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Research Article

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Molecular genetic analysis of *Salacia reticulata*, a threatened medicinal plant for the study of genetic diversity

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ABSTRACT

Salacia reticulata is a medicinally important perennial, woody climbing shrub belonging to the family Celastraceae. It has become a threatened plant due to its various uses as anti-diabetic and LDL cholesterol lowering effects. The plants were collected from 8 locations at 3 different forests of Southern India and established in the Botanical Garden Osmania University. The accessions were subjected to study of morphological characteristics, HPLC analysis for mangiferin content and RAPD analysis for study of genetic diversity.

The morphological studies revealed very little variation among the accessions. However, the exploration of active compound (mangiferin) variation of the collected roots and stems carried out through High Performance Liquid Chromatography (HPLC) revealed that the stems and roots of accessions of Chittoor District contained the highest content of mangiferin.

Molecular markers have been developed through RAPD analysis. The dendrogram was constructed on the basis of the similarity matrix data by Unweighted Pair Group Method (UPGMA) with average cluster analysis. Out of the 10 decamer oligonucleotide primers, nine primers gave non-reproducible polymorphic bands. Maximum number of polymorphic bands was obtained with four primers and unique bands were obtained with two oligo primers which can be used in the development of molecular IDs for the accessions. Though all the collected accessions have morphological similarity between the groups, genetically they are not 100% similar. This confirms the suitability of RAPD as an elegant and reliable tool in establishing the genetic relatedness and in study of changes that occurred in the genome sequence in the course of evolution.

Keywords: *Genetic diversity, RAPD-analysis, HPLC analysis, Salacia reticulate.*

INTRODUCTION

Salacia reticulata is a medicinally important, woody, climbing shrub commonly called as “Saptarangini” or “Kotala Himbutu”, belongs to the family Celastraceae, earlier called as Hippocrataceae. *Salacia reticulata* is found growing in the forest regions of southern India and Srilanka. In India it is found in the eastern and western ghats. From ancient times, *Salacia reticulata* was used as medicine by tribes and later ayurvedic practitioners started to use it to treat diabetes for normalizing blood sugar and insulin levels. Stem and root extracts were also used for treating haemorrhoids, rheumatism, gonorrhea, skin diseases and to support healthy blood lipids[1]. The plant also has potent antioxidant properties, and triglyceride and LDL cholesterol-lowering effects that aid in weight loss. It is used as supplementary food in Japan to prevent diabetes and obesity[2]. *Salacia reticulata* is considered threatened or vulnerable due to over exploitation for its variety of uses.

The stems and roots of *Salacia reticulata* contain mangiferin, a polyphenol that enhances the body’s sensitivity to insulin, and contains inhibitors of sugar digestion and absorption. They also contain other compounds such as salacinol and kotalanol that decrease the absorption of carbohydrates from the intestine[3].

The active constituents of the medicinal plants depend upon the different agro climatic zones in which they grow. Estimation of genetic diversity of plants grown in different geographical conditions is important in designing crop improvement programmes for germplasm conservation strategies. Molecular marker analysis is a powerful tool in the genetic study of populations and the Random Amplified Polymorphic DNA (RAPD) technique is suitable for the analysis of genetic diversity in natural populations[4].

In the present study, accessions of *Salacia reticulata* collected from different places of Eastern India were grown in the Botanical garden at Osmania University and subjected to morphological, biochemical and molecular analysis with RAPD markers to assess genetic diversity at genome level.

MATERIAL AND METHODS

Survey

A survey was carried out in different forests of Andhra Pradesh state of India for location and identification of *Salacia reticulata* plants and they were identified at three different forests of the districts Chittoor, Kurnool and Vizianagaram. The satellite pictures of the Andhra Pradesh State Remote Sensing Applications Centre (APSRAC) have helped in locating the plants by providing an idea of the type of soil and geology of the soil from where they could be collected. In the data of APSRAC, the geology of the soil of the hilly areas were underlined by Quartzites, Khondalites and Charnackites and plain areas by granites. The type of soil in which the plants were found growing was shallow to moderately deep and red gravelly well-drained sandy loam soil beside small flowing water streams. Permission of the Department of Forestry, Government of Andhra Pradesh was obtained to collect the plants, roots and seeds. The plants are perennial woody climbing shrubs and were collected from 8 locations, transferred and established in the Medicinal and Botanical Garden of Department of Botany, Osmania University Hyderabad, India. The accessions were subjected to study of morphological characteristics, biochemical analysis [for study of variation in the content of mangiferin through High Performance Liquid Chromatography (HPLC)] and RAPD analysis for the study of genetic diversity among the accessions.

Accessions

A total of 8 accessions of *Salacia reticulata* were collected for the study from Chittoor (G1, G2 and G3), Khammam (G4, G5 and G6) and Vizianagaram districts (in the southern Orissa border) (G7 and G8) of Andhra Pradesh state of India and are being maintained in the 'Medicinal and Botanical garden', Department of Botany, Osmania University.

Morphological analysis

The 8 accessions of *Salacia reticulata* were subjected to morphological analysis comprising the study of plant height, shape, size and colour of leaf, inflorescence, colour of the flower and fruit and 100-seed-weight.

HPLC analysis to estimate mangiferin

To estimate the variation in mangiferin content, the dried roots and stems of *Salacia reticulata* collected from the three districts viz. Chittoor, Khammam and Vizianagaram were chopped into small pieces, shade dried and powdered finely. The powder was subjected to extraction with methanol. The methanolic extracts of root and stem of *Salacia reticulata* were prepared and the resultant gum after concentration was subjected to High Performance Liquid Chromatography (HPLC) for quantitative and qualitative studies. The HPLC was carried out with Octadecyl Silance (ODS) column C18 Phenomenex, Type: Luna 5µ C 18(2) with the following parameters:

Mobile phase : Solvent-A (Buffer): Dissolve 0.136 gms of Potassium di-hydrogen orthophosphate (KH_2PO_4) in 500ml HPLC grade water, add 0.5ml of orthophosphoric acid (H_3PO_4) and make upto 1000ml.
Solvent-B: 100% Acetonitrile

Column	:ODS (Octadecyl silance) C18 Phenomenex: Type: Luna 5µC18(2), Size: 250 x 4.60mm 5µ micron
Flow Rate	:1.5
Wave Length	:254nm
Standard Preparation	:0.125mg/ml of Mangiferin (in 10% DMF, 90% Methanol)
Sample Preparation	:~10mg/ml of (in 10% DMF, 90% Methanol)
Isocratic condition	:25 % acetonitrile in Buffer

RAPD analysis

Molecular marker analysis is a powerful tool for the genetic study of populations and the Random Amplified Polymorphic DNA (RAPD) technique is suitable for the analysis of genetic diversity in natural populations. The RAPD analysis involves the study of variation in the sequence of the DNA of different DNA samples. This comprises the use of random primers to carry out polymerase chain reaction (PCR) followed by the analysis of the electrophoresed data by studying the different bands of DNA. The bands obtained from different samples are compared usually with the help of special software. For the RAPD analysis, genomic DNA was isolated from all the accessions followed by the PCR and data analysis with special software to detect the similarities and variations between accessions.

Isolation of genomic DNA

The genomic DNA was isolated from the leaves of all the accessions of *Salacia reticulata* with slight modifications of the CTAB method[5]. Fresh and young leaves (1 g) were collected and immediately placed in liquid nitrogen and ground to fine powder. To this powder, 5ml of CTAB buffer (4M NaCl, 0.5M EDTA, 1M Tris HCl, 10% CTAB, PVP) was added and incubated at 65° C for 60 minutes followed by extraction with two volumes of chloroform and isoamyl alcohol (24:1 v/v) for total cleaning of DNA and removal of residual protein. The DNA was precipitated with equal volume of chilled isopropanol, followed by two washes with 70% ethanol. The pelleted DNA was air-dried and resuspended in Tris EDTA (10 mg/ml). Quantity of the isolated DNA was estimated spectrophotometrically and its integrity was evaluated on 0.8% agarose gel electrophoretically and used for RAPD analysis.

RAPD analysis of the genomic DNA

Random amplification was performed following a modified PCR method described by Williams et al.[6]. The reaction mixture of 25 µl each contained 1 X PCR reaction buffer (10 mM Tris–HCl, 50 mM KCl, and pH 8.3), 2 mM $MgCl_2$, 0.5 U of Taq DNA polymerase, 200 µM each of dATP, dTTP, dCTP and dGTP (reagents from Bangalore Genei, Bangalore, India), 10 pM of primer, 40 ng of template DNA and sterile double distilled water. A total of 10 primers (Operon Technologies, USA) were screened for RAPD analysis, viz. OPA01, OPA02, OPA08, OPA09, OPA20, OPB01, OPB03, OPB05, OPB19 and OPM12. PCR reaction was carried out in a DNA Thermal Cycler (MJ Research, Model No. PTC 100).

The process of amplification was based on the following sequence: Initial extended step of denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 35°C for 1 min, primer elongation at 72°C for 2 min, followed by an extended elongation step at 72°C for 10 min and finally at 4 °C.

The PCR products were mixed with 10 X loading dye (bromophenol blue and xylene) and electrophoretic separation was performed on horizontal 1.5% agarose gel in Tris Boric acid EDTA (1 X) buffer with a constant voltage of 80 V.A.. A 50 bp DNA ladder was used as the DNA size marker. The fragments were visualized under a UV transilluminator after staining the gel with ethidium bromide (0.5µg/ml) and photographed with a Digital camera. Each experiment was repeated twice.

Data analysis

A digital Imaging System (Alpha Innotech Gel Documentation system) was used to analyze the data obtained from the electrophoresis of amplified products. To determine the genetic variability, a matrix was constructed and analyzed using the software program Numerical Taxonomy System (NTSYS-PC), Applied Biostatistics, Inc., New York, USA, software version 2.02e[7].

Genetic distances were calculated using Jaccard's similarity coefficient[8] and the dendrograms were built by cluster method using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm[9].

RESULTS

Morphological analysis

All of the collected *Salacia reticulata* accessions growing in the Botanical Garden of Department of Botany were subjected to morphological analysis. The accessions are more or less morphologically similar except for a few instances. The details are presented below:

- The height of the 2-year-old plants ranged from 170-191 cm with higher values in the accessions collected from Chittoor district (G1, G2 and G3) and the highest value was recorded in the G3 accession (191 cm).
- The leaves were opposite, glabrous and shining, ovate or obovate-oblong, coriaceous with the length of the older leaf blade reaching up to highest values of 11-13 cm in the accessions collected from Chittoor district (G1, G2 and G3).
- The plants attained maturity in 2 years and flowered (about 30-50 per branch) during the months of January-February. The inflorescence was axillary in fascicles and flowers small, fascicled on woody branches on axillary tubercles or cymes. Colour of the flower was yellowish green, petals ovate, acute and sepals glabrous, with three stamens continuous with the disk. Highest number of flowers viz. 50 per branch were found in the G7 and G8 accessions of Vizianagaram district.
- The fruits were big, tuberculate, orange to bright pinkish orange with 1-3 seeds immersed in the pulp, seeds after drying were brown in colour and 100-seed-weight varied from 31.8 to 33.9 g with the highest 100-seed-weight recorded in the G5 accession (33.9 g) of Khammam district.

HPLC analysis to estimate mangiferin

The mangiferin content in roots of G1, G2 and G3 accessions of Chittoor forest ranged from 0.79 to 0.89 % and 0.04 to 0.06 % in stems respectively. Whereas, mangiferin content of roots of G4, G5 and G6 accessions of Khammam forest ranged from 0.28 to 0.29 % and 0.009 to 0.01% in stems respectively. The content of mangiferin in roots of G7 and G8 accessions of Vizianagaram forest ranged from 0.19 to 0.21 % and 0.01 to 0.02% respectively. Therefore, the roots contained higher amount of mangiferin and the G1, G2 and G3 accessions of Chittoor forest contained the highest amount of mangiferin.

RAPD analysis

The PCR protocol adopted in the study resulted in reproducible pattern of amplicons using specific combinations of accession and primer. Only the primers which displayed reproducible, scorable and clear bands were considered for analysis. The banding patterns were scored based on the presence or absence of clear, visible and reproducible bands[10]. A matrix of genetic similarity was obtained between the individuals by using Rohlf formula[7]. The average of similarity matrix was used to generate a tree for cluster analysis by UPGMA.

Out of the ten Operon primers used for amplification, only nine showed clear banding, seven primers exhibited polymorphism in eight accessions. The results were analyzed based on the principle that a band is considered to be polymorphic if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals or accessions. The most intense monomorphic band was used as reference to calibrate different lanes in case of each primer for the amount of DNA present, in absence of monomorphic bands, the band with maximum frequency in each accession was considered for calibration.

Maximum number of bands were obtained with primer OPB1, followed by OPB3 (Table-1 and Figure-1). A sum total of 327 bands were amplified with respect to all nine primers. About 103 bands were polymorphic and 224 were monomorphic (Table-1). Higher number of polymorphic bands were obtained with primers OPB5, followed by OPB19, OPB1 and OPB3 (Table-1). Interestingly, few unique bands were observed with OPB19 and OPB5 primers, which can be used in the development of molecular IDs for the accessions. Reproducible results were obtained in this study with the specific primers in the replicates.

The dendrogram (Figure-2) based on similarity matrix showed distinct separation of the collected accessions, though morphologically they were similar and indistinguishable. Accessions G1 and G2 were 58% similar whereas, 54% similarity was observed in case of the G7 and G8 accessions. Analysis reveals that the accessions G1, G2 and G3 which were collected from different locations of Chittoor district are grouped in cluster I with more than 48% similarity. Accessions G7 and G8 collected from Vizianagaram district with 60% similarity were grouped in cluster II. Accessions G5 and G6 collected from Khammam district are 40% similar and are placed in cluster III. Accession G4 which was collected from Khammam district was showing maximum similarity with G7 and G8 so, G4, G7 and G8 are placed in the same cluster.

Table-1: DNA sequence of RAPD primers and the number of polymorphic bands produced in the RAPD analysis of the 8 accessions of *Salacia reticulata*.

S. No.	Primer	Sequence 5'-------- 3'	Total number of bands	Polymorphic bands
1	OPA 01	CAGGCCCTTC	30	9
2	OPA02	TGCCGAGCTG	24	8
3	OPA08	GTGACGTAGG	36	9
4	OPA09	GGGTAACGCC	32	8
5	OPA20	GTTGCGATCC	44	10
6	OPB01	GTTTCGCTCC	46	14
7	OPB03	CATCCCCCTG	34	11
8	OPB05	TGCGCCCTTC	40	18
9	OPB19	ACCCCCGAAG	41	16
10	OPM12	GGGACGTTGG	---	--

Fig.1: RAPD polymorphic banding pattern of eight accessions (G1-G8) with different primers a. OPB 1, b. OPB 5, c. OPB 19 and d. OPB 3

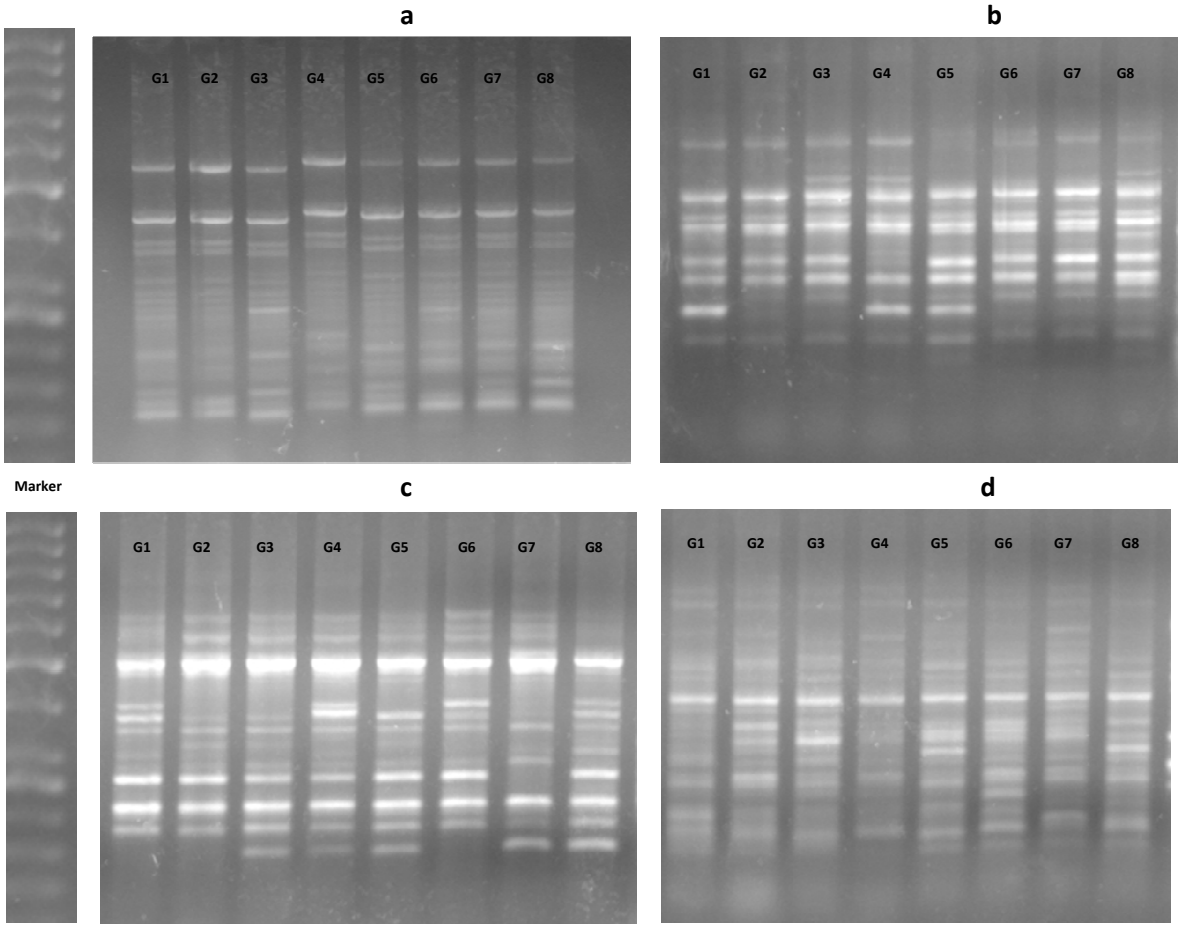
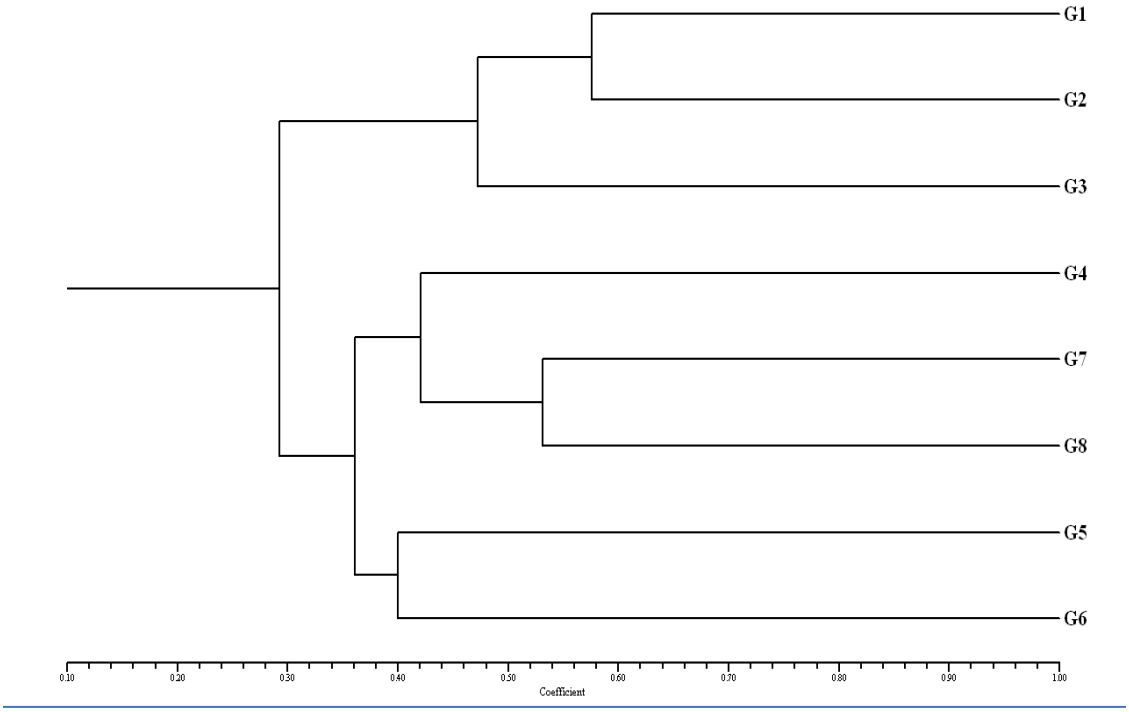


Figure-2: Dendrogram (RAPD analysis) of the eight accessions of *Salacia reticulata*



DISCUSSION

The height of the plants collected from Chittoor district (G1, G2, and G3) was highest when compared to the other accessions, and the length of the leaf blade was also highest when compared to the others. However, maximum number of flowers (50 / branch) were observed in G7 and G8 accessions of Vizianagaram district. Although the G1, G2 and G3 accessions were taller than the rest, the yield in terms of number of flowers and 100-seed-weight was higher in the latter with the highest 100-seed-weight recorded in the G5 accession (33.9 g) of Khammam district.

The aqueous and methanolic extracts of root and stem was reported to produce many pharmacologically active compounds[3]. HPLC analysis in the present study establish that the roots and stems collected from Chittoor district were found to contain the highest percentage of mangiferin when compared to those of other districts.

RAPD is the most suitable tool to assess the genetic diversity by observing the percentage of polymorphism obtained by the decamer primers. RAPD is applicable to those species where not much genomic information is available[4], which is very much true in case of *Salacia reticulata.*

RAPD has proved to be useful in the present study in molecular profiling of different accessions of *Salacia reticulata.* Maximum number of bands were obtained with primer OPB1, followed by OPB3. A sum total of 327 bands were amplified with respect to all nine primers. About 103 bands were polymorphic and 224 were monomorphic. Analysis of the grouping through UPGMA showed that the 8 accessions can be grouped into 3 clusters based on their similarity percentage. The accessions from Chittoor district were grouped in cluster I with more than 48% similarity. Accessions from Vizianagaram district were grouped in cluster II with 60% similarity. However, only two accessions (G5 and G6) from Khammam district were placed in cluster III with 40% similarity and accession G4 (from Khammam district), which showed maximum similarity with G7 and G8 of Vizianagaram district was therefore placed in the same cluster (II).

When the results are compared with published reports, the distribution patterns are in agreement with their findings[11, 12] on the genetic variation in tree species. The degree of polymorphism obtained presently is higher in *Salacia reticulata* when compared to that of *D. nigra*[13] and the percentage of polymorphism was low when compared with *Maytenus ilicifolia*[14].

Though the accessions have morphological similarity, the genetic similarity between the groups I, II and III was only 30%, which shows that the RAPD is the most suitable tool to assess the genetic diversity in the species. Similar type of findings was observed in genus *Terminalia*[4] and in *Azima tetracantha*[15] and also significantly in our earlier study on Jatropha[16].

CONCLUSION

The extent of polymorphism observed among the accessions of *Salacia reticulata* was high which indicates that RAPD is the most suitable marker to assess genetic diversity in this plant confirming the suitability of RAPD as an elegant and reliable tool in molecular diagnosis. The highest amount of mangiferin was recorded in the G1, G2 and G3 accessions of Chittoor district which were 48% similar in terms of the dendrogram and were grouped in the same cluster. The dendrogram also establishes the genetic relatedness among different accessions and quantum of changes that occurred in the genome sequence in the course of evolution. Further, the present study is the very first report on molecular analysis of this species, which can be used for its genetic improvement and conservation.

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