimethyl Ammonium Bromide (CTAB) extraction employing polyvinyl pyrrolidone. The yield of DNA ranged from 1-2µg/µl per gram of the leaf tissue and purity (ratio) was between 1.6-1.7, indicating minimal levels of contaminating metabolites. The isolated DNA was used for RAPD analysis. Reproducible and amplifiable products were observed in all PCR reactions that are polymorphic. The analysis revealed that these two plants showed genomic similarities, hence, it may be useful in paving a gateway for further research to design new modified drugs for malaria.

Key words: *Eucalyptus globulus* Labill. (Eucalyptus), *Artemisia annua* L. (Artemisia), Cetyltrimethyl Ammonium Bromide (CTAB), Random Amplification of Polymorphic DNA (RAPD)

Introduction

Malaria is the most prevalent among the insect-borne diseases. Every year, it kills between one and two million people, with as many as 300–500 million people being infected. Although human malaria is transmitted by female Anopheles mosquitoes, it has four Plasmodium species as its aetiological agents - *P. falciparum*, *P. vivax*, *P. ovale* and *P. malaria*; the most widespread and severe disease, is caused by *P. falciparum*, which transiently infects the liver before invading red blood cells of the mammalian host. Experts estimated that as many as 40% of India's malaria cases is caused by

Plasmodium falciparum. The first antimalarial drug (David and Jacoby, 2005) quinine, was isolated from the bark of Cinchona species (Rubiaceae) in 1820. It is one of the oldest and most important antimalarial drug, that is still used today. In 1940, another antimalarial drug chloroquine (Peters, 1982), was synthesized and until recently, this was the only drug, used for the treatment of malaria. Unfortunately, *P. falciparum* became resistant to chloroquine (Fogh *et al.*, 1979). Treatment of chloroquine-resistant malaria was done with alternative drugs or drug combinations, which were rather expensive and sometimes toxic. Furthermore, these combinations were not always based on pharmacokinetic principles due to inadequate knowledge of metabolism and mechanism of action of most antimalarial drugs.

Several research groups are now working to develop new active compounds as an alternative to chloroquine, especially from artemisinin (Abdin *et al.*, 2003), a plant-based

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antimalarial drug isolated from the Chinese plant, *Artemisia annua* L. *Artemisia* (family Asteraceae) species is a source of highly valuable phytochemicals and essential oils that are used in the treatment of different diseases. *Artemisia annua* (Klayman, 1993; Tu *et al.*, 1981) is used for the production of antimalarial and possible antibacterial agents and also serves as a natural pesticide. Artemisinin extracted from the leaves and flowers act as an antimalarial agent against *Plasmodium falciparum* and *Plasmodium vivax* including the multidrug resistant strains. The proposed mechanism (Krishna *et al.*, 2004; Haynes and Krishna, 2004) of action of artemisinin involves cleavage of endoperoxide bridges by iron producing free radicals (hypervalent iron-oxo species, epoxides, aldehydes, and dicarbonyl compounds) which damage biological macromolecules causing oxidative stress in the cells of the parasite. Further, research also proved the activity of Eucalyptus against malaria. Eucalyptus is a diverse genus of flowering trees in the myrtle family, Myrtaceae. Eucalyptus (Bachir and Benali, 2008) has attracted attention from global development researchers and environmentalists. It is a fast-growing source of wood, its oil can be used as a natural insecticide, and it is sometimes used to drain swamps and, thereby, reduce the risk of malaria. Therefore, these plants may prove to be the source of new antimalarial drugs.

RAPD (Sangwan *et al.*, 1999) stands for Random Amplification of Polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified (Jones *et al.*, 1997) are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR (Welsh and McClelland, 1990; Williams *et al.*, 1990), using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be obtained from a RAPD reaction. In recent years, RAPD is used to characterize, and trace, the phylogeny of diverse plant and animal species. Unlike traditional PCR analysis, RAPD (pronounced "rapid") does not require any specific knowledge of the DNA sequence of the target plant: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence.

Materials and Methods

Plant materials

Fresh and young leaf samples of *Artemisia annua* L. and *Eucalyptus globulus* Labill. were collected from different regions of Tirupathi. The leaves were brought in aluminum foils in an ice box and stored at -20°C and screened for the presence of DNA.

Chemicals and Reagents

1% (w/v) Poly Vinyl Pyrrolidone, Chloroform:Isoamylalcohol (24:1), 10% CTAB, 1% CTAB, 50 mM Tris-HCl (pH 8.0), 95% ethanol, 3M sodium acetate, 100 mg/ml Ribonuclease, Chloroform: Phenol(1:1), Agarose, Ethidium Bromide (10mg/ml), 0.025% Bromophenol Blue, 10% Glycerol.

- 2x TAB Buffer (CB): 2% CTAB (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl
 - Buffer (CPB): 10 mM EDTA (pH 8.0)
 - Salt-TE buffer (HTE): 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl
 - TE buffer (LTE): 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)
 - 50x TAE BUFFER: 2M-Tris-HCl, 1M-Acetate, 50mM-EDTA
- i. *Isolation of genomic DNA from Artemisia annua* L. by CTAB Method (Milligan, 1998).

The harvested leaves were rinsed with autoclaved distilled water and blot dried. Leaves (2 g) were crushed into a pre-chilled mortar and pestle to a fine powder in liquid nitrogen. Using 3 volumes of freshly prepared extraction buffer, the extract was incubated at 65°C for 30 min. Centrifuge at 10,000 rpm for 15 min. and collect supernatant. Extract with equal volumes of CHCl₃: IAA (24:1) and collect upper aqueous phase after centrifugation. Add one-tenth volume of warm 10% CTAB (55°C) solution and re- extract with CHCl₃: IAA as above. Add at least 1.5 volume of CPB, mix gently, observe precipitate and collect by centrifugation. Dissolve pellet in HTE and precipitate DNA by adding 2 volumes of ethanol. Dissolve pellet in LTE and incubate with 100 mg/ml of DNase free Ribonuclease for 1 h at 30°C. Extract with CHCl₃: IAA and add one-tenth volume of 3M sodium acetate. Precipitate with 2 volumes of ethanol. Centrifuge at 8000 rpm for 10min. Collect the pellet and add sterile distilled water according to the pellet. The isolated genomic DNA was primarily analysed using agarose gel electrophoresis.

- ii. *Isolation of genomic DNA from Eucalyptus globulus* Labill. by Phenol Chloroform Method (Lalueza, *et al.*, 2000)

Weigh 2g of plant leaf and add 10 ml of extraction buffer to it. Grind it well in a mortar by using pestle. Transfer the solution in to a boiling test tube and keep it in the water bath for one hour at 65°C. After incubation, filter the sample through cheese cloth. To the sample, add 1/20 volume of 3M sodium acetate and 2.5 volumes of absolute ethanol. Incubate this at -20°C for 15 minutes. Centrifuge the sample at 10,000 rpm for 10 minutes at 4°C. Collect the pellet and add sterile distilled water to it. Now add phenol, chloroform to the above solution in 1:1 ratio. Centrifuge this at 10,000 rpm for 10 minutes at 4°C. Collect the aqueous layer and add double the volumes of absolute ethanol to it, and incubate this at -20°C for 15 minutes. Centrifuge this at 10,000 rpm for 10 minutes at 4°C. Collect the pellet and add sterile distilled water according to the pellet. The isolated genomic DNA was further analysed using agarose gel electrophoresis.

iii. *Visualization of isolated DNA by agarose gel Electrophoresis* (Martin, 1996)

Make a 1% agarose solution by dissolving 0.2g agarose in 20ml of 1x TAE. Boil the solution to dissolve the agarose. Let the solution cool down to room temperature and add 2 µl ethidium bromide stocks for 20 ml gel solution. Stir the solution to disperse the ethidium bromide, and then pour it into the gel rack. Insert the comb at one side of the gel, about 5-10 mm from the end of the gel. When the gel has cooled down and become solid, carefully remove the comb. The holes that remain in the gel are the wells or slots. Put the gel, together with the rack, into a tank with 1x TAE buffer. Add 5µl of Bromophenol blue to the 10µl of Sample and load it into the wells. Allow them to electrophorese for a while at 100 volts. Observe the gel (Figure 1) under UV-trans illuminator for the presence of DNA/RNA.

iv. *DNA Purification by alcohol precipitation*

Add 1µl of Ribonuclease to 10 µl of DNA mixture and incubate at 37°C for one hour. To 25µl of DNA mixture add 1/10th volume of 3M Sodium acetate (pH-5.2) and 2.5Vol.absolute alcohol (99%) *i.e.*, for 25µl of DNA 2.5µl of 3M Sodium acetate and 62.5 µl of 99% ethanol. Keep it, on ice for 10 mins. Centrifuge this, at 12,000 rpm for 15- 20 minutes. Collect the pellet and wash it with 150µl of 70% ethyl alcohol. Keep it at room temp. for 15 -20 minutes. Air dry the pellet and dissolves it in 20-50µl of Nuclease free or sterile distilled water.

Qualitative and quantitative estimation of DNA

Take 2 ml distilled water in a cuvette and calibrate the spectrophotometer at 260nm as well as 280nm. This acts as a Blank. Add 10 ml of each DNA sample to 1990ml distilled water and mix well. Note the Optical Density values at 260nm and 280nm. Calculate the OD_{260}/OD_{280} ratio. The concentration is calculated using the following formula (Table 1).

$$\text{Concentration} = OD_{260} \times 50\mu\text{g/ml} \times \text{Dilution factor}$$

Restriction digestion

Place the vial containing restriction enzyme on ice. Thaw the vials containing control DNA, test samples and assay buffer.

Results

Table 1. Qualitative and quantitative estimation of isolated DNA samples

S. No.	Plant Material	(Optical density) OD at 260 nm	(Optical density) OD at 280 nm	Concentration of DNA (µg/µl)	Purity of DNA ratio (260nm / 280nm)
1.	<i>Artemisia annua</i>	0.055	0.030	550	1.83
2.	<i>Eucalyptus globules</i>	0.041	0.022	410	1.86

Label five tubes as control, Test sample 1, Test sample 2, Test Sample 3 and Test sample 4, respectively. Add 34 ìl of distilled water to each of the five tubes. Add 10 ìl each of control and test samples to the respective labeled tubes. Add 5 µl of the enzyme buffer to each tube. Add 1 ìl of the enzyme to each tube. Mix by tapping or gentle vortexing and incubate the vials at 37°C for 1 hour. Following restriction digestion (Kwong-Kwok Wong *et al.*, 2000) add 5 µl of gel loading buffer to each of the vials. Load the samples on the 1.5% agarose gel with 10 ìl of ready to use DNA marker. Note down the order in which the samples are loaded. Run the samples at 100 volts for 1-2 hours till the tracking dye (bromophenol blue) reaches 3/4th of the length of the gel. Visualize the gel under Gel Doc.

RAPD –PCR Analysis (Ausubel, 1987)

Prepare a cocktail of PCR reaction mix for the number of species to be tested. The variable components (control Genomic DNA and test DNA) are to be added separately.

Add the following reagents to one PCR vial in the following order:

- DNA to be tested 1µl (100ng/µl)
- Random primer (10 pm/µl): 1µl.
- PCR Master mix (22 µl)

The random primers used in this method are:

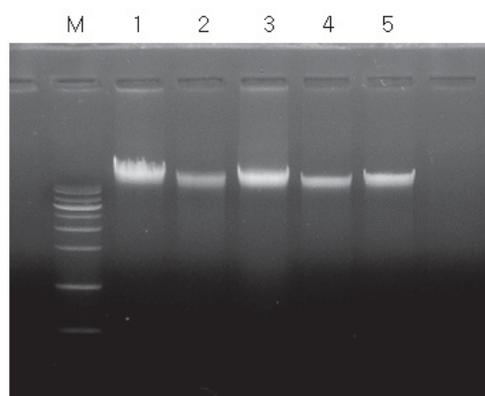
- OPA-10 (5'-GTGATCGCAG-3')
- OPC-4 (5'-CCGCATCTAC-3')
- OPB-6 (5'-TGCTCTGCCC-3')

Mix the contents uniformly and gently. All the above additions are to be done on ice. Centrifuge the samples briefly (6000 rpm for 30 sec. at 4°C) to bring down the contents of the tube. Carry out the amplification, using a thermocycler for 35 cycles according to the following condition (Table 2). After the amplification reaction, analyze the samples on 1.5% Agarose gels(Figure 2).

Table 2. Thermocycler conditions for RAPD-PCR analysis

Initial denaturation	Denaturation	Annealing	Extension	Final extension
94°C (3 minutes)	94°C (30 seconds)	45°C (1 : 30 sec)	72°C (1 : 45 sec)	72°C (7 minutes)

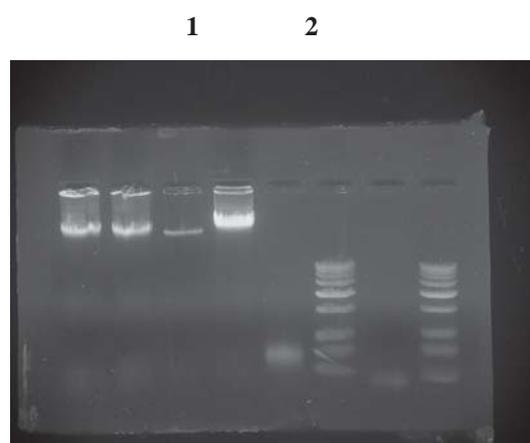
DNA is extracted from *Artemisia annua* and *Eucalyptus globulus* is by CTAB and Phenol chloroform method, respectively (Figure 1). The isolated DNA was used for RAPD analysis. Reproducible and amplifiable products were observed in all PCR reactions that are polymorphic by RAPD analysis. The genomic constitution of the isolated DNA from both the plants is found to be similar by observing the bands, obtained after RAPD Analysis (Figure 2).

**Figure 1.** Extraction of DNA from plant samples

Lane: 1: DNA sample of *Artemisia annua*

Lane: 2: DNA sample of *Eucalyptus globulus*

Lane: M: 100bp DNA Ladder

**Figure 2.** RAPD profile of purified *Artemisia annua* and *Eucalyptus globulus* DNA on 1.5% agarose gel electrophoresis.

Conclusion

The present study clearly indicated that RAPD markers could be effectively used for genetic diversity among the antimalarial plants of Indian origin at genomic level. The result of this study indicates reliable and effective means of assessing genetic variation in antimalarial species and obtaining specific polymorphic profile. The genomic constitution of *Eucalyptus* plant showed few similarities with *Artemisia*, hence, it may be a potential plant to carry further research in obtaining active constituents against malaria. As the bands obtained after RAPD analysis of both plants showed few similarities, a combination of both plants can be considered for further drug development for treatment of malaria. The current research is useful for further research to design new drugs from higher antimalarial plants & importance of polymorphic studies.

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