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Efficacy of RAPD markers for molecular diversity analysis of *Withania somnifera* (L) Dunal in central India.

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Abstract

Withania somnifera is an important plant having medicinal properties. In this study, we investigated the efficacy of RAPD markers for detection of genetic variation present in eleven individuals of *W. somnifera*. A sub set of 4 RAPD primers (OPA-03, OPA-06, OPA-12 and OPA-16) were used. All four primers successfully amplified and clear banding profile was obtained. The average polymorphic information content (PIC) was 0.466±0.024 ranging from 0.4405 to 0.4983 and the lowest and highest PIC value were recorded for primer OPA-03 (0.4405) and OPA-12 (0.4983).The range of similarity coefficient was 0.46–0.95, which indicated that the genetic distance among the *W. somnifera* accessions was high. Jaccard's coefficient were calculated and the similarity values were further used to construct a phonetic dendrogram. The dendrogram generated by UPGMA (unweighted pair group method of arithmetic averages) showed genetic diversity of different *W. somnifera* genotypes.

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

Introduction

Withania somnifera is an important medicinal plant and also known as Indian Ginseng (Lv and Wang, 2015). The medicinal properties of *W. somnifera* have been attributed to several classes of withanolides, steroidal lactones such as withaferin, and other alkaloids (Mirjalili et al., 2009). Genetic variability assessment is the key to the selection, genetic improvement, conservation and management. In order to improve yield of medicinal plant their genetic analysis are the most important and urgent tasks 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 plays key role for any breeding and improvement programme (Dekkers and Hospital, 2002). Although the cultivation of medicinal plants has been known for centuries, but their germplasm collection and utilization in breeding has been very limited.

Extreme degree of variability were observed in *W. somnifera* with respect to morphological characteristics and growth habit 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. The RAPD technique has been successfully applied for the study of genetic variation and relationship in plant populations (Dawson et al., 1993). RAPD has many other applications, including the identification of cultivars/varieties; introgression studies; determination of parentage; phylogenetic analysis and construction of genetic maps.

Polymorphisms enerated by random amplified polymorphic DNA analysis were used for fingerprinting by Connolly et al. (1994). RAPD marker based amplification does not require genome sequence information and leads to multiloci and highly polymorphous patterns (Nagaoka and Ogihara, 1997). Therefore in this study, we investigated the efficacy of RAPD markers for molecular diversity analysis of *Withania somnifera* (L) Dunal.

Materials and Methods

Plant materials

Withania somnifera leaves 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 the 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 (1990). 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 mMTris-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 resuspended in 0.5 ml TE buffer. DNA concentration was determined by electrophoresis along with Lambda /Hind III DNA in 0.8 % agarose.

PCR Amplification using RAPD primers

Extracted DNA was used in subsequent PCR amplifications, which were performed in a programmable thermocycler (ESCO). The amplification of genomic DNA was done by using 4 random decamer nucleotide primers (Table 2).

Table 2: Sequence of Random Operon Primers used in the Study.

S. No.	Code	5’ to 3’ primer sequence	GC content %	Total bp
1	OPA-03	5’ AGTCAGCCAC 3’	60	10
2	OPA-06	5’ GGTCCCTGAC 3’	70	10
3	OPA-12	5’ TCGGCGATAG 3’	60	10
4	OPA-16	5’ AGCCAGCGAA 3’	60	10

Each sample was amplified in a reaction mixture containing genomic DNA 50 ng, Taq polymerase 1U (Sigma Co.), 15 p moles of RAPD primers (Sigma), 1X PCR buffer (50 mMKCl, 10 mMTris-HCl, 0.1% Triton X-100) 2 mM$MgCl_2$ and 10µM of each dNTP (Sigma,). The cycling parameters were 4 min at 94 ^{0}C for pre-denaturation, 40 cycles each of 45 sec at 94 ^{0}C for denaturation, 1 min for annealing at 36^{0}C for RAPD Primers, 2 min at 72 ^{0}C for extension and a final extension at 72 ^{0}C for 5 min. The mixture was cooled to 4 ^{0}C and stored at 20 ^{0}C 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 RAPD analyses were scored qualitatively for presence or absence. Only clear and apparently unambiguous bands were scored.

Data analysis

Jacard similarity coefficient were used to estimate genetic similarities between the cultivars and it was measured by NTSYS-PC version 1.8 (Exeter Software, Setauket, NY, U. S. A.) software package (Rohlf, 1993). On the basis of distance matrix data, the dendrograms were constructed by using the un-weighted pair-group method with an arithmetic average (UPGMA) subprogram of NTSYS-PC (Rohlf, 1993). The polymorphism information content (PIC) was also calculated. The polymorphic information content (PIC) of each RAPD marker was determined as described by Weir (1990). PIC = 1 – Ó Pi, where Pi is the frequency of the ith allele in the examined samples.

Results and Discussion

Genetic diversity analysis provides important information for any crop improvement programme. DNA markers are very effective tools for analyzing genetic diversity in any crop improvement programme (Dekkers and Hospital, 2002).The RAPD markers has been extensively usedas genetic markers for assessment of genetic diversity and for their pedigree analysis (Dawson et al., 1993). RAPD markers are mostly dominant and were used for fingerprinting of various plant genotypes(Connolly et al., 1994). Present study is an effort to find out the efficacy of RAPD markers for the assessment of molecular diversity amongst different *Withania somnifera* accessions of Central India.

OPA-03, OPA-06, OPA-12 and OPA-16 were selected on the basis of sharp and clear banding pattern for final RAPD PCR analysis of *Withania somnifera* genotypes. The PCR reaction was carried out using a single decamer primer at a time. All these four RAPD primers resulted with amplification product. Maximum number of bands i.e. 21 were scored by primer OPA-03, while minimum number of bands i.e. 10 were produced by primer OPA-16.A total of 21 bands were scored for the OPA-03 primers out of which all 21 bands are polymorphic. OPA- 06 primers resulted into 18 bands and all bands were polymorphic. Similarly OPA-12 and OPA-16 primers resulted into 16 and 10 bands respectively, and all bands were polymorphic. So all these four RAPD primers showed 100% polymorphism.

The average polymorphic information content (PIC) was 0.466±0.024 ranging from 0.4405 to 0.4983 and the lowest and highest PIC value were recorded for primer OPA-03 (0.4405) and OPA-12 (0.4983). There is significant correlation between total number of bands amplified and polymorphic bands by a primers while there is no significant correlation between total number of band and polymorphic band per primer with PIC value of the primers.Based on electrophoretic banding pattern of RAPD primers, 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). Cluster analysis revealed that all the accessions of *Withaniasomnifera* under study were grouped in two major groups. The first major group consisted of subgroup ‘A’ having accessions namely WS1, WS9, WS10 and WS11.

Similarity coefficient values for 11 *Withania somnifera* accessions were calculated (Table 3). The range of similarity coefficient was 0.46–0.95, which indicated that the genetic distance among the Ashwagandha accessions was high.This result is in contrast with the studies on *Azadirachtaindica* (Singh et al. 1999) 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.

The subgroup 'B' included WS2. The second major group consisted of two small subgroups. WS3, WS4 and WS5 accessions were present in the one subgroup. WS6 was present in different sub groups. The average percentage of polymorphisms i.e., 100% was found to be relatively higher when compared to the other endangered species, stating that it should be able to adapt to the environmental variations.

Table 3: Jaccard's similarity coefficient values among *Withania somnifera* accessions.

	WS1	WS2	WS3	WS4	WS5	WS6	WS7	WS8	WS9	WS10	WS11
WS1	1.00										
WS2	0.71	1.00									
WS3	0.48	0.48	1.00								
WS4	0.48	0.38	0.81	1.00							
WS5	0.43	0.34	0.76	0.86	1.00						
WS6	0.62	0.43	0.67	0.76	0.62	1.00					
WS7	0.90	0.71	0.48	0.38	0.34	0.52	1.00				
WS8	0.86	0.57	0.34	0.34	0.29	0.48	0.76	1.00			
WS9	0.95	0.76	0.52	0.43	0.38	0.57	0.95	0.81	1.00		
WS10	0.86	0.67	0.52	0.43	0.38	0.57	0.86	0.81	0.91	1.00	
WS11	0.86	0.76	0.62	0.52	0.38	0.67	0.86	0.71	0.91	0.81	1.00

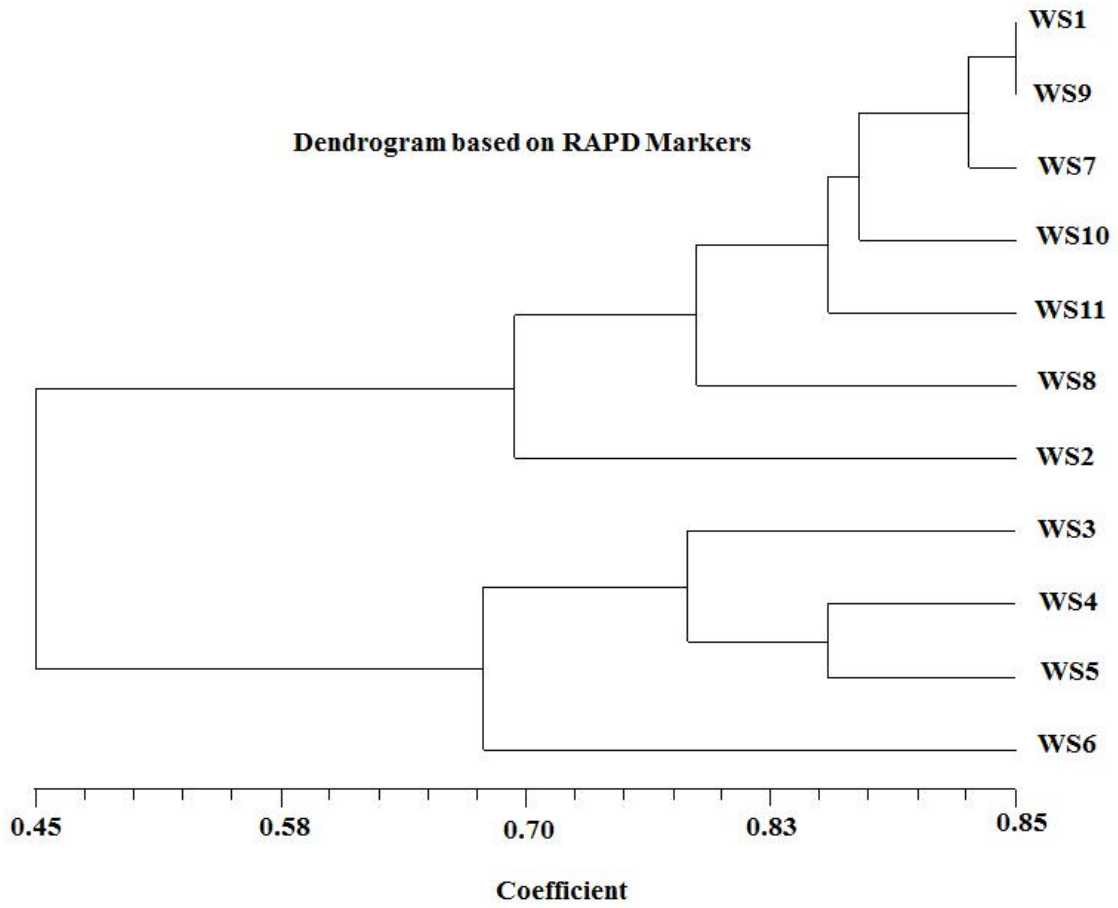

Fig. 1. Dendrograms constructed using Jaccard's similarity coefficient and UPGMA clustering for 11 genotypes of *W. somnifera* based on RAPD polymorphic data.

In conclusion, RAPD markers are useful for detection of genetic variability and relationship amongst different genotypes. It is also useful for screening of duplicate accessions, which can be eliminated from the germplasm banks.

Conflict of Interest

There is no conflict of interest among the authors.

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