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DNA ISOLATION AND OPTIMIZATION OF PCR CONDITIONS IN *GYMNEMA SYLVESTRE* BY USING INTER SIMPLE SEQUENCE REPEAT (ISSR) MARKERS

SUNITA KHATAK*, NITIN CHAUHAN and DEEPAK KUMAR MALIK

Department of Biotechnology, University Institute of Engineering & Technology,
Kurukshetra, University, KURUKSHETRA – 136119 (Haryana) INDIA

ABSTRACT

The diverse medicinal properties of *Gymnema sylvestre* was done to understand the genetic diversity among various genotypes by using ISSR primers. Initially 20 primers were used out of which 15 showed the reproducible bands. A total of 105 bands were distinguished across the selected primers and statistical analysis showed 99 bands were polymorphic and 6 were monomorphic bands. The cluster analysis of the genotypes based on UPGMA divided the five genotypes into two main clusters, cluster I having GS-03 genotype of *Gymnema sylvestre* and other having rest of all four genotypes. The results of the present study can be used for molecular breeding and improvement of *G. sylvestre* for various desired traits through hybridization in future.

Key words: *Gymnema sylvestre*, ISSR, Genetic diversity, Polymorphism.

INTRODUCTION

G. sylvestre is a slow growing, perennial, woody climber, distributed throughout the India, in dry forests up to 600 m height. It is mainly present in the tropical forest of Central and Southern India. The antidiabetic array of molecules has been identified as a group of closely related gymnemic acids after it was successfully isolated and purified from the leaves of *Gymnema sylvestre*[1]. *G. sylvestre* is an anti-diabetic agent besides this, in prevailing systems of medicine, the plant is used in the treatment of dyspepsia, constipation[2], jaundice, haemorrhoids[3], renal and vesicle calculi[4], cardiopathy, asthma[5], bronchitis, amenorrhoea and leucoderma[6]. Markers are certain indicators that are used to indicate certain or specific things. Markers are morphological and genetic markers. A genetic marker is a gene or a DNA sequence that can be used to identify individuals or species[7]. The

*Author for correspondence; E-mail: sunkhatak@rediffmail.com

morphological markers, molecular markers are independent of environmental factors and shows higher level of polymorphism[8]. The PCR based marker techniques like ISSR (Inter Simple Sequence Repeats) play an important role in the genetic diversity analysis. A large number of reports have appeared in the literature using ISSR patterns for differentiating varieties, species etc. of crop plants. Our aim in this study was to optimization of PCR conditions in *Gymnema sylvestre* by using (ISSR) markers.

EXPERIMENTAL

Materials and method

Plant material and genomic DNA extraction

Five genotypes of *Gymnema sylvestre* were used in the present study for analysis of genetic diversity. The different genotypes were obtained from medical and aromatic plants section, Chaudhary Charan Singh Haryana Agriculture University, Hisar. The samples were coded as (*Gymnema Sylvestre*) GS 01-05. The genomic DNA of five varieties of *G. Sylvestre* was extracted from young leaf by using a modified CTAB extraction method of Murray and Thompson[9], modified by Saghai-Maroof et al.[10] as shown in Fig. 1.

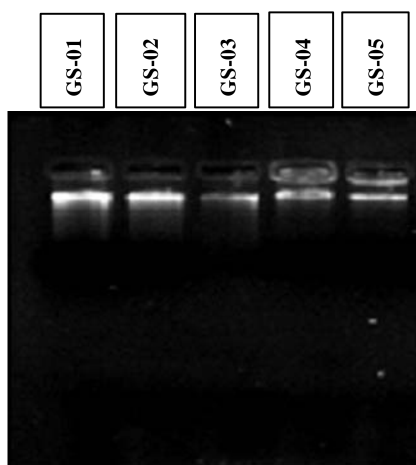

Fig 1: Agarose gel electrophoresis of genomic DNA of five genotypes of *G. sylvestre* (GS-01, GS02, GS-03, GS-04 and GS-05)

ISSR analysis

The conditions to carry out amplification of *Gymnema sylvestre* by using PCR were optimized. The best amplified products were obtained by using 20 µL of reaction mixture,

which specifically contained 100 ng templates DNA, 1.5 μM of primer, 0.20 μM of each dNTP. 2.5 mM of $MgCl_2$, 1X Taq Polymerase buffer, 2.4 U Taq Polymerase. PCR amplification was performed to fulfill 30 cycles after an initial denaturation at 94°C for 5 mins. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at annealing temperature and extension at 72°C for 2 mins, following by extension cycle for 5 minutes at 72°C in the final cycle. The various annealing temperatures of 45°C, 48°C and 55°C produced variable banding patterns. A set of 20 random primers (Bangalore Genei, India) with more than 50% GC content were elected out of which 15 primers were selected for data analysis. Samples of 10 µL ISSR-PCR product were analyzed by electrophoresis on 1.5% agarose gel in 1XTBE buffer. The sizes of DNA fragments were estimated by comparison with standard ladder (100-3000 bp; Bangalore Genei, India) containing 5 µg/mL of ethidium bromide as shown in Fig. 2. Then the gels were visualized, photographed and analysed. ISSR patters were analysed by scoring presence (1) or absence (0) of bands for estimation of similarity among all tested samples. The matrix of similarity (Jaccard) and similarity of Coefficients[11] were calculated and the dendrogram obtained by clustering according to the Unweighted Pair-Group Method with Arithmetic averages (UPGMA) using online software "DendroUPGMA: A dendrogram construction utility".

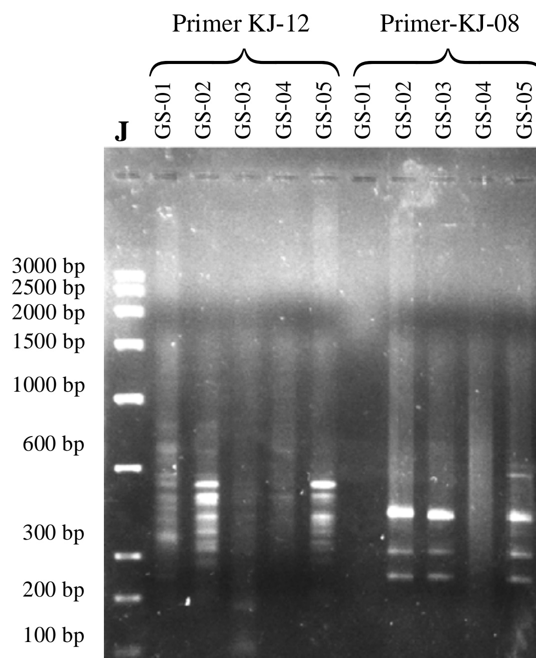

Fig. 2: Agarose gel electrophoretic separation of PCR amplified eighteen genotypes of *G. sylvestre* with primer KJ-12 & KJ-08. Lane Jindicates Quantum PCR medium range marker

RESULTS AND DISCUSSION

In present investigation 15 ISSR primers generated 105 clear bands, out of which 99 bands were polymorphic as shown in Table 1. The percentage polymorphism in *G. sylvestre* was found to be 94.285 ± 25.723%. Similar results were reported by Xia et al.[12] The data obtained using ISSR banding pattern was further used to construct similarity matrices of the 5 *G. sylvestre* genotypes using online software "Dendro UPGMA: A dendrogram construction utility". The Jaccard similarity coefficient of all the genotypes ranged from 0.105 (GS-01 and GS-02) to 0.593 (GS-04 and GS-05). The average similarity across all the genotypes was found to be 0.32 indicating a moderate level of genetic similarity among the genotypes as shown in Table 2.

Table 1: DNA amplification bands and polymorphism generated in *G. sylvestre* genotypes using ISSR primer

S. No.	Primer	Genotypes amplified	Total bands	Polymorphic	Monomorphic	(%) Polymorphism
1	BH-01	4	8	8	0	**100.00**
2	BH-03	5	8	8	1	87.50
3	BH-04	4	10	10	0	100.00
4	BH-05	4	6	6	0	100.00
5	BH-09	4	6	6	0	100.00
6	BH-10	1	1	1	0	100.00
7	BH-15	5	1	0	1	**0.0**
8	BH-18	3	7	7	0	100.00
9	KJ-08	3	6	6	0	100.00
10	KJ-12	5	15	14	1	93.3
11	KJ-13	2	8	8	0	100.00
12	KJ-14	5	10	8	2	80.00
13	KJ-19	4	6	6	0	100.00
14	KJ-20	5	6	5	1	83
15	KJ-21	4	7	7	0	100.00
Total			**105**	**99**	**6**	**94.285 ± 25.723**

Table 2: Similarity matrix computed with Jaccard coefficient

	GS-01	GS-02	GS-03	GS-04	GS-05
GS-01	**1**	0.512	0.105	0.375	**0.372**
GS-02		**1**	0.239	0.471	0.461
GS-03			**1**	0.183	0.215
GS-04				**1**	0.593
GS-05					**1**

Wide range of similarity values suggested that selected genotypes of *G. sylvestre* represents a genetically diverse population. Shahnawaz et al.[13] differentiation between 22 geographically different population of the *G. sylvestre* with an average similarity of 0.325 and the dendrogram based on similarity matrix revealed moderate level of genetic relatedness among 5 genotypes. The amplified bands in 5 genotypes of *G. sylvestre* using 15 primers varied from 1 to 15 per primer with an average of 7 ± 3.401 and an average of 6.6 polymorphic bands per primer were produced. The variation in number of bands produced per primer may be due to specificity of a primer for priming site. The unique alleles are characteristic of a particular genotype which separates it from other genotypes. These can be utilized as fingerprints for identification of a particular genotype. 36 of total unique alleles were detected in *G. sylvestre* with an average of 2.4 unique alleles per primer shown in Table 3.

Table 3: List of primers capable of amplifying unique alleles from different genotypes of *G. sylvestre* using ISSR primers

S. No.	Primer	No. of unique alleles	Allele size (bp)	Genotypes
1.	BH-03	2	680	GS-O1
			446	GS-05
2.	BH-04	1	579	GS-01
3.	BH-05	1	474	GS-01
4.	BH-09	4	665	GS-04
			545	GS-01
			268	GS-02
			123	GS-01
5.	BH-10	1	331	GS-01

Cont…

S. No.	Primer	No. of unique alleles	Allele size (bp)	Genotypes
6.	BH-18	3	436	GS-02
			276	GS-01
			185	GS-02
7.	KJ-08	3	615	GS-05
			573	GS-05
			370	GS-05
8.	KJ-12	4	640	GS-01
			455	GS-01
			250	GS-01
			187	GS-03
9.	KJ-13	7	371	GS-03
			340	GS-01
			276	GS-03
			262	GS-01
			248	GS-03
			218	GS-03
			174	GS-03
10.	KJ-14	2	654	GS-01
			465	GS-01
11.	KJ-19	2	300	GS-01
			185	GS-03
12.	KJ-20	3	466	GS-01
			340	GS-02
			220	GS-02

The maximum number of unique alleles i.e. seven in number was generated by primer KJ-13 at 262 bp, 339.93 bp in GS-01 and 371.31 bp, 275.86 bp, 248 bp, 218 bp, and 173.61 bp in GS-03.

Cluster analysis of the genotypes based on UPGMA (Unweighted Pair Group Method with Arithmetic Average) divided the five genotypes into two main clusters, with

first cluster having only GS-03 genotype of *G. sylvestre* and other having rest of all four genotypes as revealed in Fig. 3. The second cluster was further divided into two sub-clusters. Singh et al.[14] conducted a genetic diversity relationship study in wild species of *Brassicaceae* and allied genera andobtained dendrogram by the UPGMA analysis of 70 cross-transferable STMS markers clearly distinguishing the taxa and resulted in a definitive grouping among genera and species corresponding well with their recognized taxonomy.

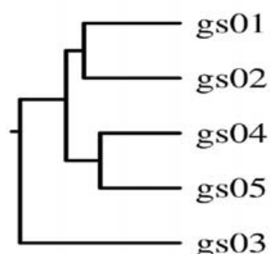

Fig. 3: Dendrogram of genetic variability of *G. sylvestre* constructed with UPGMA clustering method using 32 primers

The estimated similarity coefficients, using the Jaccard index amongst the eleven species ranged between 0.133 (*L. sativum*/*D. tenuisiliqua*) and 0.785 (*D. tenuisiliqua*/ *D. assurgens*). The major Group I was represented by the tribe *Brassiceae* while Group II contained the members of two closely related tribes *Lepidieae* and *Camelineae*. Hence, the ISSR markers showed existence of wide genetic variability within the species.

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