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Original Article

GENETIC DIVERSITY ASSESSED THROUGH RAPD MARKERS IN *SYZYGIUM ALTERNIFOLIUM* (WT) WALP

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ABSTRACT

Objective: The present study has been carried out to evaluate the genetic diversity in a representative population of *Syzigium alternifolium* from six different accessions of chittoor district.

Methods: Genomic DNA isolation, Polymerase Chain Reaction (PCR) amplication by using RAPD (OPJ, OPM series) primers, Agarose gel Electrophoresis, Jaccard's similarity coefficients, and UPGMA Dendrogram.

Results: Initially, 20 decamer primers were screened, a total of 28 bands were obtained from seven reproducible primers, with a mean of four amplified bands per primer. The primers OPJ6 and OPM2 resulted 100 % polymorphism. The plant species showed an average of 70.99% polymorphism. Cluster analysis (UPGMA) was used to generate a dendrogram based on Jaccard's similarity coefficients. A maximum similarity value of 76.5% was observed two accessions of 3 and 2 followed by 2 and 1 i.e. 71.4%. Whereas 27.8% similarity value was observed between accessions 5 and 3 and, which were found to be genetically most diverse.

Conclusion: The results suggest that, *S. alternifolium* possess the high level of genetic diversity, as compared to other endangered plant species. Present study concludes, RAPD is a potential molecular marker to evaluate the genetic diversity of *S. alternifolium* species.

Keywords: *Syzigium alternifolium*, Endemic, Endangered, Genetic diversity, RAPD markers and Polymorphism.

INTRODUCTION

Syzygium alternifolium (Wt.) Walp. (Myrtaceae) is a semi-evergreen tree species of dry deciduous forest in the Southern Eastern Ghats of India. It is endemic to tropical dry deciduous forests of Kurnool, Cuddapah and Chittoor districts of Andhra Pradesh, Chengalpattu and North Arcot districts of Tamil Nadu and Bangalore District in Karnataka in India [1].

S. alternifolium is an aromatic medium-sized tree up to 12 m tall, with slightly fissured, grayish bark. It is locally known as mogi or movi. The leaves are dark green, shining appearance on the dorsal side; berries are dark purple, globose and show considerable variation in size, shape and taste [2]. *S. alternifolium* is a fruit tree of great timber, medicinal and economic importance. Timber is used for making furniture and agricultural implements. Tender shoots, fruits and leaf juice are used to treat dysentery, seeds for diabetes and stem bark for gastric ulcers. Flowers yield honey and possess antibiotic properties. The ripe fruits are used in making squashes and jellies. Fruit juice is used to cure stomach-ache and ulcers while the external application of fruit pulp reduces rheumatic pains [3-6]. The plant tops are used to cure skin diseases as it has excellent anti-fungal properties [3]. *S. alternifolium* was reported to possess antimicrobial activity [7], hypoglycemic and antihyperglycemic activity [5] but, there are no reports on molecular characterization.

S. alternifolium is now attained endangered status because of its declining population with in the wild habitat. The reasons are cut down of trees for local uses and collection of fruits leaving less possibility for the plant to repopulate itself in its natural area. [8]. The ability of a species adapts to environmental changes depends greatly on the genetic diversity in the species [9-10]. Understanding the genetic variation within populations is essential to establish the proper conservation strategies for plant species, the preservation of genetic diversity of the populations being a fundamental goal of conservation biology [11]. DNA marker techniques based on PCR amplification have become increasingly important, to study the genetic relationships among the plant. Various approaches are available for DNA fingerprinting such as Amplified fragment length polymorphism (AFLP), Restriction fragment length polymorphism (RFLP), Simple sequence repeats (SSRs) and Randomly amplified polymorphic DNA (RAPD).

The RAPD technique, a quick and relatively inexpensive method, is extensively used to analyze the genetic variability in bacteria, fungi and plants [12]. Despite of lower reproducibility, the RAPD method is generally more desirable because of cost-effectiveness studies involving a smaller number of samples. A large number of reports have appeared in the literature using RAPD patterns for differentiating varieties, species, etc. of medicinal, rare, endemic and endangered plants. These type of studies were done in *Asparagus* species [13], *Draba dorneri* [14], *Catharanthus roseus* [15], *Tylophora rotundifolia* [16], *Grevillea scapigera* [17]. Wherein subtle differences in the banding patterns have been used as an index to differentiate varieties and assess genetic variability. The present paper deals with the genetic variability in populations of *S. alternifolium*. Until now, to the best of our knowledge this is the first description of the field of genetic variability in *S. alternifolium*.

MATERIALS AND METHODS

Plant material

A total of six accessions of *S. alternifolium* were collected from various geographic locations of Chittoor district, Andhra Pradesh, India. Fresh and young leaf samples were collected and stored in zip lock 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 [18] 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 was 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-20 °C in small aliquots.

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 37 °C 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 re checked 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; $MgCl_2$, 5 mm; dNTP (Genei), 100 µm each of dATP, dGTP, dCTP, dTTP; Primer (Operon Bio-technologies, Cologne, Germany), 1 μm; buffer (Genei), The PCR was performed using a palmcycler with the following temperature profile: initial denaturation at 94^0C for 2 min, followed by 45 cycles of denaturation at 92^0C for 1 min; annealing at 37^0C for1 min; extension at 72^0C for 2 min with final elongation at 72^0C for 5 min.

Primer survey and selection

The preliminary primer screening was carried out using 20 primers from the OPJ and OPM 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. Each amplification product was considered as a RAPD marker and recorded across for all samples. Data were 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 bands that could be reproducibly detected in the gel and '0' for the absence of a band. The data matrix thus generated was used to calculate Jaccard's similarity co-efficient for each pairwise comparison. The similarity co-efficients were subjected to the unweighted pair-group method on arithmetic averages (UPGMA) of cluster analysis to group the isolates based on their overall similarities. The SPSS 10.0 package was used for cluster analysis and subsequent dendogram preparation.

RESULTS

Six individual populations of *S. alternifolium* were collected from different areas ((table 1). In the investigation 20 random decamer oligonucleotide primers from OPJ series of operan technology inc., USA was screened, of these 20 primers, only seven primers showed reproducible and scorable bands, 13 sub-optimal or non amplified primers were discarded. The amplification pattern of the reproducible primers was represented in fig.-1. The details of the RAPD analysis were given in the Table-2. The RAPD profiles of six accessions were compared individually for each primer. All these seven primers resulted in the amplification of 28 bands, of which 23 bands were polymorphic, and 5 bands were monomorphic. The mean number of amplified bands per primer was only 4.

Table 1: *S. alternifolium* accessions collected from different localities

S. No.	Locality	Area of study	Geographical location
1	LOC 1	Narayana giri (Tirumala)	13° 40' 46.4622"E, 79° 19' 59.484"N
2	LOC2	Nelakona (Talakona)	13° 48' 41.6808"E, 79° 12' 30.4446"N
3	LOC3	Waterfalls (Talakona)	13° 48' 41.6988"E, 79° 12' 56.9988"N
4	LOC4	Papavinasanam (Tirumala)	13° 43' 12.2412"E, 79° 20' 40.2606"N
5	LOC5	Sanralla metta (Tirumala)	13° 39' 32.1012"E, 79° 23' 20.83"N
6	LOC6	Balapalli check post (Chittoor district)	13° 51' 53.2728"E, 79° 25' 2.013"N

Table 2: Total number of amplified fragments and number of polymorphic fragments generated by 7 random primers in *S. alternifolium* samples

Name of the primer	Sequence 5' to 3'	Total number of bands	Polymorphic bands	% of polymorphism
J6	TCGTTCCGCA	4	4	100
J9	TGAGCCTCGC	3	2	66.8
J15	TGTAGCAGGG	1	0	0
J16	CTGCTTAGGG	3	2	66.8
J18	TGGTCGCAGA	6	5	83.33
J19	GGACACCACT	5	4	80
M2	GGGGGATGAG	6	6	100
		28	23	70.99%

Fig. 1 represents the RAPD profiles for each primer. Two primers OPJ6 and OPM2 produced 100% discrimination power. Primers OPJ18, OPJ19, OPJ9, OPJ16, OPJ15 and their polymorphisms are 83.33%, 80%, 66.8% and 0% The maximum number of polymorphic bands (6) were obtained with primers OPM2, OPJ18 and the minimum number (1) were obtained with primer OPJ15. The polymorphic percentage ranged from 0% to as high as 100%. The size of the amplified fragments varied between 250 and 9000bp. Primers OPJ6, OPM2 showed 100% polymorphism when compared to other primers.

Table 3: Jaccard's similarity co-efficients of the amplified bands for *S. alternifolium* species

	Matrix file input					
	Loc 1	Loc 2	Loc 3	Loc 4	Loc 5	Loc 6
Loc 1	1.000					
Loc 2	.714	1.000				
Loc 3	.636	.765	1.000			
Loc 4	.560	.571	.500	1.000		
Loc 5	.261	.353	.278	.368	1.000	
Loc 6	.522	.450	.318	.524	.467	1.000

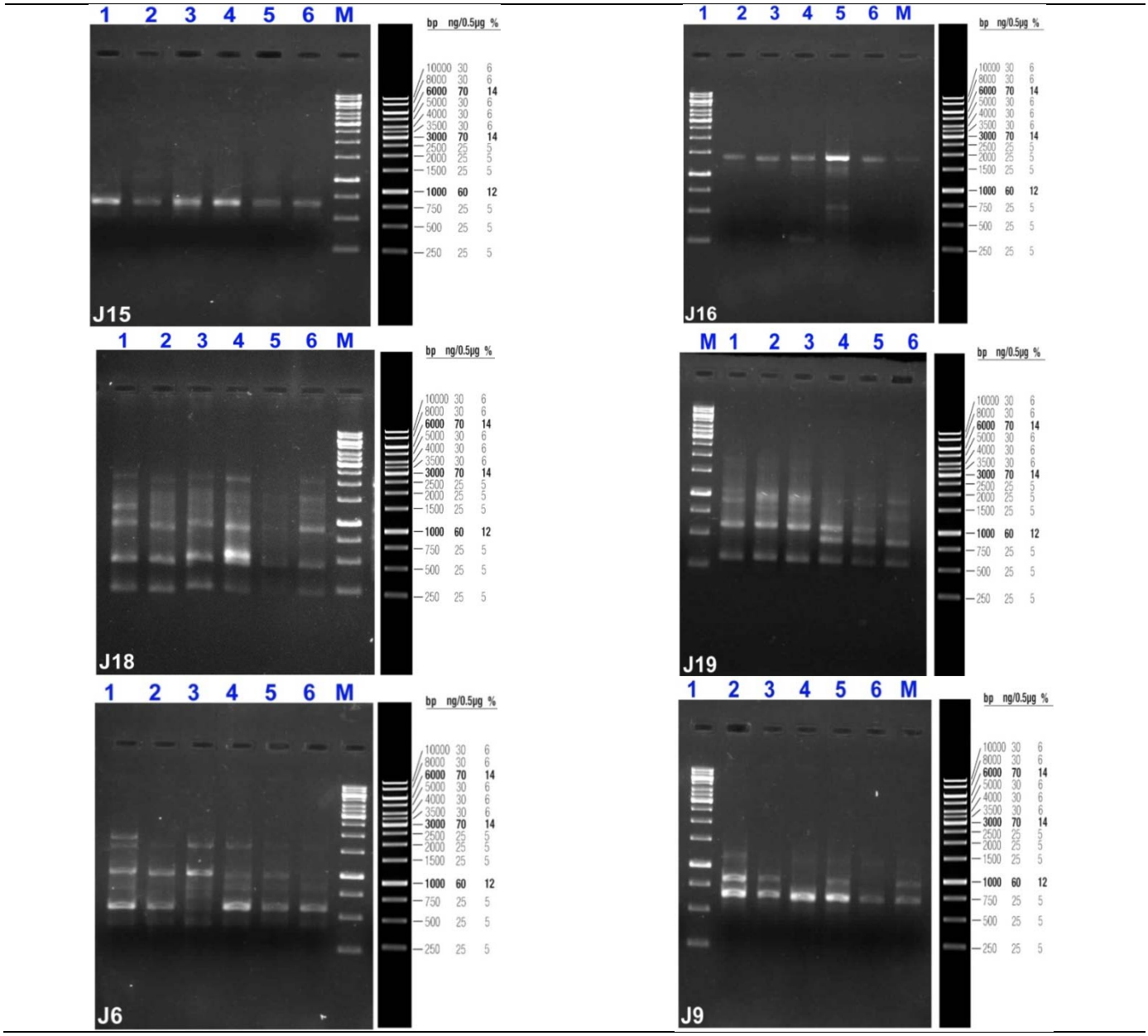

Fig. 1: The RAPD profiles of *S. alternifolium* generated by OPJ series primers. Samples 1-6 *S. Alternifolium* Individuals. M-1 kb DNA ladder

Jaccard's similarity matrix was prepared based on RAPD data. and the dendrogram, clustering (Figure. 2) based on similarity coefficients generated by the Unweighted pair group method using arithmetic means (UPGMA), using SPSS (11.0) software. Jaccard's similarity co-efficients of the amplified bands for *S. alternifolium* species (table 3). A maximum similarity value of 76.5% was observed between accessions 3 and 2 accessions, followed by 71.4% between 2 and 1 and a minimum of 27.8% between 5 and 3. An average similarity value observed across the each accession was 12.14%. The dendrogram constructed (six accessions into the single cluster) based on the similarity co-efficient, the cluster having 2, 3 and 1 accessions are genetically similar. But accession 4 in this cluster has different similarity value.571. Whereas the accession 6 and 5 did not resemble with any other in the cluster formed a separate branch.

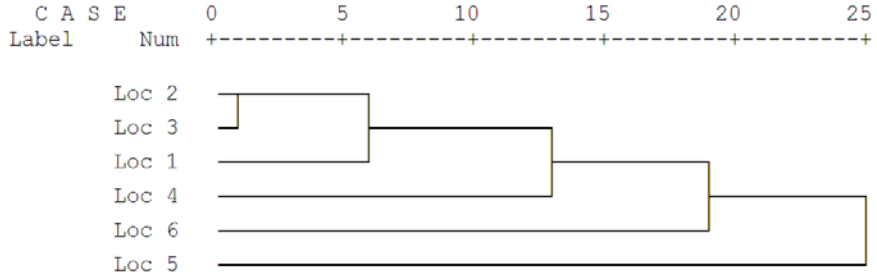

Fig. 2: UPGMA Dendrogram of the genetic similarity of six individuals of *S. alternifolium*, constructed based on Jaccard's similarity co-efficients

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 the distribution range, breeding system, and the way that its seeds disperse. Cross pollinating plant species generally exhibits high level of genetic diversity compare to self-pollinating species. *S. alternifolium* is self-incompatible and an obligate out-crosser. The flowers are rich in nector, provide reward to the visiting insects, ensures cross pollination. The endangered status of the plant is mainly due to overexploitation because it is used by the local people for making agricultural implements, furniture and also used as medicine to treat gastric ulcers and rheumatism. The ability of the plant to repopulate itself is limited by the collection of fruits by locals due to their edible nature, short viability of seeds, high seedling mortality due to water stress, nutrient deficiency and erratic rainfall or interval of drought within the rainy season.

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 [20-21], some others have showed opposite findings [22]. The percentage of polymorphism in this species was high as compared to other endangered plants, such as *Lactoris fernandeziana* (Lactoridaceae) 24.5% [23], *Cathaya argyrophylla* 32% [24], *Paeonia suffruticosa* 22.5% and *P. rockii* 27.6% [25], and *Dacydium pierrei* 33.3% [26]. This shows that the species, Genetic diversity is not low, and it should be able to fit the environmental variation. Out breeding nature of *S. alternifolium* is one of the reasons for exhibiting high genetic diversity. In the present study, the plants from 1, 2, 3 accessions are genetically similar, than 4, 5, 6 accessions, 5 and 6 accessions are genetically diverse.

CONCLUSION

The present study is the first report in the field of genetic variability in *S. alternifolium* which might be helpful for further studies in the area of molecular characterization. Based on results obtained we conclude that, *S. alternifolium* possess the high level of genetic diversity, as compared to other endangered species. Further studies need to be carried out in the wide range of populations in other geographical locations of Eastern Ghats, for better assessment of genetic variability and to establish proper conservation strategies of this endemic and endangered plant.

CONFLICT OF INTERESTS

Declared None

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