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Molecular diversity in *chakkarakolli* (*Gymnema sylvestre* R. Br.) assessed through isozyme and RAPD analysis

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Abstract

Eighteen phenotypically and biochemically distinct *Gymnema* accessions representing different geographical regions of Kerala were characterized using isozymes and RAPD markers. In the isozyme analysis, three enzyme systems *viz.*, malate dehydrogenase, esterase, and RUBISCO were studied. Five out of the eight resolved loci (62.5%) were polymorphic with the number of alleles expressed at the polymorphic loci being 10. RUBISCO activity was monomorphic across the accessions. In the RAPD assay, 123 amplified products were generated using 15 selected random primers. Of these, 90 bands were polymorphic with the total frequency of polymorphic markers as high as 73.2%. Specific bands were obtained for five accessions. Jaccard's coefficient ranged between 0.72 and 0.85 indicating a moderate level of variability. Cluster analysis of RAPD data using Unweighted Pair Group Method of Arithmetic Averages produced two major clusters and three sub-clusters. Overall, molecular fingerprinting revealed the existence of considerable genetic variations in the *Gymnema* germplasm collection from Kerala.

Keywords: Poly acrylamide gel electrophoresis, Random amplified polymorphic DNA, Molecular markers.

Introduction

The indigenous medicinal herb, *Gymnema sylvestre* R.Br., is a potential natural alternative to chemical means of blood sugar regulation (Siddhiqui et al., 2000). Coincidentally, several products containing *Gymnema* alone or in combination with other antidiabetic herbal medicines are marketed in India. In view of its importance and the genetic diversity of this species in Kerala, we attempted to characterize 18 accessions of *Gymnema*, which were earlier identified as variants based on morphological and biochemical evaluations (Nair and Keshavachandran, 2006), using isozyme and RAPD markers. Both isozyme markers and genetic fingerprinting using RAPD have been widely used for molecular characterization of plant species (Reyes et al., 1998; Singh et al., 2004; Keshavachandran et al., 2005). Yet, there has been no previous 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 ecotypes.

Materials and Methods

Eighteen morphologically and biochemically distinct accessions of *Gymnema* collected from different parts of Kerala ('Adapurutti', 'Erumayoor 69' and 70, 'Kottayi 149', 'Kozhinjampara 51' and 61, 'Kuthanoor 108', 'Kuzhalmannom 89', 'Mathur 127', 'Mundur', 'Nelliampathi', 'Pambadi 116', 'Pambadi', 'Panniyur', 'Peringottukurishi 137', 'Thodupuzha', 'Valiyathovala', and 'Walayar') were subjected to molecular characterization during June to September 2004. The materials were selected based on leaf morphology and saponin content after an initial screening of 93 accessions that were maintained at Vellanikkara for three years (Nair and Keshavachandran, 2006).

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Enzyme extraction and isozyme analysis

Fully opened leaves from the apex were collected in the morning and stored at 4°C. The samples were chilled with liquid nitrogen, homogenized using ice-cold extraction buffer (0.1 M Tris HCl, PVPP5%, sucrose 10%, 10 mM DTT, Triton X 100 at 0.1% and 10 mM 2-Mercaptoethanol), and centrifuged to collect the enzyme extract. Isozyme banding patterns were observed for three different enzyme systems *viz.*, malate dehydrogenase, esterase, and RUBISCO. The isozymes were separated by electrophoresis in a vertical polyacrylamide gel run in a Mini-Protein System (Bio-Rad, USA). Native polyacrylamide gel electrophoresis (PAGE) was performed in 6% stacking gel and 8% resolving gel. The tank buffer used and the migration conditions followed for the three enzyme systems were as per Reyes et al. (1998). Electrophoresis was performed at 4°C and continued until the migration indicator reached the far end of the gel. The gels were incubated in suitable substrate solutions (Reyes et al., 1998) to obtain bands corresponding to enzyme activity. After sufficient destaining, the gels were photographed and the relative position of each visualized band was drawn schematically for analysis.

For each of the three enzyme systems, the number of isozyme loci and the number of alleles within each locus were identified. Banding pattern was scored and cluster analysis of genotypes based on genetic distances conducted using the Unweighted Pair Group Method of Arithmetic Average (UPGMA; Sneath and Sokal, 1973). The Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) software package was used to get the phenetic cluster (Rohlf, 1990).

Isolation of genomic DNA and RAPD assay

Four to 5 fully opened leaves from the apical tip were collected from field-grown plants of each accession. Total genomic DNA was isolated as per the protocol developed by Rogers and Bendich (1994) with suitable modifications. The crude DNA was purified by treatment with RNase A and Proteinase K followed by phenol: chloroform extraction. The DNA concentration was estimated with UV-spectrophotometer (Spectronic Genesys-5, Spectronic Instruments, USA). Aliquots from this sample after suitable dilution were used for polymerase chain reaction (PCR).

The PCR amplification was performed according to the protocol outlined by Williams et al. (1990) with some modifications. PCR conditions were optimized by varying concentrations of template DNA, *Taq* DNA polymerase, dNTPs, and primer. Varying the concentration of DNA from 20 to 50 ng revealed that 50 ng of DNA gave maximum number of reproducible bands and it was used for all analysis. 1U of *Taq* DNA polymerase enzyme, 100 pmoles each of dNTPs and 20 pmoles of primer produced the best amplification. A higher or lower concentration resulted in either sub-optimal or complete lack of amplification. A non-discrete size range of amplification products appearing as a smear on the gel were converted into discretely sized bands by reducing the concentration of template DNA and *Taq* DNA polymerase enzyme.

Initial screening was done with random decamer primers (Operon Technologies Inc., USA) using genomic DNA from the accession 'Pambadi'. Sixty random primers from four different Operon primer kits were used and 15 primers that gave good amplification with five or more discrete bands were selected for RAPD profiling. Reproducibility of the selected primers was tested by repeating the PCR amplification twice under the same amplification conditions. A 25 µl volume reaction mixture containing 50 ng template DNA, 100 µM each of dATP, dCTP, dGTP and dTTP, 20 pM primer, 1U *Taq* DNA polymerase enzyme, 1X Assay buffer, and 2.5 mM $MgCl_2$ was prepared. A control PCR tube containing all the components but no genomic DNA was also run with each primer to check for contamination. Polymerase chain reaction was carried out in the thermal cycler (model PTC 200 of MJ Research, USA) programmed for an initial denaturing period of 94°C for 3 min. followed by 40 cycles of 1 min. denaturation at 92°C, 1 min. primer annealing at 37°C, and 2 min. primer extension at 72°C. The reaction products were mixed with 4 µl of 6X loading dye and resolved in 1.2 % (w/v)

agarose gel containing ethidium bromide in a horizontal electrophoresis tank containing 1X TAE buffer (Sambrook et al., 1989). The λDNA/*Eco*RI/*Hin*dIII double digest was used as molecular weight marker. The gel was visualized under UV light on a transilluminator and documented using the 'Quantity One' software of the Biorad Gel Documentation system. The reliability of the polymorphic bands was tested by repeating the assay twice.

Amplification profiles of 18 genotypes were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of a particular band. The data was analyzed using NTSYS software package (Rohlf, 1990). The SIMQUAL programme was used to calculate Jaccard's coefficient. Clustering was done using Sequential Agglomerative Heirarchial Nested Clustering (SAHN) routine and a dendrogram constructed using UPGMA (Sneath and Sokal, 1973) with NTSYS package.

Results and Discussion

Isozyme markers of Gymnema *accessions*

Three zones of activity were observed for the enzymes malate dehydrogenase and esterase. RUBISCO activity, however, was evident over the entire gel slice but only two zones of activity could be clearly detected. The number of polymorphic loci and the alleles expressed are summarized in Table 1. Two of the malate dehydrogenase loci were polymeric and the enzyme activity is shown in Fig.1. For esterase, all the three loci were polymeric, while RUBISCO activity was monomorphic. The quaternary structures of malate dehydrogenase and esterase are dimeric in many species (May, 1994) and so segregation may have contributed to differential banding patterns. Looking at the quaternary structure of RUBISCO, it is an octa-dimer. The complex pattern of banding obtained probably reflects the structural complexity of the enzyme. Overall, 10 alleles were identified over the five polymorphic loci, yielding an average of two alleles per polymorphic locus. In a similar study, Broyles (1998) observed 3.5 alleles per polymorphic locus in *Asclepias exaltata* populations.

The genetic similarity values estimated based on the scored data ranged between 0.47 and 1.00 and the cluster analysis showed that the 18 *Gymnema* accessions formed

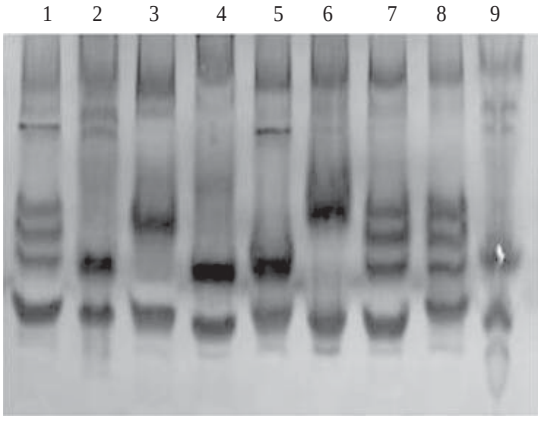

Figure 1. Isozyme banding pattern produced by the enzyme malate dehydrogenase of 18 *Gymnema* accessions from different geographical regions of Kerala grown at Vellanikkara (Samples 1-Pambadi, 2-Mundur, 3-Valiyathovala, 4-Nelliampathi, 5-Walayar, 6-Adapuruthi, 7-Panniyur, 8-Todupuzha and 9-Kuthannoor 108).

Table 1. The number of enzyme loci, polymorphic loci and alleles expressed for each of the three enzymes of 18 *Gymnema* accessions from different geographical regions of Kerala grown at Vellanikkara.

Enzyme	Number of		
	Total loci	Polymorphic loci	Alleles expressed by polymorphic loci
Malate-dehydrogenase	3	2	5
Esterase	3	3	5
RUBISCO	2	0	0
Total	8	5	10

Percentage of polymorphic loci = 62.5%

two distinct groups at 47% (Fig. 2). While one of these groups had only a single accession ('Panniyur'), the other group had 17 accessions, which could be subdivided into two clusters of five and 12 accessions each with further sub-clustering.

Overall, the electrophoretic survey reflected a moderate to high level of genetic variability among the accessions studied. Since the accessions covered a broad geographic range enzymatic differentiation is expected to exist among them. Interestingly, the accessions from nearby locations in Palakkad like 'Erumayoor 70', 'Kottayi 149' and 'Kuthannoor 108' showed 100% similarity and some others like 'Nelliampathy' and 'Valiyathovala' from high range areas also clustered together, implying that genetic variability may have some dependence on the ecoclimatic factors. Presumably, variable selection pressures may have caused the accessions from different areas to become more diverse.

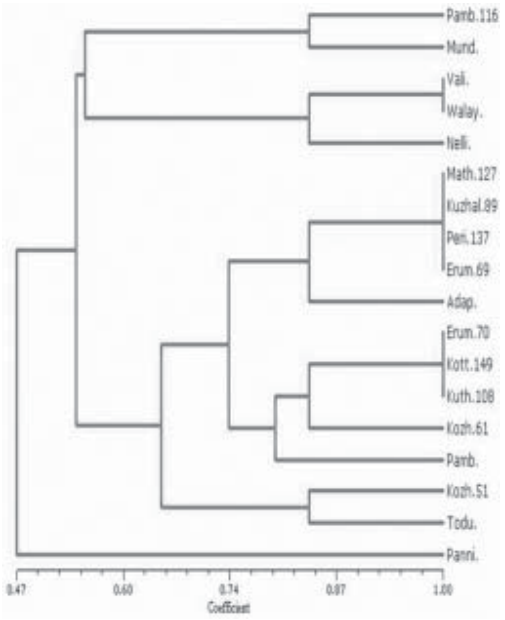

Figure 2. Dendrogram based on isozyme analysis of 18 *Gymnema* accessions from different geographical regions of Kerala grown at Vellanikkara (Pamb. 116 – Pambadi 116, Mund. – Mundur, Vali. – Valiyathovala, Walay. – Walayar, Nelli. – Nelliampathi, Math. 127 – Mathur 127, Kuzhal. 89 – Kuzhalmannom 89, Peri. 137 – Peringottukurushi 137, Erum. 69 – Erumayoor 69, Adap. – Adapurutti, Erum.70 – Erumayoor 70, Kott. 149 – Kottayi 149, Kuth.108 – Kuthannoor 108, Kozh. 61 – Kozhinjampara 61, Pamb. – Pambadi, Kozh. 51 – Kozhinjampara 51, Todu. – Todupuzha and Panni. – Panniyur).

RAPD markers

A total of 123 RAPDs were generated, of which 90 bands were polymorphic, giving an average of six polymorphic bands per primer. The amplification produced by the primer OPAH12 is shown in Fig. 3. Number of amplification products per primer ranged between 5 and 13 with OPE17 having the maximum number of bands (13). There was a notable difference in the RAPD banding pattern with the total frequency of polymorphic markers as high as 73.2%. The sequences of 15 random primers used in this study along with the number of RAPD markers generated are given in Table 2. Specific bands could be obtained for five accessions (Table 3). With combination of suitable primers, most of the accessions could be identified by having an accession-specific band, or that lacks a band which is present in all other accessions.

The 18 germplasm accessions formed two distinct clusters at 72% similarity (Fig. 4). One of these, however, included only two accessions ('Panniyur' and

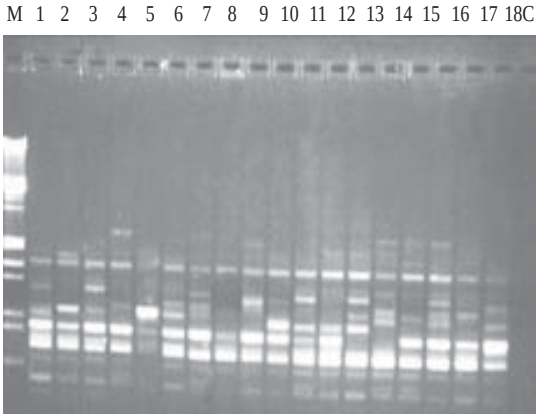

Figure 3. RAPD profile produced by the primer OPAH12 of 18 Gymnema accessions from different geographical regions of Kerala grown at Vellanikkara (Samples 1-Pambadi, 2-Mundur, 3-Valiyathovala,4-Nelliampathi,5-Panniyur, 6- Walayar, 7-Erumayoor 69, 8-Kottayi149, 9- Kozhinjampara 51, 10-Kozhinjampara 61, 11-Peringottukurushi 137,12-Kuzhalmannom 89, 13- Erumayoor 70, 14-Kuthannoor 108, 15-Todupuzha 16-Mathur 127, 17- Pambadi and 18- Adapuruthi).

Table 2. Primer sequence and amplification pattern produced by 15 random primers of 18 *Gymnema* accessions from different geographical regions of Kerala grown at Vellanikkara.

Primer	Sequence	Number of		Polymorphism (%)
		Amplification products	Polymorphic bands	
OPA 11	CAATCGCCGT	6	5	83
OPA 13	CAGAACCCAC	6	3	50
OPA 14	CTCGTGCTGG	5	4	80
OPA 15	TTCCGAACCC	10	7	70
OPA 17	GACCGCTTGT	10	9	90
OPA 18	AGGTGACCGT	9	6	67
OPAH 12	TCCAACGGCT	12	11	92
OPAH 17	CAGTGGGGAG	5	5	100
OPE 14	TGCGGCTGAG	10	5	50
OPE15	ACGCACAACC	12	7	58
OPE 17	CTACTGCCGT	13	12	92
OPE 18	GGACTGCAGA	8	5	63
OPF 13	GGCTGCAGAA	6	3	50
OPF 14	TGCTGCAGGT	5	3	60
OPF 19	CCTCTACACC	6	4	67

'Todupuzha'). In the cluster analysis based on isozyme analysis also, 'Panniyur' remained separate showing a high level of variation. The alternate cluster of 16 accessions further segregated into three subclusters each with a number of sub-sub-clusters. The highest genetic similarity was noticed between 'Peringottukurishi 137' and 'Kuzhalmannom 89' with a similarity coefficient of 0.85. These two accessions had identical isozyme banding patterns also.

The genetic divergence of 0.15 to 0.28 across the *Gymnema* accessions reflect considerable variations at the DNA level and indicates a wide and diverse genetic base of the materials studied. The sources of polymorphism also could be deletion of a priming site, insertions that render priming sites too distant to support amplification or insertions that change the size of a DNA segment without preventing amplification (Williams et al., 1990). Nonetheless, the collections originating from

Table 3. Accession specific bands generated by random primers of 18 *Gymnema* accessions from different geographical regions of Kerala grown at Vellanikkara.

Primer	Sequence	Band size (Kb)	Accession
OPA-17	GACCGCTTGT	2.34	Kottayi 149
OPA-18	AGGTGACCGT	1.23	Mundur
OPA-18	AGGTGACCGT	0.13	Valiyathovala
OPE-15	ACGCACAACC	2.06	Mundur
OPE-17	CTACTGCCGT	1.14	Walayar
OPE-17	CTACTGCCGT	0.15	Walayar
OPF-14	TGCTGCAGGT	1.10	Peringottukurushi 137

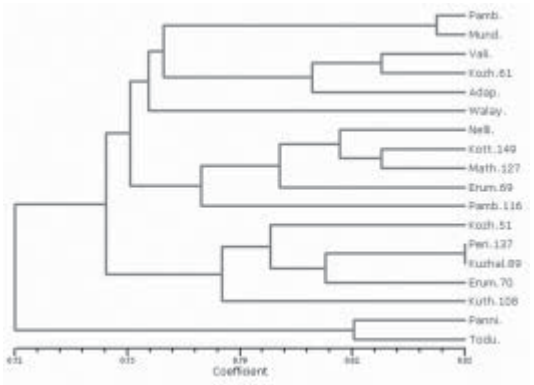

Figure 4. Dendrogram derived from the analysis of 18 *Gymnema* accessions from different geographical regions of Kerala grown at Vellanikkara using 15 random primers (Pamb. – Pambadi, Mund. – Mundur, Vali. – Valiyathovala, Kozh. 61- Kozhinjampara 61, Adap. – Adapurutti, Walay. – Walayar, Nelli. – Nelliampathi, Kott. 149 – Kottayi 149, Math. 127 – Mathur 127, Erum. 69 – Erumayoor 69, Pamb. 116 – Pambadi 116, Kozh. 51 – Kozhinjampara 51, Peri. 137 – Peringottukurushi 137, Kuzhal. 89 – Kuzhalmannom 89, Erum.70 – Erumayoor 70, Kuth.108 – Kuthannoor 108, Panni. – Panniyur and Todu. – Todupuzha).

various parts of the state did not form well-defined groups and were interspersed with each other, indicating little association between RAPD pattern and geographic origin of the accessions. This may be because of recurrent introduction from the same source or sharing of germplasm among the farming community across different geographical locations.

On a final note, the present study revealed the existence of considerable variations at the molecular level in the *Gymnema* germplasm, which confirms the results of the earlier on morphological and biochemical studies. The results could be used for identification of ideal genotypes for extraction of drugs by correlating the molecular fingerprints with desirable morphological and biochemical features. It will also help in devising strategies to protect the genetic diversity of this species.

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