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Genetic diversity of ashwagandha genotypes on the basis of morphological traits and RAPD markers

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Abstract

In genetic diversity analysis based on morphology traits and random amplified polymorphic DNA (RAPD) markers was carried out in a set of forty-eight ashwagandha genotypes selected from germplasm maintained at Directorate of Medicinal and Aeromatic Plants Research, Boriavi, Anand (Gujarat) and Department of Plant Breeding and Genetics of MaharanaPratap University of Agriculture and Technology, Udaipur (Rajasthan). Observations were recorded on six different phenotypic traits to study the morphological diversity among the collected germplasm. The genotypes were grouped into seven clusters.Intercluster distance was maximum between clusters II and VII followed by clusters I and II hence they were genetically most diverse. Among the seven characters studied, root dry weight per plant contributed the maximum accounting for 35.52 per cent of total divergence. Further, in RAPD marker analysis, 8 primers were used to study the diversity at molecular level in ashwagandha genotypes. The dendrogram constructed on the basis of these marker data was able to classify all the genotypes of ashwagandha into clusters indicating molecular divergence in the population. Ashwagandha genotypes RAS 32 and RAS 57 had the highest similarity, while genotypes JA 20 and IC 283942 had minimum similarity. Thus genotypes, JA 20 and IC 283942 were most diverse with respect to RAPD markers. The study indicated that the RAPD marker analysis can be a useful tool for the assessment of diversity among ashwagandha genotypes, which will aid its further utilization in crop improvement programs.

Keywords: RAPD, clusters, ashwagandha genotypes, diversity

Introduction

Ashwagandha, Indian Ginseng, and Winter Cherry has been an important shrub in the Ayurvedic and indigenous medical systems for over 3000 years. These plants are under-shrub of the family Solanaceae. The species is widely distributed from the Southern Mediterranean region to the Canary Islands and to South and East Africa; from Palestine up to India, covering Israel, Jordan, Egypt, Sudan, Iran, Afghanistan and Pakistan. Another species of this genus *i.e.*, *Withaniacoagulance*grows widely in India and Pakistan (Gilani *et al*., 2009) [3]. In India the plant grows wild in North Western regions extending to mountainous regions of Punjab, Himachal Pradesh and Jammu up to an altitude of 1500 m. In India this species is cultivated in Rajasthan, Madhya Pradesh, Andhra Pradesh and Uttar Pradesh (Kaul, 1957 and Kumar *et al*., 2007) [6, 9].

The world market for herbal medicines including herbal products and raw materials has been estimated to have an annual growth rate between 5 to 15 per cent.|grow to US $ 5 trillion by the year 2050 (Joshi *et al*., 2004) [5]. In India, this crop is cultivated over an area of approximately ten thousand hectares with dried root production of 8000 tonnes. The annual demand of dried roots of this plant which increased from 7028 tonnes (2001-02) to 9127 tonnes (2004-05) necessitated the increase in its cultivation and higher production (Kubsad *et al.*, 2009) [8].

The roots and leaves of *W. somnifera* have been used in the Indian traditional system of medicine, Ayurveda and the plant is marketed world over because of its medicinal properties. The role of *W. somnifera* to modulate a variety of biological responses has been reported. It is importantly used as adaptogen, tonic, anti-inflammatory, anti-tumor, anti-stress and antioxidant *etc.*

The genus *Withania*has been estimated to consist of 26 species distributed widely in South Asia and the Eastern Mediterranean area (Kaul 1957 and Kumar *et al*., 2007) [6, 9]. Of these, only two species, *W. somnifera* and *W. coagulance*, have been reported in India. Ashwagandha is an erect, evergreen tomentose shrub grown up to a height of 75 to 100 cm with extensive branching. The leaves and roots of the plant have medicinal properties. The species is cross-pollinated in nature with complex floral biology (Vandana Singh 2009) [15].

Material and Methods

The experimental materials for the present investigation consisted of 48 genotypes of *Withaniasomnifera*. These were obtained from the Directorate of Medicinal and Aeromatic Plants Research, Boriavi, Anand (Gujarat) and Department of Plant Breeding and Genetics of MaharanaPratap University of

Agriculture and Technology, Udaipur (Rajasthan). The genotypes are listed in Table 1

Table 1: List of genotypes

Sr. No	Genotype	Sr. No	Genotype	Sr. No	Genotype
1.	MWS 311	17.	RAS 23	33.	IC 310320-B
2.	MWS 316	18.	RAS 15	34.	K 86
3.	MWS 226	19.	RAS 33	35.	JA 134
4.	MWS 205	20.	RAS 67	36.	JA 20
5.	MWS 322	21.	RAS 11	37.	MPAS 2
6.	MWS 302	22.	RAS 29	38.	MPAS 3
7.	MWS 201	23.	RAS 32	39.	MPAS 4
8.	MWS 217	24.	RAS 57	40.	MPAS 5
9.	MWS 329	25.	RAS 55	41.	MPAS 6
10.	MWS 309	26.	RAS 65	42.	MPAS 7
11.	MWS 101	27.	IC 286632	43.	MPAS 10
12.	MWS 204	28.	IC 283662	44.	MPAS 12
13.	MWS 208	29.	IC 283942	45.	MPAS 15
14.	RAS 18	30.	IC 283966	46.	MPAS 16
15.	RAS 16	31.	IC 310595	47.	SKN Local
16.	RAS 21	32.	IC 310620-A	48	*Withaniacoaglance*

The experiment was laid out in a Randomized Block Design with three replications. Each entry was planted in single rows of 1.0 meter length placed at 30 cm apart. The sowing was carried out by line sowing. The experiment was shown on first week of August, 2011 at the botanical garden of the Department of Genetics and Plant Breeding. All recommended agronomic practices were followed to raise the crop.

Observations were recorded on five randomly selected competitive plants for each entry tagged before flowering in each replication for all the agronomical characters i.e Plant height (cm), Days of flowering, Root length (cm), Root diameter (mm), Number of primary root/branches, Root yield per plant (g)

The data pertaining to various characters was used for analysis of variance by using following linear additive statistical model (Panse and Sukhatme, 1978).

The data pertaining to various characters was used for analysis of variance by using linear additive statistical model (Panse and Sukhatme, 1978). Genetic divergence with respect to six different phenotypic traits and one biochemical traits was estimated in the population using Mahalanobis's D^2 statistics. The clustering was done on the basis of similar response so that genotypes within a cluster are homogeneous (considering all variables simultaneously) and genotypes falling in different clusters are heterogeneous. The limit for divergence within groups was based upon the range of all n(n-1)/2 D^2 values. However, the group formation proceeds from one to the next, this limit gradually increases. In case of overlapping, determination of clustering may become a case of common sense. The recurrent exercise of grouping of genotypes leads to satisfactory cluster formation. In general, divergence between clusters should not be less than that within clusters. After the formulation of the clusters, average intracluster distance (D) was calculated. The intercluster distance was calculated by measuring the distance between clusters I and II, between clusters I and III, between clusters II and III and so on. Likewise, one by one cluster was taken and their distances from other clusters were calculated.

- **Genomic DNA extraction**

DNA extraction protocol was standardized for ashwagandha from young leaves of 21 to 25 days old seedlings following CTAB (Cetyltrimethyl Ammonium Bromide) method of Doyle and Doyle (1990) [2] with some modifications by using PVP. The quality of DNA was checked on 0.8 % (w/v) agarose gel. The quantification of DNA was carried out using spectrophotometer. The good quality DNA samples with a ratio of 1.8 – 2.0 at O.D. 260 / 280 nm in spectrophotometric measurements were retained for PCR amplification.

- **RAPD analysis**

PCR amplification of the genomic DNA was carried out in 200 µl thin walled PCR tubes. The reaction volume of 25 µl was subjected to amplification through PCR in a thermal cycler (Eppendorf) along with the control (without template DNA). Each 25 µL reaction volume contained about 2.5 µL of 10X buffer, 1 µL of MgCl2, 0.375 µL of dNTPs, 2.5 µL of primer, 0.2 µL of Taq Polymerase, 16.925 µL of sterile distilled water, and 1.5 µL of total genomic DNA. The thermocycler was programmed as 5 min at 94ºC for initial denaturation, (1 min at 94ºC for denaturation, 1.30 min at 37ºC for annealing, 2 min at 72ºC for extension- 35 cycles), 10 min at 72ºC for final extension. PCR products were loaded on to 1.2 percent (w/v) agarose gel. The band profiles were visualized and documented using Alpha InnotechFlour Chem. FC2 gel documentation system.

Data scoring and analysis

Data on RAPD markers as presence or absence of bands of PCR amplified DNA fragments were scored. The data were subjected to statistical analysis for the calculation of Jaccard's similarity coefficient and cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using NTSYS-PC based software (Jaccard, 1908, Sokal and Michener, 1958 and Rohlf, 2000) [4, 14, 23]. The polymorphism percentage was calculated as per the method suggested by Blair *et al.*, (1999) [1]. Polymorphism information content (PIC) values were also calculated for each RAPD primer.

Result and Discussion

The analysis of variance for different characters in ashwagandha field experiment revealed highly significant differences among genotypes for all characters, indicating the presence of considerable amount of variation in the material.

Phenotypic characters are genetically informative; they impart the understanding of the genetic distance within and between the populations of same species and permit to classify them in groups showing qualitative differences between them (Marzougui *et al.*, 2007) [10]. On the basis of the magnitude of the D^2 values, the genotypes were grouped into seven clusters. In general, intra cluster distances were lower than the inter cluster distances. Thus, the genotypes included within a cluster tended to diverse less from each other (Table 2). The distance (D^2) between two clusters ranged from 3.91 to 14.46 which was an indicator of diversity present in the material evaluated. The genetic diversity among the different clusters can be attributed to the combined effects of geographical differences, genetic drift, spontaneous variation, history of selection, heterogeneity and selection under diverse environments (Murty and Arunachalam, 1966) [12].

Table 2: Composition of cluster based on D^2 values of forty seven genotypes of Ashwagandha

Cluster	No. of genotypes	Name of genotypes
I	3	MWS 309, RAS 55, IC 310320 B
II	5	MWS 311, MWS 316, MWS 329, MWS 101, MWS 204
III	6	MWS 322, RAS 29, MPAS 2, MPAS 5, MPAS 10, IC310595
IV	6	MWS 302, MWS 208, RAS 21, RAS 23, RAS 65, IC 310620 A
V	10	MWS 201, RAS 16, RAS 15, RAS 67, MPAS 4, MPAS 6, MPAS 15, IC 283942, IC283966, JA 134
VI	13	MWS 226, MWS 205, MWS 217, RAS 18, RAS 33, RAS 11, RAS 32, RAS 57, MPAS 3, MPAS 16, SKN Local, IC 286632, JA20
VII	4	K 86, MPAS 7, MPAS 12, IC 283662

In present study, the cluster VI was the largest having 13 genotypes while cluster I was the smallest with only three genotypes (Table 2). The numbers within one cluster possessed similar morphological and biochemical characteristics. Similarity among the genotypes within a cluster might be due to common parental lines. The maximum inter-cluster distance (D^2= 50.83) was between clusters II and VII followed by that between II and II (D^2 = 41.31). These two clusters were the most diverse among all.

It can besuggested that inter-mating between the genotypes included in these two clusters may give more and better recombinants in the segregating generations. Cluster means for different traits are presented in Table 3.Important feature of cluster I was higher root length (17.00 cm). Higher number of primary branches (4.43) and higher amount of total withanolides (0.17 g/100 g) in roots (table 4) these results may be utilized in formation of core collection and classification of germplasm for various breeding objectives. The contribution of individual characters to the total divergence was worked out.

Table 3: Average inter and intra-cluster distance ($D = D^2$) values in ashwagandha

	I	II	III	IV	V	VI	VII
I	14.46	41.31	20.12	17.83	30.56	26.83	38.85
II		8.93	19.71	12.31	17.35	10.16	50.85
III			3.91	10.16	5.79	8.65	14.58
IV				5.98	13.47	7.75	34.78
V					5.28	8,97	18.73
VI						5.81	30.38
VII							12.14

Table 4: Cluster mean for seven characters in Ashwagandha

Cluster	Plant height (cm)	Days to flowering	Root length (cm)	Root diameter (mm)	No. of primary branches	Root dry weight/ plant (g)	Total withanolides content (g/100 g)
I	43.11	82.33	17.00	7.28	4.43	1.69	0.17
II	33.11	86.46	12.98	5.67	2.26	0.75	0.15
III	43.42	82.94	16.12	7.31	2.8	1.38	0.16
IV	37.87	82.39	14.62	6.50	3.11	0.95	0.17
V	44.83	76.79	15.6	7.30	2.31	1.19	0.16
VI	38.75	89.82	14.94	6.32	2.28	1.01	0.16
VII	45.7	83.25	16.90	8.45	2.58	2.38	0.15
Mean	40.81	83.81	15.30	6.82	2.67	1.38	0.16

Important feature of cluster I was higher root length (17.00 cm). Higher number of primary branches (4.43) and higher amount of total withanolides (0.17 g/100 g) in roots. Members of cluster III had dwarfness of height (33.11 cm) and lower root dry weight per plant (0.75 g). The clusters III and IV were found average in most of the characters. The cluster V was characterised by minimum days for initiation of flowering (76.79). The highest days to flowering was observed in cluster VI. The root dry weight per plant (2.38 g), root diameter (8.35 mm) and tallness of plant (45.7 cm) was registered by the member of cluster VII.

Table 5: Per cent contribution of different characters to total genetic divergence

Sr. No.	Character	Number of character times character ranked first	% contribution
1.	Plant height (cm)	91	8.40
2.	Days to flowering	119	11.00
3.	Root length (cm)	74	6.84
4.	Root diameter (mm)	40	3.70
5.	Number of primary branches	231	21.36
6.	Root dry weight per plant (g)	384	35.52
7.	Total withanolides content	142	13.13
Total		1081	

These traits may play an important role for germplasm collection and evaluation. It was observed that root dry weight (35.52 %) was the main contributor to the total divergence. Number of primary branches (21.36 %) and total withanolides content (13.13 %) contributed moderately and days to flowering (11.00) plant height (8.4 %) and root length (6.84 %) shown small contribution. Root diameter (3.70 %) shown very low contribution (table 5).

The contribution of individual characters to the total divergence (Table 5) revealed that the root yield per plant contributed maximum towards genetic divergence. The genetic divergence was also partly due to number of primary branches, total withanolides content and days to flowering. These traits may play an important role for germplasm collection and evaluation. The characters *viz.*, root length; root diameter had low contribution towards genetic divergence. This information provides guideline about the order of priority for collection of germplasm.

Molecular marker analysis

Amount and purity of extracted genomic DNA play crucial role for proper amplification in PCR. In present study, an average concentration of DNA was 854.57 ng/µl.DNA was found optimum for identical RAPD profiles. RAPD markers were used to examine the level of genetic diversity in a population composed of 48 genotypes of ashwagandha.

All the eight primers in the present study revealed polymorphism (Table 6). The PIC values for 8 primers varied from 0.5211to 0.7925. In RAPD analysis, polymorphism results from mutation or rearrangements either at or between the primer binding sites (Xu, 2010) [10].

Table 6: Polymorphism obtained with different RAPD primers generated from *Withaniasomnifera* (L.) Dunal *and Withaniacoagulance*

Sr. No.	Seq. Name	Range of BandSize (bp)	TotalPoly-morphicBands	Total MonomorphicBands	Total Bands	% Polymorphism	PIC
1.	OPA 12	203.14-1741.27	7	0	7	100	0.7727
2.	OPA 13	254.65-858.02	4	0	4	100	0.7033
3.	OPA 6	257.82-1808.91	7	0	7	100	0.7925
4.	OPA 20	216.39-1373.84	4	0	4	100	0.7147
5.	OPA 5	353.51-2055.20	5	0	5	100	0.7793
6.	OPB 13	327.29-1004.74	3	0	3	100	0.5211
7.	OPD 13	237.90-1435.16	4	0	4	100s	0.7208
8.	OPH 5	342.75-1269.27	4	0	4	100	0.6782
Total:			38	0	38	100	0.7103

Cluster analysis based on the presence or absence of band was performed by Jaccard’s similarity coefficients, following unweighted pair group method with arithemetic averages (UPGMA). Genetic similarity among the genotypes ranged from 0.071 to 0.833, indicating high genetic divergence. Ashwagandha genotypes RAS 32 and RAS 57 had the highest similarity, while genotypes JA 20 and IC 283942 had minimum similarity. The range of co-efficient values suggested that *W.somnifera*germplasm collection represented a diverse population.

Dendrogram was prepared by using Jaccard similarity co-efficients through NYSYS-PC based software (Figure 1).The dendrogram was able to distinguish all theashwagandha genotypes.Two genotypes RAS 57 and RAS 32 had highest similarity with maximum co-efficient value.

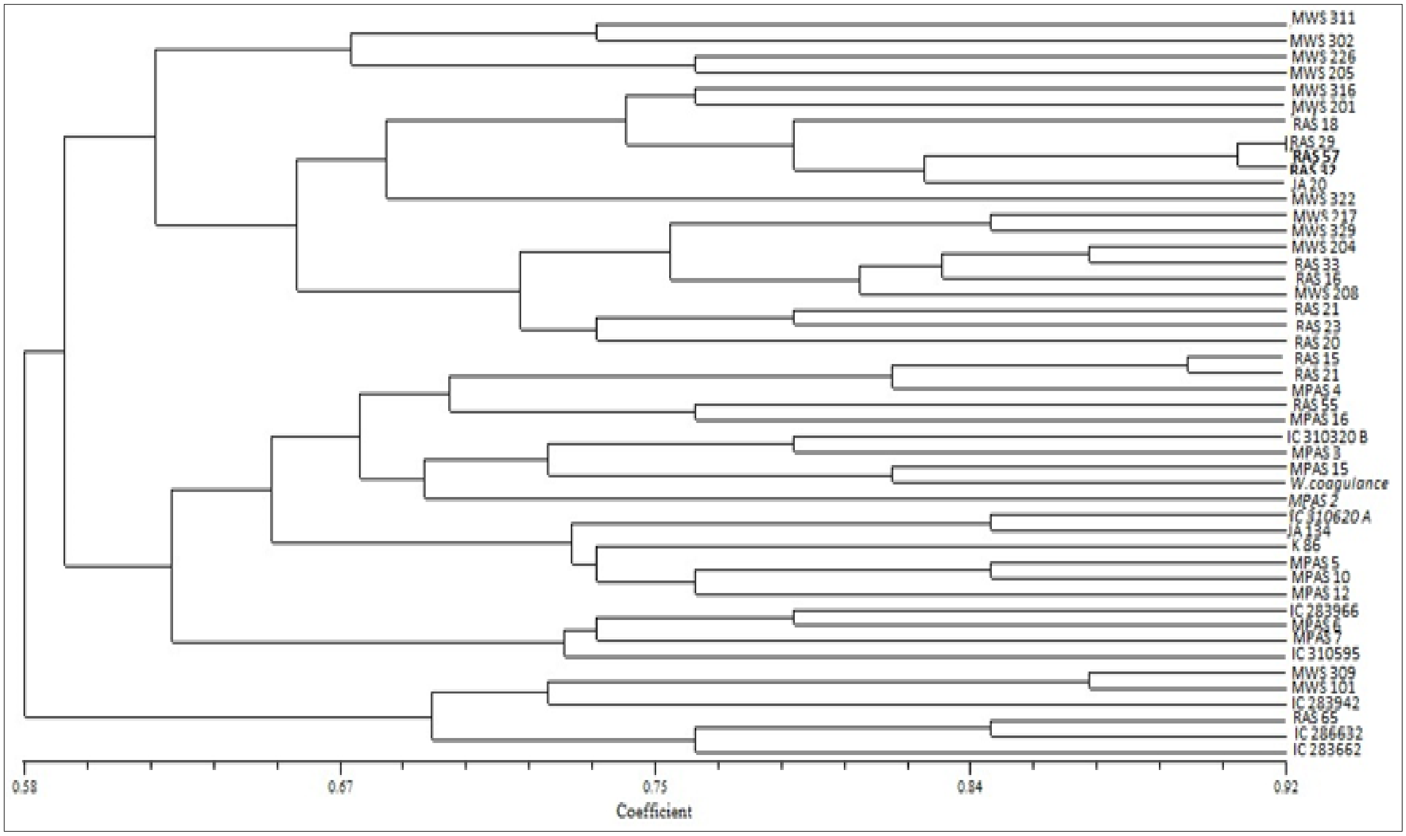

Fig 1

In present investigation, the diversity with respect to polygenic morphological traits and RAPD markers was estimated. The genotypes were classified on the basis of D^2 values as well as molecular polymorphism based UPGMA dendrogram. The seven clusters were composed on the basis of D^2 values, while five clusters were formed by UPGMA dendrogram. When comparison was made between these two methods, it revealed only partial similarity between them. Only a few genotypes such as RAS-55 and IC 310320-B showed similarity at morphological and molecular level. The members within a cluster of dendrogram may be diverse morphologically. Obviously, the RAPD markers were not meant to amplify flanking sights of any of the loci controlling the polygenic morphological traits. There was no possibility of any positive correlation between morphological and molecular markers especially RAPDs because of their genetic nature (Zhang *et al.*, 2010) [17]. Nevertheless, the genetic relationship observed using molecular markers may provide information on the history and biology of cultivars, but it do not necessarily reflect what may be observed with respect to agronomic traits (Metais *et al.*, 2000) [11]. Thus, the clustering of genotypes by D^2 analysis and molecular marker based UPGMA dendrogram was not identical. It can be concluded that in present study, the diversity revealed by morphological and molecular markers was not comparable. The same view was also reported by Khan *et al.* (2011) [11].

In both, D^2 statistics and biochemical revealed diversity in the ashwagandha population under study with respect to morphological. This result may be useful for ashwagandhagermplasm management and breeding program.

Conclusion

Ashwagandha genotypes RAS 32 and RAS 57 had the highest similarity, while genotypes JA 20 and IC 283942 had minimum similarity. Thus genotypes, JA 20 and IC 283942 were most diverse with respect to RAPD markers. The study indicated that the RAPD marker analysis can be a useful tool for the assessment of diversity among ashwagandha genotypes, which will aid its further utilization in crop improvement programs.

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