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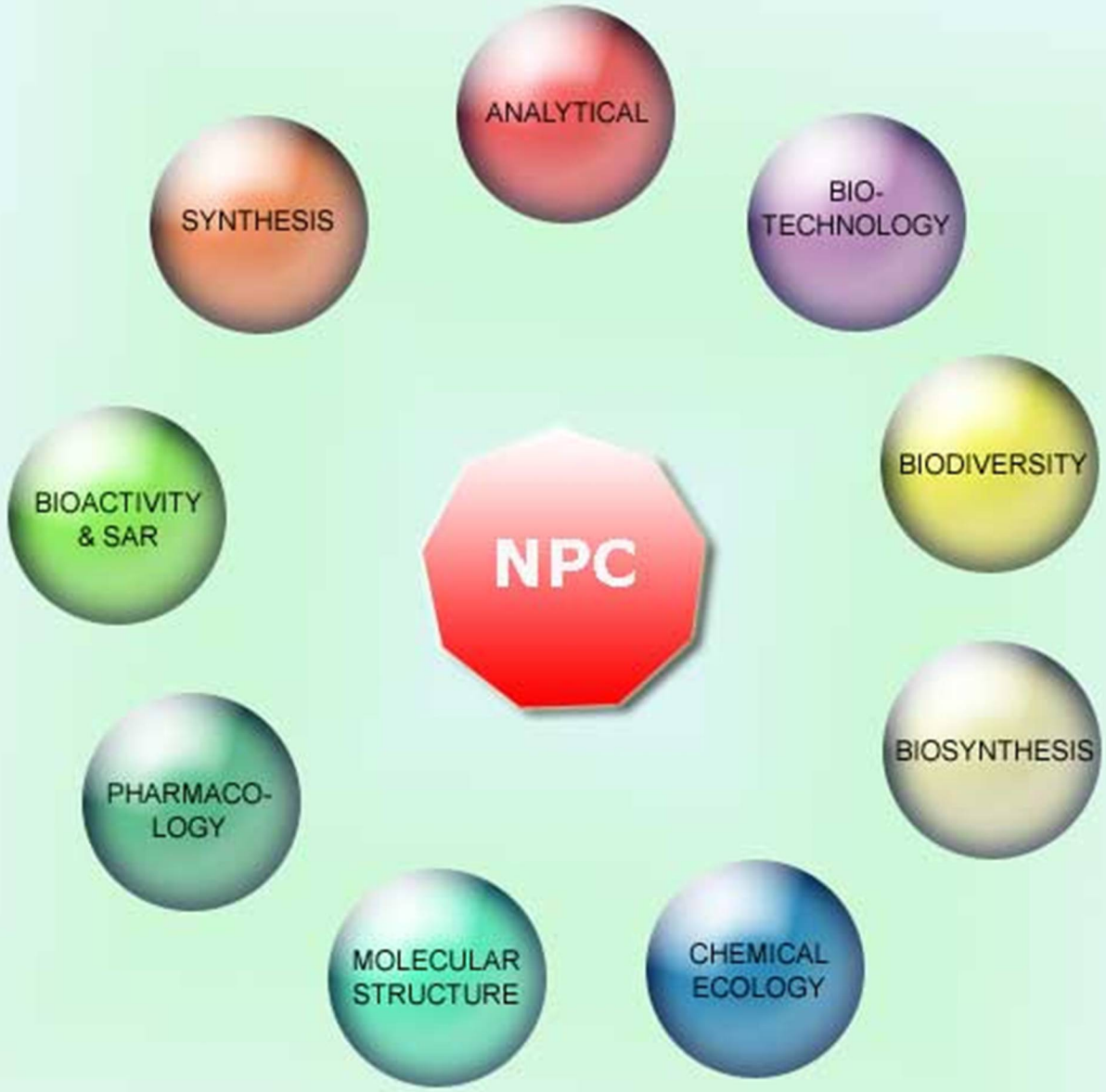

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AFLP Marking and Polymorphism among Progenies of *Gymnema sylvestre*: an Important Medicinal Plant of India

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The level of polymorphism among twelve selected progenies of *Gymnema sylvestre* was investigated through AFLP markers by multiplexing PCR reactions using 64 (8x8) primer combinations. Fourteen primer combinations were selected as the most suitable combination for *G. sylvestre*. Analysis of the 12 progenies with these 14 primer pairs produced 1689 fragments of which 972 (57.5%) were polymorphic and 485 (28.7%) were unique to a particular genotype. The number of fragments produced by individual primer pairs was in the range of 55 to 225. Out of these, polymorphic fragments were in the range of 34 (E-ACC/M-CAC) to 157 (E-AGG/M-CAG) and unique bands observed were 8 (E-ACC / M-CAC) to 69 (E-AGG/M-CAC). Different primer combinations detected different levels of polymorphism, ranging from 33% (E-AGG/ M-CAC) to 69.8% (E-AGG/ M-CAC). From the observations, it appears that the primer combinations E-AGG/M-CAC, E-AGG/CTG, E-AGG/CAG and E-ACA / CAT were the most informative for the detection of polymorphism among the progenies compared with others, since they produced a high number of unique fragments. The similarity coefficient ranged from 0.212 to 0.731. High similarity was observed between progeny S8 and S9 (73%) and high divergence between progenies S3 and S11. Among the selected progeny, S9 was found to be the most similar to the parent (63%), while genotype S11 was the most distant (36.9%).

Keywords: AFLP, DNA polymorphism, DNA markers, *Gymnema sylvestre*, Gurmar, triterpenoid saponins, glycosides, hypoglycemia.

Gymnema sylvestre R Br., (family Asclepiadaceae), is a large, stout, woody climber used as a stomachic, diuretic, and laxative, and for the treatment of sore throat and diabetes [1,2]. Leaves of this species, when chewed, have a unique property of antagonizing the sweet taste of sugar [3]. The leaf extract was found to contain glycosides of gymnemic acid possessing hypoglycemic activity [4,5]. The genetic variability in *G. sylvestre* (Chakkarakolli) was assessed using morphological and biochemical markers by Nair and Keshavachandran [6].

Advances in molecular biology during the last decade have provided a new class for studying variations at the DNA level through development of DNA markers. These genetic markers were found to be extremely useful in differentiating individuals compared with either phenotypic or protein markers. The DNA markers have been used to evaluate genetic diversity in different crop species [7]. They detect variations in the amplified DNA regions as in the case of RAPD (Random Amplified Polymorphic DNA), STSP (Sequence Tagged Site Polymorphism) and AFLP (Amplified Fragment Length Polymorphism). The AFLP technique combines the power of restriction fragment length polymorphism (RFLP) with the flexibility of PCR-based technology. The fingerprints are produced without prior knowledge of sequence by using a limited set of primers. This technique has been extensively used and well preferred to other DNA based markers because of its high multiplex ratio and non-requirement of prior sequence information [8]. These markers consist largely of non coding DNA [9]. The AFLP markers that make up the fingerprint are often concentrated in the centromeric regions [10]. The patterns obtained from different strains are polymorphic due to mutations in the restriction sites, mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and insertion and deletions within the amplified fragments [11].

AFLP markers have found genetic variation below the species level, particularly in the investigation of population structure and differentiation [12]. In this study, AFLP was employed to detect the genetic divergence of progeny of *G. sylvestre* from the parent. Therefore, the major objective of the present study was to investigate, the level of polymorphism among parent and progeny of *G. sylvestre* by AFLP.

Analysis of 12 *G. sylvestre* progenies with the 14 primer pairs revealed a total of 1689 bands (Table 1) of which 972 (57.5%) were polymorphic and 485 (28.7%) were unique

Table 1: AFLP primer combinations, total number of bands, unique, monomorphic and polymorphic fragments generated by each primer combination used in the study of *G. sylvestre* accessions

Serial No.	Primer combination EcoRI / MseI	Total number of bands	Number of polymorphic bands	Number of monomorphic bands	Number of unique bands	Percent Polymorphism
1	ACA/CAC	96	52	31	13	54.2
2	AGG / CAC	132	44	19	69	33.3
3	**ACC / CAC**	**55**	**34**	**13**	**8**	**61.8**
4	**ACA / CTA**	**146**	**101**	**16**	**29**	**69.2**
5	AGG / CTA	106	64	8	34	60.4
6	ACC / CTA	99	53	22	24	53.5
7	**ACA / CAT**	**178**	**113**	**13**	**52**	**63.5**
8	AGG / CAT	131	84	10	37	61.1
9	ACA / CAG	108	66	16	26	59.6
10	**AGG / CAG**	**225**	**157**	**14**	**54**	**69.8**
11	ACC / CAG	72	41	14	18	56.9
12	ACA / CTG	102	49	20	33	48.0
13	AGG / CTG	154	70	17	66	45.5
14	ACC / CTG	85	43	20	22	50.6
Total		**1689**	**972**	**233**	**485**	
Polymorphism%			**57.5**	**13.8**	**28.7**	

Table 2: AFLP similarity coefficients for twelve genotypes of *G. sylvestre*

	P	S2	S3	S4	S5	S7	S8	S9	S10	S11	S12	S13
P	1.000											
S2	0.572	1.000										
S3	0.414	0.466	1.000									
S4	0.530	0.587	0.586	1.000								
S5	0.535	0.642	0.519	**0.705**	1.000							
S7	0.546	0.476	0.354	0.498	0.607	1.000						
S8	0.589	0.531	0.435	0.570	0.548	0.689	1.000					
S9	0.630	0.555	0.454	0.538	0.567	0.629	**0.731**	1.000				
S10	0.438	0.495	0.335	0.508	0.561	0.498	0.528	0.610	1.000			
S11	0.369	0.346	**0.212**	0.368	0.412	0.435	0.390	0.429	0.462	1.000		
S12	0.549	0.505	0.401	0.482	0.594	0.577	0.567	0.610	0.452	0.473	1.000	
S13	0.473	0.539	0.332	0.482	0.484	0.362	0.429	0.478	0.489	0.321	0.432	1.000

to a particular genotype. The fragment sizes determined by comparing the amplicons with size standard DNA ranged from 30 – 400 bp and only a size more than 50 bp was considered for scoring the presence and absences of fragments. Data in Table I show that the total number of fragments detected by individual primer pairs ranged from 55 (E-ACA/ M-CAC) to 225 (E-AGG/M- CAG), and the number of polymorphic fragments from 34 (E-ACC/ M-CAC) to 157 (E-AGG/M-CAG). Also, individual primer combinations gave a range of 8 to 69 unique bands. Different primer combinations detected different levels of polymorphism ranging from 33% detected by primer combination E-AGG/ M-CAC to 69.8% for the combination E-AGG/M-CAC. From the results, it appears that the primer combinations E-AGG/M-CAC, E-AGG/CTG, E-AGG/CAG and E-ACA/CAT were most informative in detecting polymorphism among the genotypes compared with others since they have produced high numbers of unique fragments.

Genetic similarity and cluster analysis

A genetic similarity matrix was generated based on correlation coefficients using AFLP data for the assessment of genetic relatedness among the 12 progenies. The similarity coefficients ranged from 0.212 to 0.731. The high similarity was between progenies S8 and S9 (73%) and high divergence was between progenies S3 and S11 (Table 2). Among the selected progenies, S9 was found to be the most similar to Parent (63%), followed by

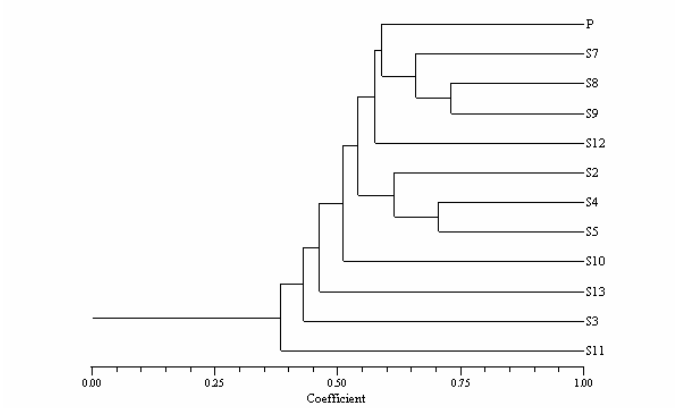

Figure 1: A dendrogram generated based on AFLP data using UPGMA cluster analysis among 12 genotypes of *Gymnema sylvestre*

progeny S8 (58%), while progeny S11 was the most distant (36.9%). Clustering of progenies based on genetic similarity is displayed in Figure 1, in which progeny S8 and S9 sub-cluster together sharing 73% of the fragments. Similarly, progeny S4 and S5 sub-clustered together sharing 70% of similarity. This study assessed the level of polymorphism among *Gymnema* progenies as well as the potential of the AFLP technique in analyzing the genetic variation in closely related genotypes.

The AFLP showed enough sensitivity to detect the polymorphism among parent and seed raised progenies of

G. sylvestre at the molecular level, which will be further used to discriminate the parent and progeny. AFLP has been proved to be the most powerful and reliable marker and revealed much higher levels of polymorphism irrespective of complexity of genome [13-15]. The results obtained will provide a basis for identification and development of molecular markers in this important antidiabetic plant of India, the natural resources of which are fast disappearing due to its overexploitation [16].

Experimental

Amplified fragment length polymorphism

Plant material and genomic DNA isolation: The plant materials comprised of twelve genotypes (one parent and 11 seed progenies) of *Gymnema sylvestre* maintained at CIMAP Conservatory, Lucknow, India. Leaves were collected from the plants and DNA isolated from leaf tissue according to the protocol described by Khanuja *et al.* [17] and quantified by loading on agraose gel together with known amounts of Lambda Hind III Eco RI DNA marker.

DNA restriction and ligation reactions: Genomic DNA was restricted with 2 restriction endonucleases, EcoRI and Tru 9I (an isoschizomer of MseI), and double stranded adaptor was ligated to the ends of DNA fragments, generating template DNA for subsequent PCR amplifications. Restriction and ligation reactions were carried out simultaneously in a single reaction [13]. To carry out the reaction, an enzyme master mix was prepared (for 10 reactions) containing 1 µL (10X) T_4 DNA ligase buffer, 1 µL (0.5 M) NaCl, 0.5 µL (1 mg/mL) BSA, 1 µL Tru 9I (10U/ µL), 4.25 µL EcoRI (12 U/µL), and 0.5 µL T_4 DNA ligase (20U/µL), and the volume was adjusted to 10 µL by addition of 1.75 µL double distilled water. The restriction-ligation reaction consisted of 300 ng of DNA (5.5 µL), 1 µL 10X T_4 DNA ligase buffer, 1.0 µL 0.5 M NaCl, 0.5 µL (1mg/mL) BSA, 1 µL MseI Adaptors (Applied Biosystems), 1 µL EcoRI adaptors (Applied Biosystems) and 1 µL enzyme master mix, as described above. The reaction mix was incubated overnight at room temperature and subsequently diluted 20-fold with $T_{10}E_{0.1}$ (10 mM Tris and 0.1 mM EDTA) buffer. The ligated adaptors served as the primer binding site for the low-level selection in pre-selective amplification of the restriction fragments.

PCR amplifications

Preselective and selective amplification: The MseI complementary primer had a 3′- C and the EcoRI complementary primer a 3′-A. Only the genomic fragments having adaptor on each end amplified exponentially during PCR. The pre-selective amplification mixture was prepared by adding 4 µL of 20 fold diluted DNA from the restriction ligation reaction, 0.5 µL AFLP pre-selective primers (EcoRI, Applied Biosystems), 0.5 µL AFLP pre-selective primer (MseI, Applied Biosystems), and 15 µL AFLP core mix. The pre-selective amplification was carried out in a thermal cycler programmed at 72°C for 2 min, followed by 20 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 2 min, 60°C for 30 min and finally incubated at 4°C. The amplified DNA was diluted 20 fold with $T_{10}E_{0.1}$ buffer and selective amplifications were carried out using different combinations of MseI and EcoRI primers. Sixteen out of the available AFLP primers (8 fluorescent labeled EcoRI and 8 unlabeled MseI) were chosen for amplifications. The EcoRI primers contained 3 selective nucleotides with the sequence 5′ -[Dye-primer-Axx]- 3′, and the MseI primers had the selective nucleotides starting with C 5′ -[primer-Cxx]- 3′. The explorer gel for all 64 reactions was run with sample P (Parent) to determine the most responsive primer pairs that generate the greater number of fragments. Multiplexing PCRs was designed to set up all 64 (8 X 8) reactions in 24 tubes. Selective amplification reactions contained 3 µL of 20- fold diluted pre-selective amplification reaction products, 15 µL AFLP core mix, 1 µL MseI primer 5′ -[primer-Cxx]- 3′, 1.5 µL EcoRI primers 5′ -[primer-Cxx]- 3′ (0.5 µL of 3 primers each were pooled here). Selective amplification was carried out in a thermal cycler programmed at 94°C for 2 min, followed by 10 cycles of 94°C for 20 sec, 66°C for 30 sec and 72°C for 2 min with a subsequent hold for 30 min at 60°C and final incubation at 4°C.

Gel electrophoresis of PCR amplicons: Samples were loaded on 5% polyacrylamide gel on an ABI Prism 377 DNA sequencer (Applied Biosystem).The selective amplification reaction product (3 µL) was mixed with 4 µL loading buffer (10% ROX 500 size standard, 10% blue dextran, 80% deionized formamide), from which 1.5 µL was finally loaded on the gel. The data for explorer gel was processed to determine the most efficient primer combinations providing the maximum number of fragments to carry out the AFLP reactions for the whole samples. The most suitable primer combinations for these samples under study were E-ACA /M- CAC, E-AGG/ M-CAC, E-ACC/ M-CAC, E-ACA/ M-CTA, E-AGG / M-CTA, E-ACC/ M-CTA, E-ACA/ M-CAT, E-AGG/ M-CAT, E-ACA/ M-CAG, E-AGG / M-CAG, E-ACC/ M-CAG, E-ACA/ M-CTG, E-AGG / M-CTG, E-ACC/ M-CTG. All samples were then subjected to selective amplification with these primer combinations, as above.

AFLP data analysis: Data were analyzed by scoring presence and absence of bands from the amplified products and similarity matrixes were obtained using software SPSS for windows (Jaccard correlations) and averages of the pooled similarity of the whole primers were used for clustering based on the UPGMA (Unweighted Pair Group Method with Arithmetic average) method using NTSY2.1 software program.

Analysis of amplified fragment length polymorphism: The AFLP data were converted from binary data matrix to a diagonal matrix format (Table 2) using SPSS v10.0

Software by calculating the similarities through Jaccard's coefficient (1908) [18]. The genetic similarities (GS) were estimated between the 8 primer combinations according to Jaccard's coefficient. These AFLP data were clustered using the NTSYS – pc statistical package v.17 [19]. A dendrogram was constructed employing the UPGMA (Unweighted Pair Grouping Method of Arithmetic averages) method according to Sneath and Sokal [20] to group the individuals into discrete clusters (Figure 1).

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