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Molecular Diversity Analysis of *Withania Somnifera* (L) Dunal in Central India using ISSR Markers

Prakash Tiwari[1*] and Abhilasha Shrivastava[2]

[1]School of Environmental Biology, Awadhesh Pratap Singh University Rewa (M.P.), India

[2]Department of Botany and Biotechnology, Government P.G. Science College Rewa (M.P.), India

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Abstract: *Withania somnifera* is an important medicinal properties. The objective of the present study was to investigate the intraspecific genetic variation present in eleven individuals of *W. somnifera* using ISSR (inter simple sequence repeat) marker technique. A sub set of 4 ISSR primers were screened to select the good ISSR primers in Ashwagandha. All four primers successfully amplified and clear banding profile was obtained. The average polymorphic information content (PIC) was 0.70 ±0.02 ranging from 0.462 (UBC-80) to 0.495 (UBC-811). There is highly significant correlation between total number of amplified band and polymorphic bands (r= 0.905 p> 0.001). The information about genetic variation determined from ISSR data was employed to estimate similarity matrix value based on Jaccard's coefficient. The similarity values were further used to construct a phenetic dendrogram revealing the genetic relationships. The dendrogram generated by UPGMA (unweighted pair group method of arithmetic averages) distinguished different genotypes of *W. somnifera*.

Keywords: *Withania somnifera*, ISSR, Genetic variation.

INTRODUCTION

Ashwagandha is medicinally important plant and also known as Indian Ginseng [1]. The medicinal properties of *W. somnifera* have been attributed to several classes of withanolides, steroidal lactones such as withaferin, and other alkaloids [2]. The assessment of the genetic variability is the key to the selection, genetic improvement, conservation and management of useful accessions in gene banks to avoid redundancy. In order to improve yield of medicinal and aromatic plants' germplasm and their genetic analysis are the most important and urgent tasks concern of plant scientists and need is greatest in country particularly in Madhya Pradesh. Where genetic diversity is great and the existence of many species are threatened. Genetic resources are a building block of any breeding and improvement programme [3]. Although the cultivation of medicinal plants has been known for centuries, their germplasm collection, evaluation and utilization in breeding has been very limited. It was observed that an extreme degree of variability existed in *W. somnifera* with respect to growth habit and morphological characteristics of plants in different parts of India and in other countries. Several PCR-based markers have been used to provide information on genetic variation in plant species. Initially, RAPD (random amplified polymorphic DNA) markers were employed for genetic analyses, but problems regarding reproducibility were reported [4].

Inter-Simple Sequence Repeat Markers (ISSR, anchored microsatellites) use simple sequence repeats anchored at the 5' or 3' end by a short arbitrary sequence as PCR primers [5`]. ISSRs are ideal as markers for genetic mapping and population studies because of their abundance, and the high degree of polymorphism between individuals within a population of closely related genotypes [6]. Those properties indicate their potential role as good supplements for RAPD-based genome analysis [7]. Such amplification does not require genome sequence information and leads to multiloci and highly polymorphous patterns [8]. Therefore in this study, our aim was to study the molecular diversity of *Withania somnifera* (L) Dunal using ISSR markers.

MATERIALS AND METHODS

Plant materials: *Withania somnifera* leaves and roots were used for the present study. Samples were collected from various locations of Madhya Pradesh. All samples were collected during the month of December and January. The plants were collected from their natural habitats (**Table-1**).

Table-1: *Withania somnifera* genotypes used in present study.

S. No.	Genotypes	*Place*
1.	WS1	*Jabalpur*
2.	WS2	*Indore*
3.	WS3	*Bhopal*
4.	WS4	*Maihar*
5.	WS5	*Shahdol*
6.	WS6	*Mandsore*
7.	WS7	*Gwaliar*
8.	WS8	*Satna*
9.	WS9	*Panna*
10.	WS10	*Rewa*
11.	WS11	*Sidhi*

DNA extraction: Total genomic DNA was extracted from young leaves by CTAB method of Doyle and Doyle [9]. One gram of fresh leaves were homogenized with pestle and mortar in liquid nitrogen. The homogenized powder was incubated in 50 ml falcon tube in 10 ml of 2 % Cetyl Trimethyl Ammonium Bromide (CTAB) buffer 2 % (w/v), 1.4 M NaCl, 20 mM Na_2EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), 0.2 % β-mercaptoethanol containing 100 mg PVP at 65 ^oC for one hour. After centrifugation supernatant was transferred to fresh tubes and RNase A was added in each then incubated at 37° C for 30 min. The suspension was then mixed with about equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1) mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform: Isoamyl alcohol (24:1) and again centrifuged. The aqueous layer was transferred to a new tube, 2/3 volume of isopropanol was added and DNA was either spooled using a pipette or sedimented by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and re-suspended in 0.5 ml TE buffer. DNA concentration was determined by electrophoresis along with Lambda /Hind III DNA in 0.8 % agarose.

PCR Amplification: Extracted DNA was used in subsequent PCR amplifications, which were performed in a programmable thermocycler (ESCO). Each sample was amplified in a reaction mixture containing genomic DNA 50 ng, Taq polymerase 5U / µl (Sigma Co.), 15 p moles of ISSR primers (Sigma), 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton X-100) $MgCl_2$ and 200µM of each dNTP mixture (Sigma,). The sequences of ISSR primers are shown in **Table-2**. The cycling parameters were 4 min at 94 ^0C for pre-denaturation, 40 cycles each of 45 sec at 94 ^0C for denaturation, 1 min for annealing at 50 ^0C for ISSR Primers, 2 min at 72 ^0C for extension and a final extension at 72 ^0C for 5 min. The mixture was cooled to 4 ^0C and stored at 20 ^0C until electrophoresis. The amplified products were separated on 1.5% agarose gel (Sigma) in 1X TAE buffer. The gels were run for 4 h at 65 V and stained with ethidium bromide and photographed under Gel documentation system (Syngene, U.K.). The PCR products from ISSR analyses were scored qualitatively for presence or absence. Only clear and apparently unambiguous bands were scored for ISSR.

Table-2: Sequence of UBC ISSR primers used in the study.

S. No.	Code	5' to 3' primer sequence	Short sequence	*Total bp*
1	UBC-809	AGAGAGAGAGAGAGAGG	5' (AG)8 G 3'	*17*
2	UBC-810	GAGAGAGAGAGAGAGAT	5' (GA)8 T 3'	*17*
3	UBC-811	GAGAGAGAGAGAGAGAC	5' (GA)8 C 3'	*17*
4	*UBC-840*	*CTCTCTCTCTCTCTCTTT*	*5' (CT)8 TT 3'*	*18*

Data analysis: Genetic similarities between the cultivars were measured by the Jacard similarity coefficient by using NTSYS-PC version 1.8 (Exeter Software, Setauket, NY, U. S. A.) software package [10]. The resultant distance matrix data was used to construct dendrograms by using the un-weighted pair-group method with an arithmetic average (UPGMA) subprogram of NTSYS-PC [10]. The polymorphism information content (PIC) was also calculated for both the marker system. The polymorphic information content (PIC) of each ISSR marker was determined as described by [11a wgvb]. PIC = 1 – Ó Pi, where Pi is the frequency of the i^{th} allele in the examined samples.

Table-3: Jaccard's similarity coefficient values among Ashwagandha accessions using ISSR primers.

	WS1	**WS2**	**WS3**	**WS4**	**WS5**	**WS6**	**WS7**	**WS8**	**WS9**	**WS10**	**WS11**
WS1	1.00										
WS2	0.89	1.00									
WS3	0.95	0.95	1.00								
WS4	0.89	0.89	0.95	1.00							
WS5	0.89	0.89	0.84	0.78	1.00						
WS6	0.78	0.78	0.73	0.78	0.78	1.00					
WS7	0.89	0.78	0.84	0.78	0.78	0.89	1.00				
WS8	0.84	0.84	0.78	0.73	0.84	0.84	0.84	1.00			
WS9	0.67	0.67	0.73	0.78	0.56	0.67	0.67	0.62	1.00		
WS10	0.45	0.34	0.39	0.45	0.45	0.45	0.45	0.28	0.56	1.00	
WS11	0.50	0.39	0.45	0.39	0.50	0.50	0.61	0.56	0.61	0.50	1.00

RESULTS AND DISCUSSION

Genetic diversity analysis is the first and foremost step in any crop improvement programme. DNA markers represent very effective tool for analyzing genetic diversity in any crop improvement programme [3]. ISSR markers have been extensively used in many crops, as genetic markers for assessment of genetic diversity and successful in characterizing individual with their pedigree. These markers are mostly dominant and detect variation in both coding as well as non-coding regions of the genome [4, 5]. Present study is an effort to find out the diversity among few geographically different Ashwagandha accessions of Central India.

A sub set of 4 ISSR primers were screened to select the good ISSR primers in Ashwagandha. All four primers successfully amplified and clear banding profile was obtained. Nucleotide sequences of these selected ISSR primers are shown (**Table-2**). Maximum number of bands i.e. 19 were scored by primer UBC-811, while minimum number of bands i.e. 8 were produced by primer UBC-809. A total of 8 bands were scored for the UBC-809 primers out of which 5 bands were polymorphic and 3 bands were monomorphic. Polymorphic percentage for UBC-809 was 62.5%. UBC-810 primers resulted into 16 bands out of which 15 bands were polymorphic and 1 bands were monomorphic. Polymorphic percentage for UBC-810 was 93.75%. Similarly UBC-811 primers resulted into 19 bands out of which 16 bands were polymorphic and 3 bands were monomorphic. Polymorphic percentage for UBC-811 was 84.21%. On the other hand, UBC-840 primers resulted into 18 bands out of which 16 bands were polymorphic and 2 bands were monomorphic. Polymorphic percentage for UBC-811 was 88.89%.

The average polymorphic information content (PIC) was 0.70 ±0.02 ranging from 0.462 (UBC-80) to 0.495 (UBC-811). There is highly significant correlation between total number of amplified band and polymorphic bands ($r= 0.905$ $p> 0.001$). There is significant correlation also between total number of amplified bands and PIC ($p> 0.05$) per primer, similarly, polymorphic bands and PIC value ($p> 0.05$) of respective primers. Furthermore, it was found that the information content in terms of percentage polymorphism detected differed from one primer to other, which did not necessarily correlate with the number of bands generated. This result is in contrast with the studies on *Azadirachta indica* [12] where 35% polymorphism was detected even though the individuals were collected from 10 different states of India. Such a high degree of polymorphism within *W. somnifera* may be explained by the fact that different morphotypes were analyzed for genetic variation.

Similarity coefficient values for 11 Ashwagandha accessions were calculated (**Table-3**). The range of similarity coefficient was 0.43–0.92, which indicated that the genetic distance among the Ashwagandha accessions was high. Based on electrophoretic banding pattern of ISSR primer pairwise genetic similarity among 11 accessions for genetic diversity were estimated and a dendrogram was generated by unweighted pair group method with "UPGMA" sub programme of "NTSYS – pc" (Figure-1). The cluster analysis grouped these accessions into two major groups, the first major group consisted of subgroup 'A' having accessions WS1, WS3, WS2, WS4 and WS5. The subgroup 'B' having accessions WS6, WS7 and WS8. In the subgroup 'C' and 'D' having accessions WS9 and WS11 respectively. The second major group included the accessions WS1.

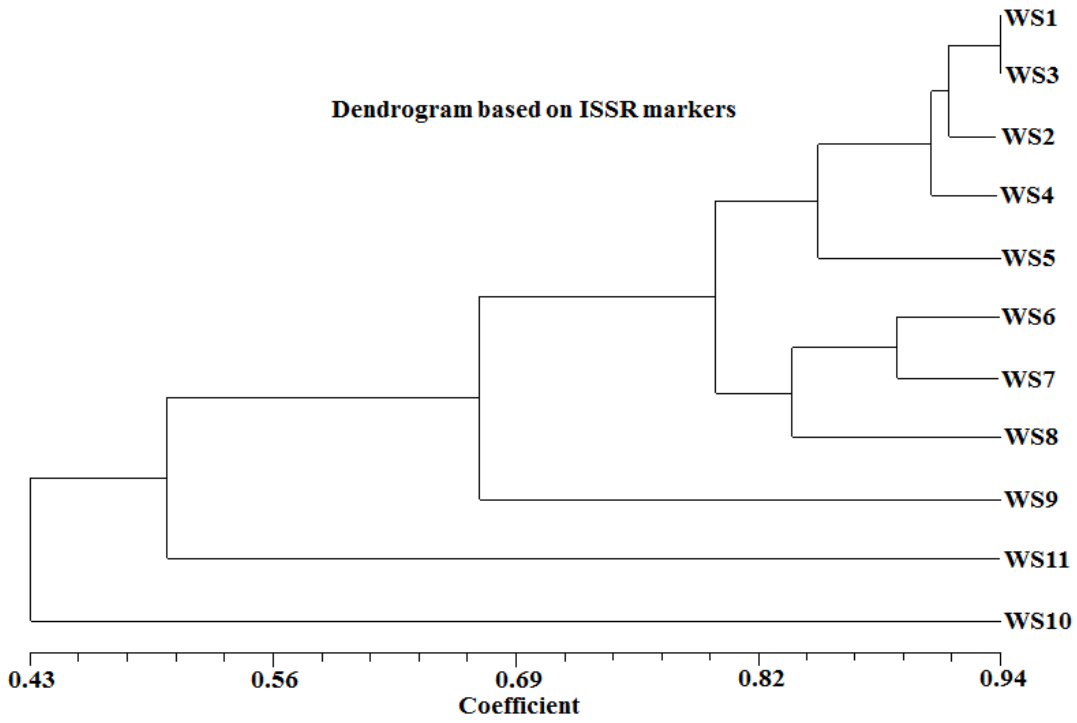

Figure 1: Dendrograms constructed using Jaccard's similarity coefficient and UPGMA clustering for 11 genotypes of W. somnifera based on ISSR polymorphic data.

In conclusion, ISSR is a useful marker for revealing genetic relationships and detecting variant genotypes. It is also an attractive method for screening of duplicate accessions, which can be eliminated from the collections in the germplasm banks.

CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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***Corresponding author: Prakash Tiwari;** School of Environmental Biology, Awadhesh Pratap Singh University Rewa (M.P.), India.