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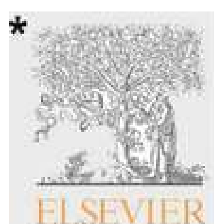

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RESEARCH ARTICLE MOLECULAR BIOLOGY

MOLECULAR CHARACTERIZATION OF 17 ACCESSIONS OF *GYMNEMA SYLVESTRE* R. BR. USING RAPD MARKERS

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ABSTRACT

Gymnema sylvestre is a large, stout, woody, vine-like plant which climbs on bushes and trees. It is known in Sanskrit- *Meshashiringi, Madhu nashinin* (Madhu=sugar, nashini= destroy), in Hindi- *Gur-mar.* The Latin name of *Gymnema Sylvestre* means “sugar destroyer” and is considered as herbal remedy for high blood sugar. It is considered to be a very effective antidiabetic plant in Indian indigenous system of medicine. In view of its importance and the genetic diversity of this species in India, we attempted to characterize 17 accessions of *Gymnema sylvestre* using Random Amplified Polymorphic DNA (RAPD). Out of 20 primers tested from OPL group, four were selected, which gave 48 clear and bright fragments, out of which 24 (50%) fragments were considered as polymorphic. The amplicons usually ranged from 500bp to 2500bp. The Dendrograms generated by UPGMA analysis were compared based on the gymnemic acid content. The major cluster I comprised 4 accessions from Warangal-I, with high gymnemic acid content (42%), Warangal-II (38.6%), Mulugu-I (40.4%) and OU-Botanical garden (31.4%). The major cluster II had two accessions from OU-Hyderabad with lower gymnemic acid content 23% and 26.6%. The major cluster III had minor clusters with 11 accessions. One cluster with samples 2, 13, 3, 14, 10, 11 and 12 had higher gymnemic acid content ranging from 32 to 39%. The results of the present study clearly suggested that anti-diabetic *Gymnema sylvestre* can be characterized through RAPD markers and high value superior accessions can be identified for product development in control of diabetes.

KEY WORDS

Gymnema sylvestre, Molecular Characterization, Accessions, RAPD markers

INTRODUCTION

Gymnema sylvestre R. Br. (Asclepiadaceae) is a large, stout, woody, vine-like plant which climbs on bushes and trees (Fig 1). It is known in sankrit- *Meshashiringi, Madhu nashinin* (Madhu=sugar, nashini= destroy), in Hindi- *Gur-mar.* The Latin name of *Gymnema Sylvestre* means "sugar destroyer" and is considered as herbal remedy for high blood sugar. The medicinally active parts of the plant are the leaves and the roots. Traditionally it was recommended for stomach problems, constipation, liver disease but the recent studies have shown that the extract of *Gymnema sylvestre* is useful in controlling blood sugar to treat type-II diabetes. It is a potential natural alternative to chemical means of blood sugar regulation[1].

The leaves of this plant have been used for over 2000 years to treat diabetes, giving it a prominent place in the indigenous system of medicines in this country. Fresh leaves when chewed exhibit a remarkable property of temporarily paralyzing the sensory perception of sweet and bitter tastes[2]. Administration of *Gymnema* lowers the blood glucose level in diabetic patients[3] and the alcoholic extracts of *Gymnema* have been shown to increase the release of insulin from pancreatic β-cells[4]. The quantity of gymnemic acid, the active principle in *Gymnema* leaves is, however, variable among accessions from different ecoclimatic regions[5]. Considerable variations also exist among the morphological traits of *Gymnema* accessions[6]. However, detailed information on the extent of variability in the *Gymnema* populations is not available. Hence, a study was undertaken to characterize the variations among the *Gymnema* germplasm accessions from diverse ecoclimatic regions of various parts of the country.

This is of particular significance in the current scenario where demand for plant-based medicines is increasing and over-exploitation of the wild resources is endangering its genetic diversity in the natural habitat[7]. Documenting the genetic variations will provide an efficient tool for identifying useful genotypes that could be used as cultivars for extraction of standard drugs. Seventeen accessions of *Gymnema* collected from various geographical regions of the country were used for the present study. Differentiation of accessions through morphological features is inefficient and inaccurate. The use of genetic markers for identification of accessions offers a viable alternative method[8]. RAPD marker has been widely used in the reconstruction of phylogenetic relationships for many organisms. The molecular biology tools are being used to produce revolutionary results.

DNA analysis offers a better choice of genetic marker than other methods. Human geneticists[9] first proposed the use of DNA markers in genetic research. Soon this approach has been implemented for the genetic analysis of plants. Application of DNA markers in forest genetics was complicated early but has been made possible recently. The great advantage of this technique is its simplicity to perform, preferably those experiments where the genotypes of a large number of individuals are to be determined at a few genetic loci. With this idea we attempted to characterize 17 accessions of *Gymnema*, which were earlier identified as variants based on morphological and biochemical evaluations[10], using isozyme and RAPD markers. Both isozyme markers and

genetic fingerprinting using RAPD have been widely used for molecular characterization of plant species[11,12,13]. Yet, there has been no sufficient report on the use of these methods to characterize genetic diversity of *Gymnema*. Hence, a study was undertaken to provide unambiguous identification of *Gymnema* germplasm and to characterize its accessions.

Figure 1
Seventeen accessions of Gymnema sylvestre grown in experimental site

MATERIALS AND METHODS

(i) Sample Collection

Seventeen accessions were collected from different parts of the country (Table. 1) and grown in experimental site. After acclimatization 1g of young leaves were harvested for DNA isolation

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Table 1
Acquisition of 17 accessions of Gymnema sylvestre from different parts of the country and the percentage of gymnemic acid

S.No	Name of the accession	Place of collection	% of Gymnemic acid
1	Panchagani	Pune-Westernghats	31.39
2	Kandala	Pune-Westernghats	35.4
3	Ambavale	Pune-Westernghats	38.34
4	Housaryghat – I	Pune-Westernghats	26.35

5	Housaryghat – II	Pune-Westernghats	23.68
6	Bhubaneswar	Bhubaneswar-Orissa	26.4
7	Warangal – I	Warangal	42
8	Warangal – II	Warangal	38.55
9	Mulugu – I	Mulugu village	40.4
10	Mulugu – II	Mulugu village	39.33
11	Mulugu – III	Mulugu village	36.8
12	Rajahmundry – I	Rajahmundry	33.4
13	Rajahmundry – II	Rajahmundry	32
14	Osmania University – I	OU, Hyderabad	28.4
15	Osmania University – II	OU, Hyderabad	26.55
16	O.U – Bridge	OU, Hyderabad	23
17	O.U – Botanical garden	OU, Hyderabad	31.39

(ii) Isolation of total genomic DNA

Total genomic DNA was extracted from seventeen accessions of *Gymnema sylvestre* using the CTAB protocol[14]. The leaf samples were ground under liquid nitrogen with a mortar and pestle along with 50mg of PVP and were made to fine powder. The powder was quickly transferred to centrifuge tubes, and to it 5 ml of freshly prepared preheated (65°C) DNA extraction buffer (10%CTAB, 4M NaCl, 0.5M EDTA (pH 8.0), 1M Tris-Hcl (pH 8.0) and 0.2% β-mercapto ethanol) was added to each tube and shaken vigorously by inversion to form slurry and was incubated in water bath at 60°C for 1 hour with intermittent shaking and swirling for every 30 minutes. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed properly by inversion for 15-20 minutes and centrifuged at 15000rpm for 10 minutes at room temperature to separate the phases (long term mixing of samples in Chloroform: Isoamyl alcohol approximately for 30 min, will help in removal of pigments and formation of brownish color in DNA sample can be omitted).

The supernatant was carefully decanted and transferred to a new tube and equal volume of chilled isopropanol or ethanol was added and gently mixed. A pellet of white fibrous structure of DNA was observed in tube. The pellet was washed with 70% Ethanol to remove the impurities, air dried and resuspended in 300µl of TE buffer. It was further treated with 5µl RNase and incubated overnight at 37°C (An overnight RNase treatment helped achieving in proper genomic DNA). DNA was kept at -70°C for long term and -20°C for short term storage.

(iii)Quantity and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a UV spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260nm to that of 280nm. DNA concentration and purity were also determined by running the samples on 0.8% agarose gel.

(iv) RAPD (Random Amplified Polymorphic DNA)-PCR Reaction

DNA amplification was done using twenty arbitrary primers (Operon Technologies, Inc., Alameda, California, USA OPL-01 to OPL-20) according to the protocol outlined by Williams et al (1990) with some modifications[8]. PCR reactions were carried out on each DNA sample in a 20 µl reaction mix containing 1µl genomic DNA (100ng), 1µl primer (1.0µl), 2µl PCR buffer (10x), 2µl Mg^{+2} buffer (1.5mM),

2μl dNTPs (250μM), 0.5μl Taq polymerase (1U) and the rest amount of sterile deionized water (11.5μl) to prepare 20μl reaction mixture.

DNA amplification was performed in an oil-free thermal cycler (Eppendorf). The reaction mix was preheated at 94ºC for four minutes followed by 35 cycles of one min denaturation at 94ºC, one min annealing at 35ºC and elongation or extension at 72ºC for 2 minutes. After the last cycle, a final extension of five minutes at 72ºC was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4ºC. The amplified products were mixed with 4 μl of 6X loading dye and resolved in 1.5% agarose gel containing ethidium bromide in a horizontal electrophoresis tank containing 1X TAE buffer[15]. DNA bands were recorded using a gel documentation system (Alpha Innotech) and the images were photographed. The reliability of the polymorphic band was tested by repeating the assay twice.

All distinct bands or fragments were thereby given identification numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using all the primers in the RAPD analysis were then pooled to create a single data matrix and used to estimate polymorphic loci, gene diversity, genetic distance (D) and constructed dendrogram using UPGMA[16] (Unweighted Pair Group Method of Arithmetic Means).

RESULTS AND DISCUSSION

DNA isolation was improved by modifying some of the steps in the original CTAB DNA isolation protocol[14] and high quality DNA was obtained from all the seventeen accessions of *Gymnema sylvestre* (Fig 2). The purity of DNA was determined by calculating the ratio of absorbance at 260nm to that of 280nm (A_{260}/A_{280}nm). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of the DNA[17,18,19]. Addition of PVP along with CTAB may bind to the polyphenollic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent[20].

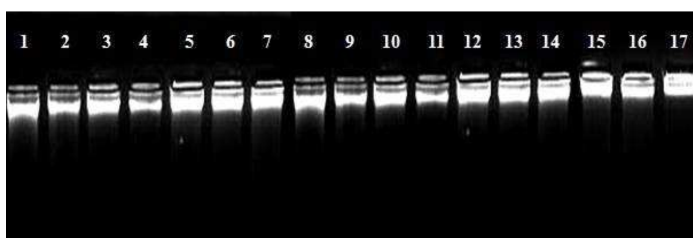

Figure 2

Genomic DNA of 17 accessions of Gymnema sylvestre using method – I (Modified Doyle and Doyle)

1	**Panchagani**	**9**	**Mulugu – I**
2	**Khandala**	**10**	**Mulugu – II**
3	**Ambavale**	**11**	**Mulugu – III**
4	**Housaryghat – I**	**12**	**Rajahmundry – I**
5	**Housaryghat – II**	**13**	**Rajahmundry – II**
6	**Bhubaneswar**	**14**	**OU – I**
7	**Warangal – I**	**15**	**OU – II**
8	**Warangal – II**	**16**	**OU – Bridge**
17	**OU – Botanical Garden**		

Tannins, terpenes and resins considered as secondary metabolites are also difficult to separate from DNA[21]. NaCl has been used in the DNA extraction buffer to remove polysaccharides[22]. Mercapto ethanol has been used in the extraction buffer to separate proteins, amino acids etc. Long-term chloroform: isoamylalcohol treatment ensured removal of chlorophyll and other coloring substances such as pigments, dyes etc. Additional precipitation steps, removed large amounts of precipitates (detergents, proteins etc) by modified speed and time of centrifugation. We found these modified steps necessary to standardize and increase the quality and quantity of genomic DNA.

An extensive variation in RAPD patterns among the accessions was demonstrated on the basis of the number of clear, reproducible and polymorphic bands ranging from about 500 to 2500 bp. Out of 20 primers screened, only 4 primers gave reproducible bands and proved useful in detecting genetic diversity among the accessions. They amplified 48 bands, 24 of which (50%) were found to be polymorphic (Table. 2). The four different primers generated various banding patterns, ranging from 8 to 14. The primer OPL-02 produced the maximum number of bands (14) out of which 8 were polymorphic. The amplicons usually ranged from 500 to 2500 bp (Fig 3). On the other hand the primers OPL-04, OPL-09 and OPL-14 generated 8, 3 and 5 polymorphic bands respectively (Fig 3a, 3b and 3c).

The profile generated by the primer OPL-02 identified the accession collected from Warangal-I, which had the high gymnemic acid content of 42%. Analysis was carried out by Jaccard's Coefficient. Dendrograms generated by UPGMA analysis were compared based on the gymnemic acid content (Fig 4) and three major clusters were identified. The major cluster I (Figure. 4a) comprised 4 samples from Warangal-I with high gymnemic acid content (42%), Warangal-II (38.6%, Mulugu-I (40.4%) and OU-Botanical garden (31.4%) The major cluster II (Fig 4b) had two samples collected from OU-II and OU-Bridge with lower gymnemic acid content of 23% and 26.6%. The major cluster III had minor clusters (Fig 4c) with 11 samples. One cluster with samples 2, 13, 3, 14, 10, 11 and 12 had higher gymnemic acid content (Fig 4d) ranging from 32 to 39%.

Table 2

List of primers with corresponding bands scored and polymorphic bands in seventeen accessions of Gymnema sylvestre

S.No.	Primer code	Base Sequence (5'→3')	Total no. of bands scored	No. of polymorphic bands
1	OPL-02	5' TGGGCGTCAA 3'	14	8
2	OPL-04	5' GACTGCACAC 3'	13	8
3	OPL-09	5' TGCGAGAGTC 3'	8	3
4	OPL-14	5' GTGACAGGCT 3'	13	5
		Total bands	48	24

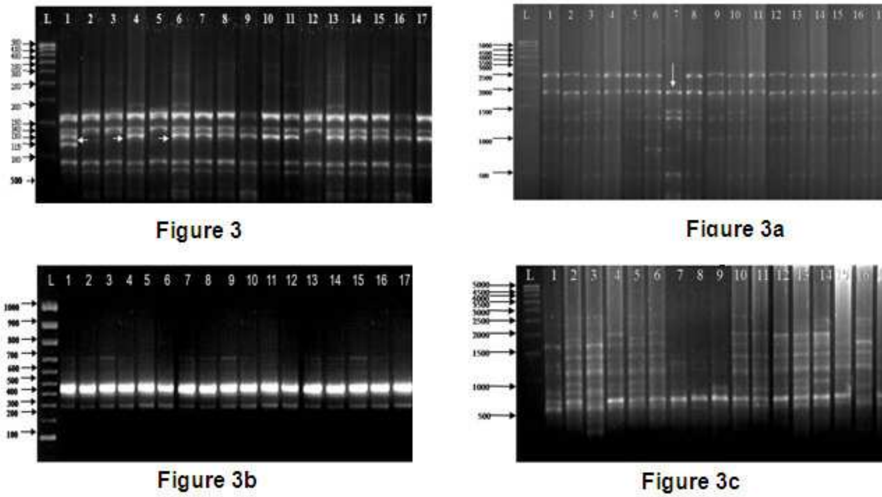

Figure 3.
RAPD profiles of 17 accessions of Gymnema Sylvestre using OPL-02 Primer. The profile identified the accession collected from Warangal - I (Lane - 7) is specific.
3a. ***RAPD profiles with OPL-04 primer showing presence of additional bands in some accessions,***
3b. ***Profiles with OPL-09 gave the monomorphic banding pattern and three bands were poly- morphic, the amplicons ranged from 300 to 500 bp,***
3c. ***Profiles with OPL-14 gave 13 bands out of which 8 were polymorphic***

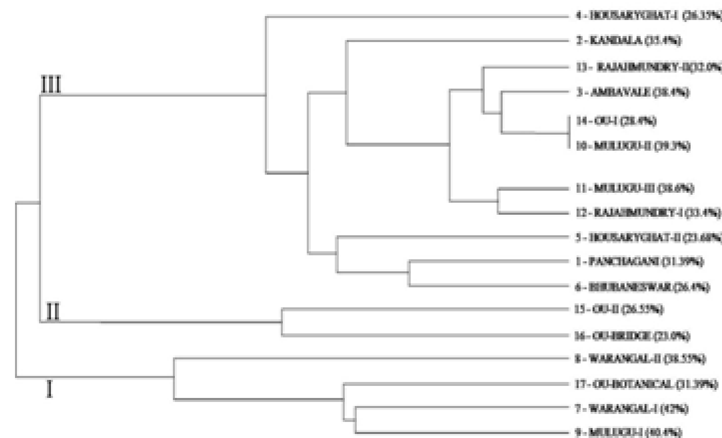

Figure 4
Dendrogram (UPGMA) pattern of RAPD analysis in seventeen accessions of G. sylvestre

Figure 4a.

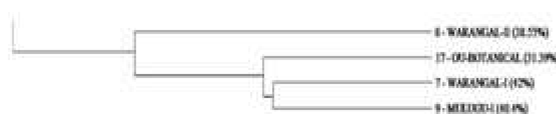

Figure 4b.

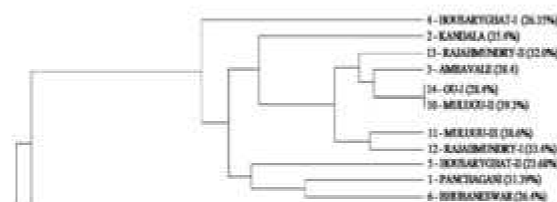

Figure 4c.

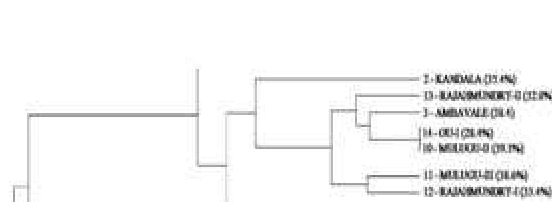

Figure 4d.

Figure 4a.

Major cluster I comprised 4 samples with high gymnemic acid content, 4b.Major cluster II had two samples with lower gymnemic acid content of 23% and 26.6%, 4c&4d. Major cluster III had minor clusters with 11 samples; one cluster with samples 2, 13, 3, 14, 10, 11 and 12 had higher gymnemic acid content ranging from 32 to 39%

CONCLUSION

The present study revealed the existence of considerable variations at the molecular level in the *Gymnema* accessions. The results could be used for identification of ideal genotypes for extraction of drugs by correlating the molecular fingerprints. The results of the present study clearly suggested that anti-diabetic *Gymnema sylvestre* can be characterized through RAPD

markers and high value superior accessions can be identified for product development in control of diabetes.

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