

## Assessment of Genetic Diversity of Ficus Species Using Rapid Markers as a Measure of Genomic Polymorphism

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**Abstract:** Random Amplified Polymorphic DNA analysis is an excellent tool as a biomarker in determining genetic purity and estimating genetic diversity in plant species. Seven species of *Ficus* genus that are locally available in Hyderabad such as *Ficus benjamina*, *Ficus microcarpa*, *Ficus elastica*, *Ficus binnebdjkii*, *Ficus benghalensis*, *Ficus religiosa* and *Ficus carica*. Those ficus cultivars were then analysed by using three oligonucleotide primer Z13 (5'GACTAAGCCC 3'), Z17 (5' CCTTCCCACT 3') and Z18 (5'AGGGTCTGTG 3'). A total of 340 amplified fragments, in which 212 (62.4%) were polymorphic fragments. The number of polymorphic bands scored per primer ranged from 9 (primer Z13) to 25 (primer Z18). Sixty out of 340 RAPD-PCR fragments were found to be useful as cultivar specific markers. Genetic similarities among the seven *Ficus* cultivars were estimated according to RAPD and cultivar distribution on the consensus tree according to banding pattern of RAPD. Results of combined data exhibited that *Ficus benghalensis* and *Ficus elastica* are related cultivars with highest similarity index. On the other hand *Ficus benghalensis* and *Ficus benjamina* are two distant related species with low similarity index.

**Keywords:** *Ficus*, RAPD marker, PCR, Polymorphism, Phoretix 1D.

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### I. Introduction

The discovery of molecular markers in recent years has greatly enhanced the scope for detailed genetic analysis and approaches to improvement of crop plants. The recently developed DNA- based markers like Restriction Fragment Length Polymorphism (RFLP) and Randomly Amplified Polymorphic DNA (RAPD) provides excellent tools to study the genetic diversity eliminate duplicates in germplasm study, genetic relationships, genetic tagging, genome mapping PVR (Plant Varietal Rights) purposes etc. These markers measure diversity at DNA level and are seldom influenced by environmental conditions as in case of morphological markers. Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the identification of genetic polymorphism. In the last decade, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. [1,2]. If a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel. RAP markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. Characterization of plants using molecular markers is an ideal approach for improvement and conservation of plant genetic resources. Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. But In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate. [3,4].

### II. Materials And Methodology

#### 2.1 Sample collection

The leaf Samples of different *Ficus* species such as *Ficus benjamina*, *Ficus microcarpa*, *Ficus elastica*, *Ficus binnebdjkii*, *Ficus benghalensis*, *Ficus religiosa* and *Ficus carica* were collected from the herbarium and local military nursery situated in Hyderabad, India.

## **2.2 Processing of leaf for DNA extraction**

Young, fresh and healthy leaves were selected and were first rinsed with distilled water. Those are then put into sodium hypochlorite solution for 2 minutes. Then they were washed in distilled water for 5-6 times. The leaves were then washed with 70% ethanol (v/v). Afterwards they were washed repeatedly upto 5-6 times in distilled water and air dried in a laminar air flow hood. The leaves were then cut into very tiny pieces with a sterile scalpel.

## **2.3 Extraction of Crude DNA**

Then DNA was extracted from plant leaf sample by Cetyl-Trimethyl ammonium Bromide Method (CTAB method). The crude DNA can be seen as a cotton cloud like structure upon addition of ice-cold isopropanol. The crude DNA was extracted by centrifugation at 12,000 rpm for 10 minutes. The pellet containing the crude DNA was dissolved in 10mM Tris-EDTA buffer. The crude DNA was stored at 4<sup>o</sup>C for further use. [5,6]

## **2.4 Purification of crude DNA**

The crude DNA was purified using liquid liquid extraction of phenol – chloroform method. The proteins will partition into the lower organic phase while the nucleic acids remain in the upper aqueous phase [7]. The purified DNA is then dissolved and stored in 10mM Tris-EDTA buffer.

## **2.5 Qualitative and Quantitative Estimation of DNA in purified Sample**

The Qualitative and Quantitative estimation was determined spectrophotometrically [8,9] The purity of the nucleic acids is determined by calculating the ratio of absorbance at both 260 and 280nm by using the following formula.  $O.D_{260\text{ nm}} / O.D_{280\text{ nm}}$ . If the ratio is equivalent to 1.8, then the DNA is pure one. If the ratio of  $O.D_{260\text{ nm}} / O.D_{280\text{ nm}}$  is greater than 1.8, then there is RNA contamination and if the ratio is smaller than 1.8, then the sample is contaminated with protein and phenol. Likewise Concentration of DNA in the sample ( $\mu\text{g/ml}$ ) =  $O.D_{260} \times 50 \times \text{dilution factor}$  (for double stranded DNA).

## **2.6 RAPD analysis**

**2.6.1 Selection of Primers:** Three RAPD primers were chosen as potentially useful, from twenty, 10-mer oligonucleotides with arbitrary sequence namely Z13 (5'GACTAAGCCC 3'), Z17 (5' CCTTCCCACT 3') and Z18 (5'AGGGTCTGTG 3')

**2.6.2 PCR Reaction:** The PCR reaction mixture consisted of 20ng genomic DNA, 5X PCR buffer (Promega), 25mM/L MgCl<sub>2</sub> (Promega), 100 $\mu$ M/L of each dNTP (Promega), 66ng/ $\mu$ l Primer and 5 U/ $\mu$ l Taq polymerase in a 25 $\mu$ l volume. The amplification protocol was carried out according to [10-12] with some modifications.

The reaction mixtures were pre-denatured at 94 °C for 5min, followed by each cycle containing denaturation step at 92<sup>o</sup>C for 30 seconds, annealing step at 40<sup>o</sup>C for 30 seconds and extension step 72<sup>o</sup>C for 90 seconds. The PCR reaction was carried out for 30 cycles, with a final extension at 72 °C for 5 min, and eventually stored at 4 °C.

**2.6.3 Agarose gel Electrophoresis:** The amplified products were then electrophoresed in 1% agarose gel with 0.5x TBE buffer. After the complete running of the gel, the gel was stained with ethidium bromide and then the banding patterns of the DNA in gel were visualized with a UV transilluminator and Gel Documentation system (BioRad).

**2.6.4 Data Analysis:** The gel images were analysed using the Phoretix 1D software. The gel image is loaded into the Phoretix 1D Software. (The Phoretix family of software for electrophoresis analysis was first produced in 1991. TotalLab was first released in 1999 representing a breakthrough in levels of automation and ease of use. Both were developed by Nonlinear Dynamics Ltd.). Lane creation is the first step involved in gel analysis, where the software shows where the lanes are on the gel image. The second step is to subtract the background intensity of the gel image, from the measurements of lanes. Third step involve the analysis, wherein detect the band in the lanes, deconvolution of profile and matches the band between the lanes. The measurement and calibration of band include the fourth step where it includes band picking and analysis of bands based on retardation factor, Molecular size, Quantity and intensity calibration and normalization. The final step is the construction of dendrogram based on the clustering present in between the two sequences of the lane each alignment and the scoring is represented by aligned as 1 and a non-alignment is given a score 0. The overall similarities are calculated and presented in the form of a dendrogram.

### III. Result And Discussion

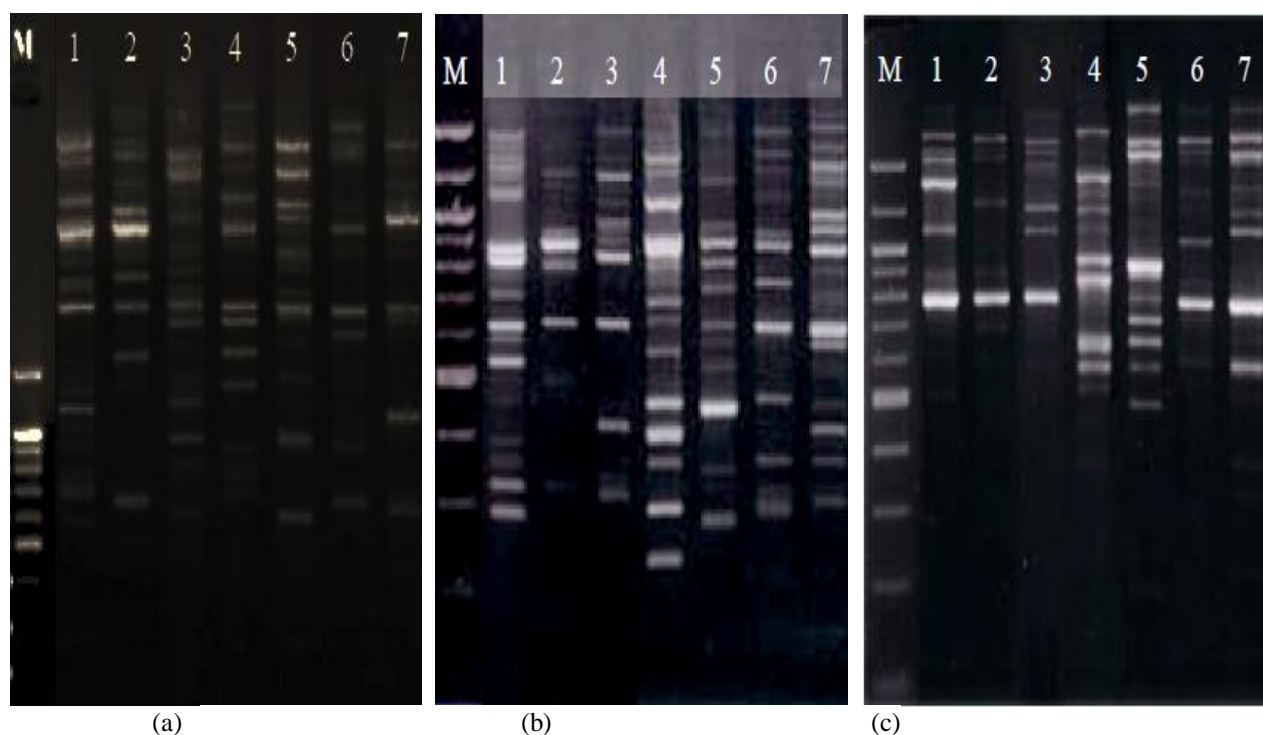
The DNA from the seven cultivars of Ficus were isolated and purified. The DNA from the seven cultivars were then qualitatively and quantitatively estimated.( TABLE 1).

**Table 1 : Qualitative and Quantitative analysis of DNA from different species of Ficus.**

Sl.no.	Cultivar	A <sub>260</sub>	A <sub>280</sub>	A <sub>260</sub> /A <sub>280</sub>	Inference pure/impure DNA	Quantity of DNA in (µg/ml)
1.	Ficus benjamina	0.024	0.014	1.714	Pure	720
2.	Ficus microcarpa	0.024	0.013	1.846	Pure	720
3.	Ficus elastica	0.025	0.014	1.785	Pure	750
4.	Ficus binnebdjkii	0.028	0.015	1.866	Pure	840
5.	Ficus benghalensis	0.026	0.014	1.857	Pure	780
6.	Ficus religiosa	0.032	0.018	1.777	Pure	960
7.	Ficus carica	0.024	0.013	1.846	Pure	720

For RAPD analysis , the primers i.e. Z13 (5'GACTAAGCCC 3'), Z17 (5' CCTTCCCACT 3') and Z18 (5'AGGGTCTGTG 3') were chosen for all the species of Ficus. and the polymerase chain reaction was carried out for 30 cycles. The results were analysed in a Gel documentation system to find out the polymorphism. (FIGURE: 1).

The name designated in the gel image are : M = DNA Marker, Lane 1: Ficus microcarpa, Lane 2: Ficus elastica, Lane 3: Ficus binnendijkii , Lane 4: Ficus benghalensis , Lane 5: Ficus benjamina, Lane 6: Ficus carica, Lane 7:Ficus religiosa. ( FIGURE 1).



**Figure 1:** Gel image of RAPD markers after PCR reaction for 30 cycles of different purified DNA from seven selected species of Ficus. From left to right : (a) Gel image for **Z-13 markers**, (b) Gel image for **Z-18 markers**, (c) Gel image for **Z-17 markers**.

RAPD data were scored as presence (1) or absence (0) bands by using of the Phoretix 1D image analysis system (Phoretix International, London) to integrate the data. Similarity indices were calculated and consensus tree was developed based on the banding patterns of the Seven cultivars in RAPD using SPSS statistical analysis program (Version 10). Individual scoring function based on band matching and dendrogram for each marker was produced. (FIGURE: 2- FIGURE: 4) The genetic relationships among the seven cultivars, at the molecular level, were determined by analyzing the dendrogram produced from clusters by UPGMA method (FIGURE: 5).

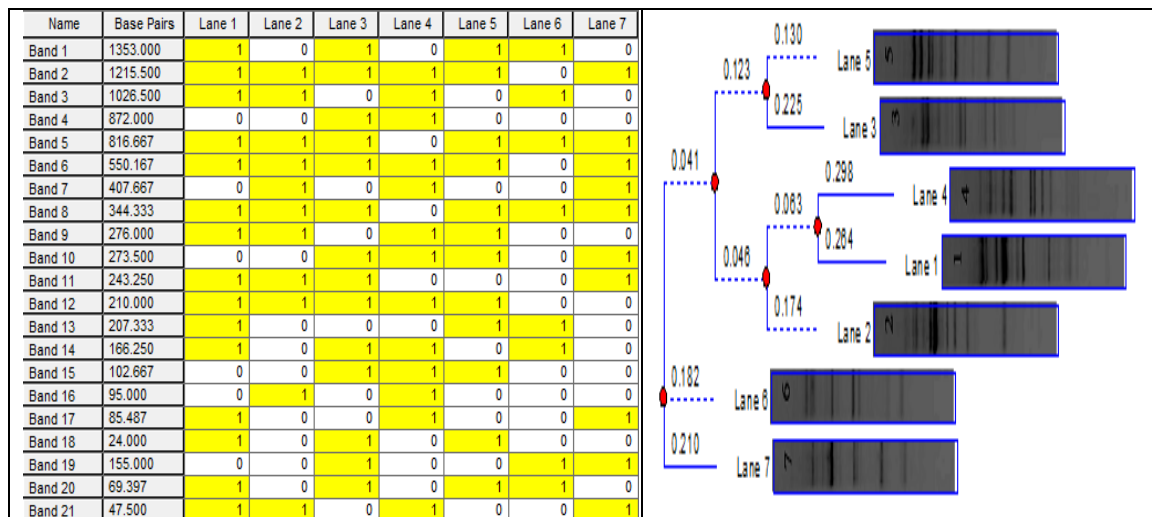


Figure 2: Scoring Function and dendrogram for Z-13 markers

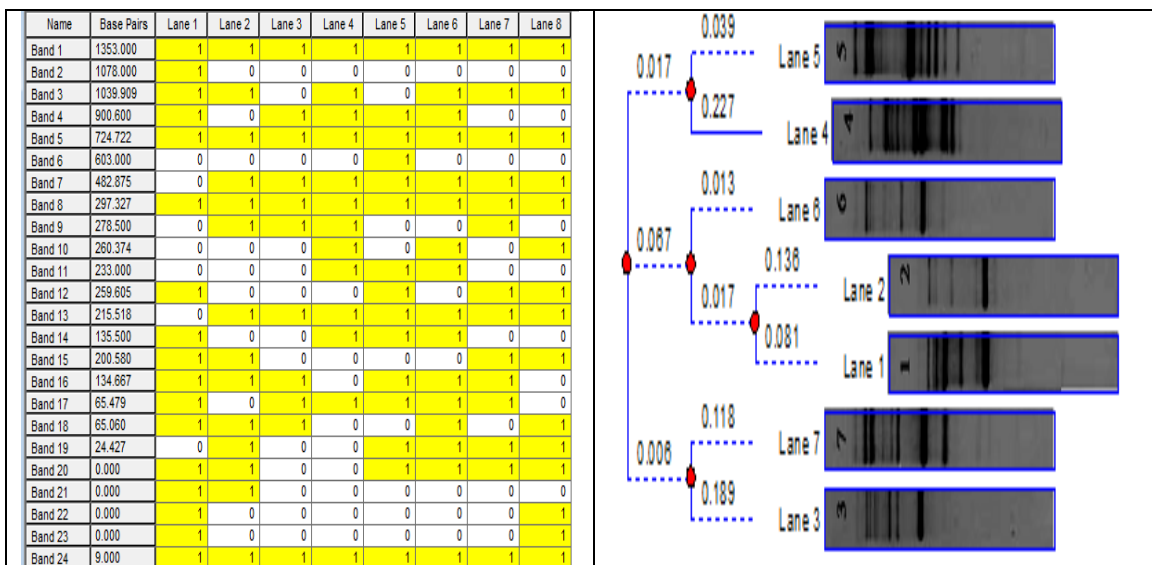


Figure 3: Scoring Function and dendrogram for Z-17 markers

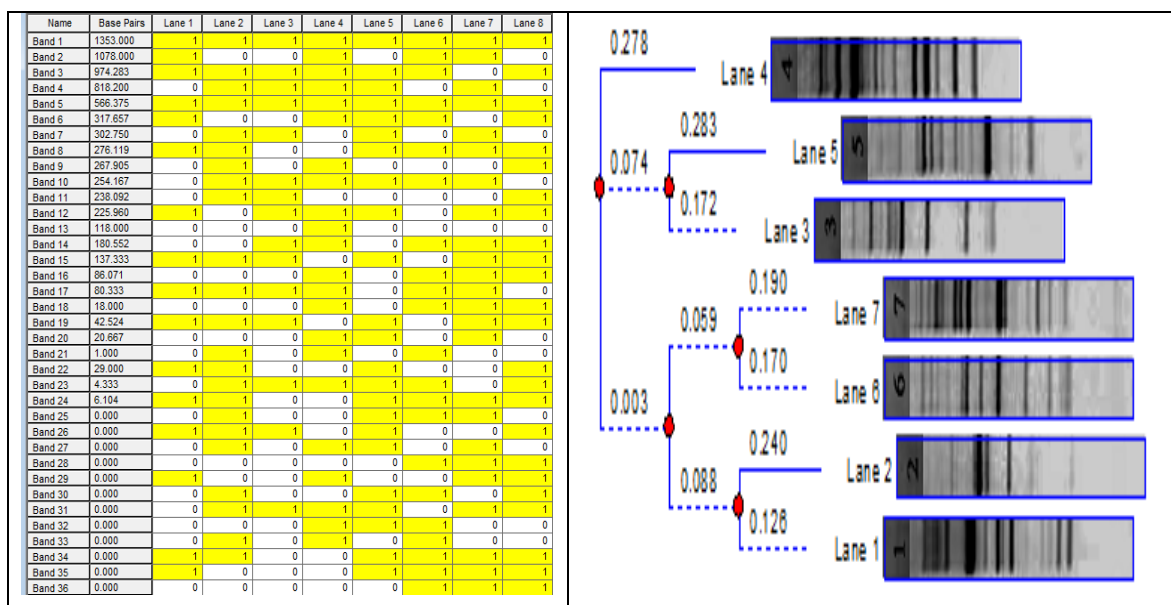
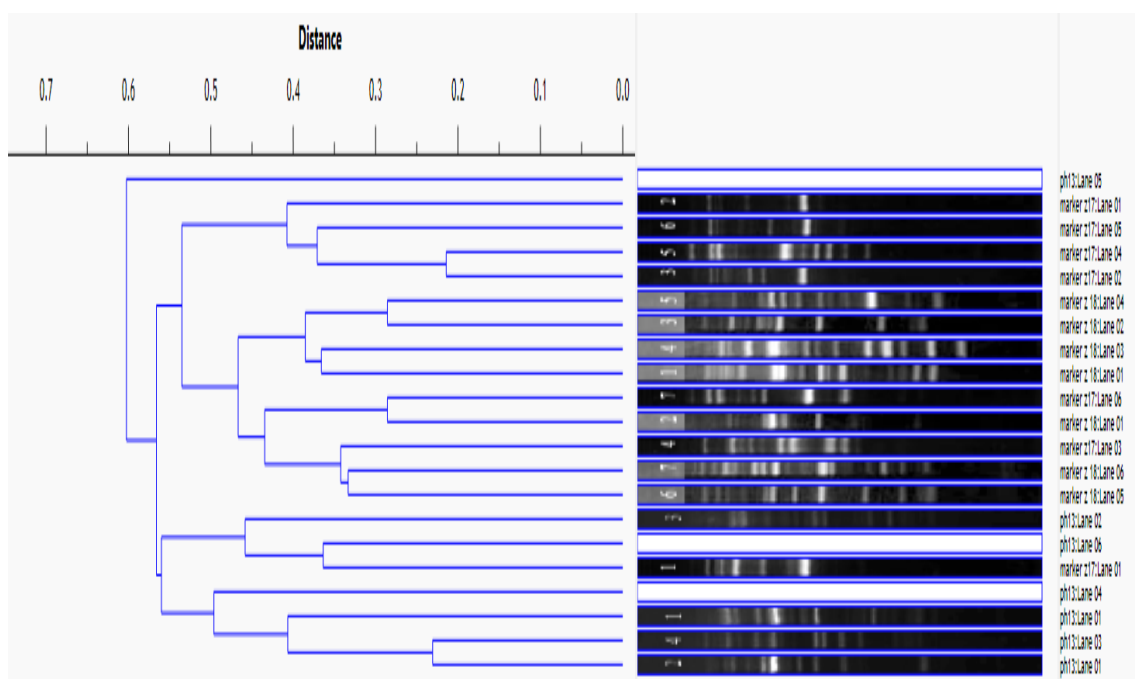


Figure 4: Scoring Function and dendrogram for Z-18 markers



**Figure 5: Final dendrogram showing polymorphic relationship between different species of ficus (UPGMA method).**

#### IV. Discussion

The seven species of Ficus plants that were identified and collected from the geographical region of Hyderabad where the leaves are collected and nuclear DNA was collected from each sample of Plant species. The extraction of the genomic DNA by CTAB method was followed by the purification of the DNA. We got higher quantity of genomic DNA in *Ficus religiosa* (960µg/ml) followed by *Ficus binnendijkii* (840µg/ml). The purified DNA sample of the seven species of Ficus were subjected to PCR reaction upto 30 cycles using three different oligonucleotide random primers such as Z-13, Z-17, Z-18 where the amplified product was observed by running them in 1% agarose gel electrophoresis and visualizing them in UV trans-illuminator or Gel Documentation system. The gel documentation gel images are then used by Phoretix 1D software where various steps are involved such as Lane Creation, Background subtraction, Lane Analysis, Calibration and Band picking, and lastly the dendrogram construction basing upon the scoring from a scoring matrix produce where it shows the polymorphism of specified DNA. The least polymorphic species of ficus were *Ficus elastica* and *Ficus bengalensis* obtained by using primer Z 17 and primer Z 18 and the most divergent species is *Ficus benjamina* from all the seven cultivars used for analysis.

#### V. Conclusion

RAPD polymorphism could be used as efficient tools for the detection of similarities and phylogenetic relationships of the studied genotypes, which could be useful in the breeding programs, such as assessing the level of genetic diversity within germplasm and cultivar identity. This can also be useful in mapping the efficacy and the pharmacological activity of the bioactive compounds of the related species of medicinal and pharmacological important plants.

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