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# Genetic Diversity Assessed yhrough RAPD Markers in Terminalia Pallida Brandis.

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**Abstract:** Random amplified polymorphic DNA markers were used to evaluate the genetic diversity in a representative population of *Terminalia pallida* from six different geographical regions of chittoor district. 20 OPA primers were used for the RAPD analysis. A total of 33 bands yielded from 7 reproducible primers, with a mean of 4.28 amplified bands per primer. The plant species showed 69.5% polymorphism. Jaccards similarity co-efficients ranged from 0.167 to 0.600. A dendrogram clustered based on the UPMGA clustering method revealed two clusters. Cluster-1 comprises 1 and 2 accessions, and cluster-2 comprises 3, 4, 5 and 6 accessions. 4 and 6 accessions are genetically more diverse than other accessions. Our results imply that *T. Pallida* possess high level of genetic diversity, compared to other endangered plant species. Present study is the first report on molecular characterization, of *T. Pallida.* It suggests RAPD is a good molecular marker to study the genetic diversity of these species.

**Keywords**: *T. pallida*, Endemic, Endangered, Genetic diversity, RAPD markers and Polymorphism.

## INTRODUCTION:

*Terminalia pallida* Brandis. (Family: Combretaceae) is a semi evergreen endemic tree species. It is known to the local people as "Tellakaraka". It occurs in the rocky hilly areas of dry deciduous forests of Chittoor, Cuddapah and Kurnool Districts in Andhra Pradesh, at 700–800 m elevation in the Eastern Ghats, but it is mainly centred at Tirumala Hills, Chittoor, Andhra Pradesh, India [1]. The fruit is used as an anti-pyretic, purgative, for diarrhoea, peptic ulcers, diabetes, venereal diseases, cough, cold, dysentery, fissures, cracks and in tanning and dyeing. It is also used as a substitute for the fruit of *T. Chebula* [2-3]. Fruit powder is used to treat diabetes by tribal people administering the drug orally twice a day for 25 days [4]. *T. pallida* is highly potential for its medicinal properties. The plant is reported to possess antioxidant, Hepatoprotective activity [5], antibacterial and antifungal activity [6].The highly potential medicinal properties of this plant is due to the presence of diverse phyto chemical constituents [7]. *T. pallida* has been given an endangered status at the global level based on IUCN criteria by the Conservation Assessment and Management Planning Workshop held at Hyderabad in 2001. It is now included in the First Red List of Medicinal Plants of Andhra Pradesh [8].

The presence of genetic diversity is crucial for improving any plant species. An understanding of the magnitude and pattern of genetic diversity has important implications in breeding programmes and for conservation of genetic resources. Morphological markers are routinely used for estimating the genetic diversity but are not successful due to strong influence of environment. Molecular markers are independent of the environmental growth conditions and physiological age of the plant enables characterization of all plant genotypes [9-10].Hence, Molecular Markers are precisely used to characterize genetic variation within and among the populations which contribute towards conservation programme concerned with the critically endangered species [11]. Different type of molecular markers have been used to ascertain DNA polymorphism but, RAPD marker system has been widely employed because of its simplicity, rapidity, low cost and non-requirement of prior sequence information for primer design [12].RAPD polymorphism reflects the variation of the whole genomic DNA and would be a better parameter to measure the pattern of genetic diversity of the rare and endangered plants [13]. As per the earlier literature concerned, a large number of reports have appeared previously, using RAPD patterns for differentiating varieties, species, etc. of, medicinal, endemic and endangered plants. These include, studies on, *Changium smyrinoides* [14], *Draba dorneri* [15], *Gymnema sylvestre* [16], *Catharanthus roseus* [17], *Dendrobium officinale*[18] etc. Wherein subtle differences in the banding patterns have been used as an index to differentiate varieties and assess genetic variability. Therefore, Random amplified polymorphic DNA (RAPD) is the marker of choice in present study. The principal aim of this present investigation was, to assess the genetic diversity within the population of *T.pallida* and to provide the baseline information for establishment of conservation strategies for the endemic species.

## MATERIALS AND METHODS

### Plant material

A total of six accessions of *Terminalia pallida* were collected from six geographic locations of Chittoor district, Andhra pradesh, India. Fresh and young leaf samples were collected and stored in ziplock bags with silica gel and transported back to the laboratory for DNA extraction.

### Genomic DNA extraction

DNA extraction was done by the procedure given by Murray [19] with slight modifications. 5 gms of leaves were grinded in liquid nitrogen, the powder was transferred into centrifuge tubes carrying 25 ml of preheated (65ºC) 2 % 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. Equal volume of Chloroform: Isoamylalcohol (24:1) was added to each tube and mixed gently. Samples were centrifuged at 10,000 rpm for 10 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. The pellets obtained were washed with 70% Ethanol and keep for drying at room temperature. Nucleic acid obtained was dissolved in sterile distilled water and stored at -20ºC in small aliquots until used for PCR amplification.

**Purification of DNA**

RNase treatment was given to remove RNA from the total nucleic acid. 2 µl of RNase from stock solution was added to nucleic acid extraction and incubated at 37ºC for an hour. DNA concentration of samples and purity was determined by taking ultraviolet absorbance at 260 nm and 280 nm in a spectrophotometer and rechecked by a running sample on 1% agarose along with 1 Kb molecular weight marker.

**PCR amplification**

**Optimization of polymerase chain reaction (PCR)**

The PCR was optimized by varying the content 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, 25 ng; Taq DNA polymerase (Genei, Bangalore, India), 0.5 units; $MgCl_2$, 5mm; dNTP (Genei), 100 µm each of dATP, dGTP, dCTP, dTTP; Primer (Operon Bio-technologies, Cologne, Germany), 1 $\mu$m; buffer (Genei), The PCR was performed using a palmcycler with the following temperature profile: initial denaturation at $94^0$C for 2 min, followed by 45 cycles of denaturation at $92^0$C for 1 min; annealing at $37^0$C for1 min; extension at $72^0$C for 2 min with final elongation at $72^0$C for 5 min.

**Primer survey and selection**

The preliminary primer screening was carried out using 20 primers from OPA series (Operon Bio-technologies) for molecular variation analysis. The primers that gave reproducible and recordable amplification were used in the analysis of variability of the accessions.

**Agarose gel electrophoresis**

To 25 µl of amplification products obtained after the PCR, 2 µl of loading dye (bromophenol blue) were added and loaded into individual wells of 1.2% agarose in 1 X Tris-acetic acid/EDTA buffer. Electrophoresis was carried out at 60 V for 3 h, and thereafter the gel was stained with ethidium bromide on a transilluminator under UV light. The 1 kb RAPD primer set M ladder (MBI, Fermentas, Germany,) was also loaded in one lane as a marker.

**Data analysis**

RAPD patterns were analysed by assigning character state, presence (1) or absence (0) for estimating the similarity among all the tested samples. The number of polymorphic bands is calculated for each population. Similarity matrices were computed based on Jaccard's [20] similarity coefficient, using the SPSS (11.0),software. UPGMA method was used for cluster analysis and constructed the dendrogram based on obtained similarity coefficients The polymorphism percentage was estimated by dividing the number of polymorphic bands over the total number of bands.

## RESULTS

Six individual populations of *T. pallida* were collected from six different areas ((Table 1). In the investigation 20 random decamer oligonucleotide primers from OPA series (operan technology inc., USA) were screened, of these 20 primers, only 7 primers showed reproducible and scorable bands, 13 sub-optimal or non amplified primers were discarded. Figure-1 represents the amplification pattern of the reproducible primers. The details of the RAPD analysis were given in the Table-2. The RAPD profiles of 6 accessions were compared individually for each primer. All these 7 primers resulted in the amplification of 33 bands, of which 23 bands were polymorphic, and 10 bands were monomorphic. The mean number of amplified bands per primer was only 4.8. The highest number of amplicons (7) was obtained for primer OPA13, followed by (5) for primer OPA4 and OPA10, (4) for primer OPA2, OPA3, OPA7, and OPA14 . The primer OPA13 produced a maximum number (5) of polymorphic bands, and OPA3, produced a minimum number (2) of polymorphic bands. The highest level of polymorphism(80%) was produced by the primer OPA10, followed by the primers OPA2,OPA 7,OPA14(75%) , OPA13(71.4%) ,and OPA 4(60%) and lowest level of polymorphism produced by OPA3(50%). All primers obtained a wide range of amplicons, ranging from 250bp to 9000bp.

**Table-1: *T. Pallida* accessions collected from different localities**

| LOCALITY | AREA OF STUDY | GEOGRAPHICAL LOCATION |
|---|---|---|
| LOC 1 | Nelakona (Talakona) | 13° 48' 41.6808"E, 79° 12' 30.4446"N |
| LOC2 | Waterfalls (Talakona) | 13° 48' 41.6988"E, 79° 12' 56.9988"N |
| LOC3 | Narayanagiri (Tirumala) | 13° 40' 46.4622"E, 79° 19' 59.484"N |
| LOC4 | Papavinasanam (Tirumala) | 13° 43' 12.2412"E, 79° 20' 40.2606"N |
| LOC5 | Sanralla metta (Tirumala) | 13° 39' 32.1012"E, 79° 23' 20.83"N |
| LOC6 | Balapalli check post (Chittoor district) | 13° 51' 53.2728"E, 79° 25' 2.013"N |

The dendrogram constructed, based on UPGMA clustering method has been shown in Figure- 2. Jaccard's similarity co-efficients of the amplified bands for *T. pallida* species has been given in Table-3. Jaccards pairwise similarity co-efficient values ranged from 0.167(accessions 1 and 6) to 0.600(accessions 1 and 2). A maximum similarity value of 60% was observed between accessions 1 and 2 accessions, followed by 52.6% between 5 and 3, 26.3% between the

accessions 4 and 1, 26.1% between 5 and 1, and a minimum of 16.7% between 6 and 1. An average similarity value observed across each accession was 38.6%. The cluster tree analysis showed that the 6 accessions were broadly classified into two main clusters .First cluster having 1 and 2 accessions exhibited high level of genetic similarity. Within the Second cluster, 3 and 5 accessions exhibited high level of genetic similarity, compared to the accessions 4 and 6, which are genetically diverse.

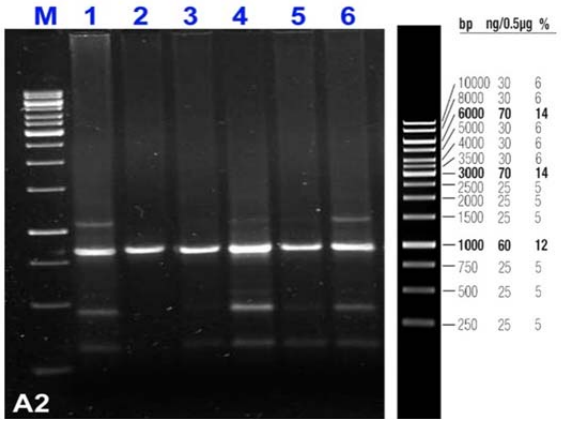
**Table-2. Total number of amplified fragments and number of polymorphic fragments generated by 7 random primers in *T. pallida* samples**

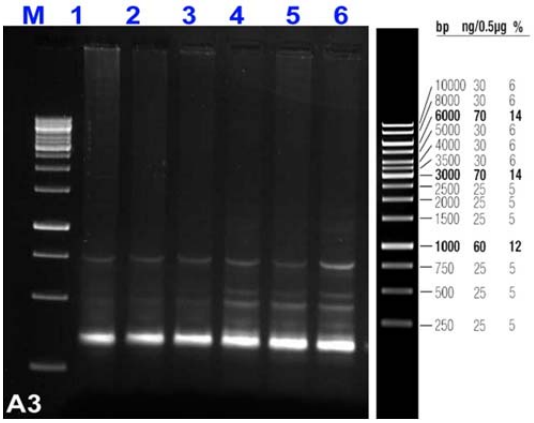
| Name of the primer | Sequence 5' to 3' | Total number of bands | Polymorphic bands | % of polymorphism |
|---|---|---|---|---|
| A2 | TGCCGAGCTG | 4 | 3 | 75% |
| A3 | AGTCAGCCAC | 4 | 2 | 50% |
| A4 | AATCGGGCTG | 5 | 3 | 60% |
| A7 | GAAACGGGTG | 4 | 3 | 75% |
| A10 | GTGATCGCAG | 5 | 4 | 80% |
| A13 | CAGCACCCAC | 7 | 5 | 71.4% |
| A14 | TCTGTGCTGG | 4 | 3 | 75% |
| | | 33 | 23 | 69.5% |

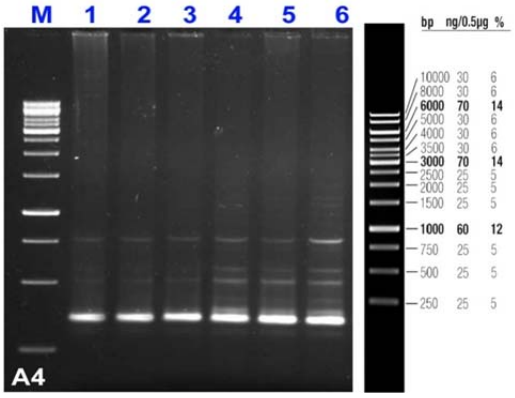
**Table-3: Jaccard's similarity co-efficients of 6 accessions of *Terminalia pallida* based on polymorphism obtained with OPA primers**

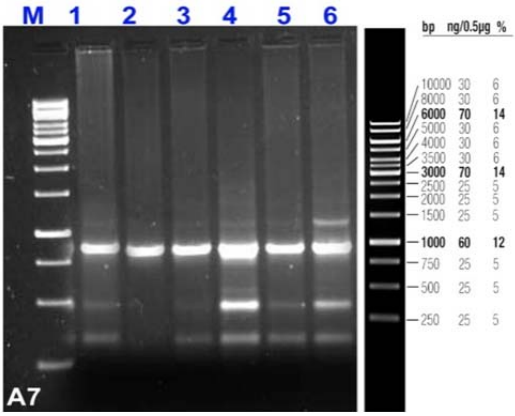
| Case | Matrix File Input | | | | | |
|---|---|---|---|---|---|---|
| | Loc 1 | Loc 2 | Loc 3 | Loc 4 | Loc 5 | Loc 6 |
| Loc 1 | 1.000 | | | | | |
| Loc 2 | .600 | 1.000 | | | | |
| Loc 3 | .316 | .381 | 1.000 | | | |
| Loc 4 | .261 | .435 | .417 | 1.000 | | |
| Loc 5 | .263 | .400 | .526 | .435 | 1.000 | |
| Loc 6 | .167 | .389 | .300 | .429 | .471 | 1.000 |

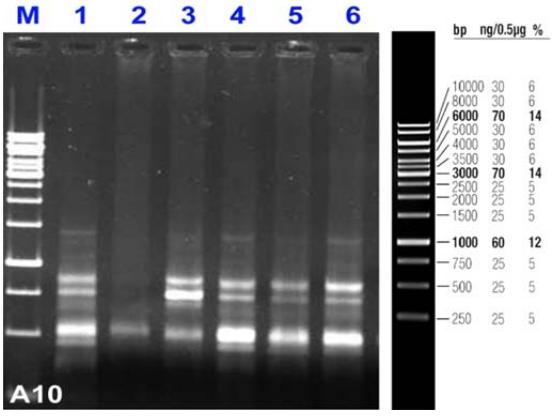
**Figure- 1: The RAPD profiles of *T. pallida* generated by OPA series primers. Samples 1 - 6 *T. pallida* individuals. M-1 kb DNA ladder.**



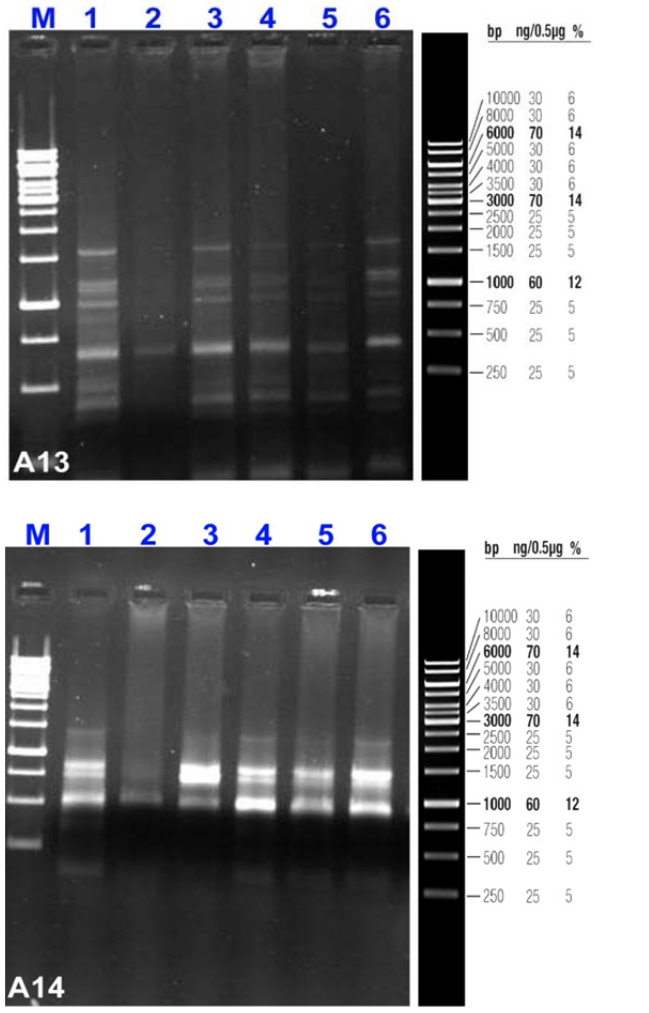
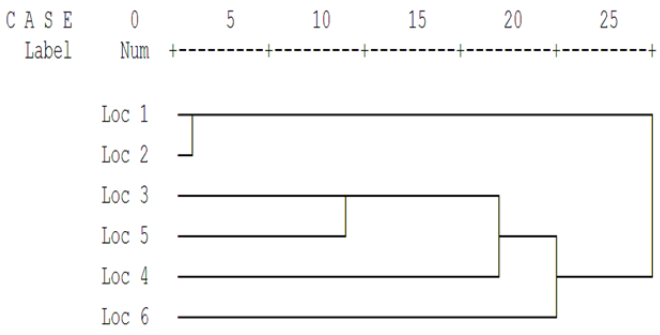



**Figure-2. Dendrogram of the six individuals from natural population of *T. pallida*, constructed using UPGMA based on Jaccard's similarity co-efficient.**




## DISCUSSION:

Medicinal plants are increasingly endangered due to over exploitation, habitat loss, habitat fragmentation and also due to the effect of diverse environmental factors and natural hazards. The plant species adaptability to the prevailing environmental conditions influenced by the genetic variability it contains. Understanding the genetic variation within populations is essential to establish the proper conservation strategies for plant species. Genetic diversity of a plant species could be affected by many factors such as distribution range, breeding system, Genetic drift, restricted gene flow and the way that its seeds disperse. Cross pollinating plant species generally exhibits high level of genetic diversity compare to self pollinating species. *T. pallida* is self-incompatible and an obligate out-crosser. The flowers are rich in nector, visited by 33 species of insects, ensures cross pollination. The endangered status of the plant is mainly due to its low population, as a result of flower and fruit predation by beetles, fruit predation by rodents and fruit fungal infestation. *T. Pallida* generally found on rocky hills, nutrient poor rocky habitat appears to be a limiting factor for its population expansion.

Among the different type of molecular markers RAPD can be considered to be essential tool for assessment of genetic variability and to study the phylogenetic relationships with in, and among the populations of varieties, species. The genetic diversity of the plants is closely related to their geographic distribution. Species with a wide geographic area generally have more genetic diversity. Many studies have demonstrated that endangered and endemic species tend to possess low levels of genetic diversity based on ISSR data [21], some others have showed opposite findings [22]. In the present study *T. Pallida* showed a high percentage of genetic polymorphism of 69.5% respectively, which was near to the percentage for *Changium smyrinoides* 69% and for *Cassia occidentalis* 71.7%[23],but higher than that of other endangered plants, such as *Lactoris fernandeziana* 24.5% [24], *Cathaya argyrophylla* 32% [25], *Paeonia suffruticosa* 22.5% and *P. rockii* 27.6% [26], and *Dacydium pierrei* 33.3% [27] . This shows that the species, Genetic diversity by itself is high compared to other endangered plant species, and the high level of denetic variability enables the plant species adopt to wide environmental variations. Self incompatibility and obligate out breeding nature of *T. Pallida* is one of the reason of its high level of genetic variability. In the present study, the plants from 1, 2, 3,and 5 accessions showed high level of genetic similarity, compared to 4 and 6 accessions, which are genetically more diverse.

## CONCLUSION

Accurate assessment of genetic diversity is prerequisite for establishment of appropriate conservation strategies, of a particular plant species. The present study is the first report in the field of genetic variability in *T. Pallida*. The present findings provide only the basic information regarding the genetic diversity in *T. pallida* which might be helpful for further studies in the area of molecular characterization. Based on results obtained we conclude that, *T.pallida* possess high level of genetic diversity, as compared to other endangered plant species. These studies indicate that RAPD is sufficiently informative and powerful to detect genetic variability in natural populations of *T. pallida.* Moreover RAPD marker will have a major impact on the conservation and improvement of the endemic and endangered tree species *T. pallida.*

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