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Development of microsatellite markers and their correlation with morphological and chemical markers in *Withania somnifera* (L.) Dunal

Punit Kumar Khanna [1]
Ratna Chandra [1]
Arun Kumar [2]
Nidhi Dogra [1]
Heena Gupta [1]
Gourav Gupta [1]
Vijeshwar Verma [1]

Authors' addresses:
[1] School of Biotechnology,
Shri Mata Vaishno Devi University,
Kakryal, Katra, Jammu and Kashmir,
182320 India.

[2] Biodiversity and Applied Botany,
Indian Institute of Integrative Medicine
(CSIR), Jammu, Jammu and Kashmir,
180001 India.

Correspondence:
Ratna Chandra
School of Biotechnology,
Shri Mata Vaishno Devi University,
Kakryal, Katra, Jammu and Kashmir,
182320 India.
Tel.: +91-01991-285691
e-mail: ratna.chandra@smvdu.ac.in

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ABSTRACT

The genetic variation and relationships among 14 *Withania* accessions were evaluated using morphological, chemical and Simple Sequence Repeat (SSR) markers. Wild accessions are more robust and better performing in morphological and chemical metabolite accumulation than cultivated one. The results revealed that out of fourteen, four primers showed distinct polymorphism, indicating the robust nature of microsatellites in revealing polymorphism. The banding pattern was recorded in the form of 0-1 data sheet which was analyzed using unweighted pair group method with arithmetic mean (UPGMA) based on Jaccard's similarity coefficient. The cluster analysis showed higher level of genetic variation among the accessions. Similarity coefficients ranged from 0.125 to 1. The dendrogram revealed 3 major distinct clusters. Higher range of similarity values for related genotypes using simple sequence repeats (SSR) provides greater confidence for the assessment of genetic diversity and relationships. The polymorphism information content (PIC) value for the SSR loci ranged from 0.0 to 0.40. Higher PIC values were associated with higher level of polymorphism. Results of this study showed a high degree of variation among analyzed accessions, indicating an important source of genetic diversity that can be used in future breeding programs.

Key words: *Withania somnifera*, morphological, chemical, markers, microsatellite

Introduction

Withania somnifera (L.) Dunal is an important medicinal plant belonging to the family Solanaceae and is commonly called Ashwagandha or Indian Ginseng. It is also known as Indian Winter Cherry. It is a valuable shrub in Ayurvedic medicine and is classified as a rasayana and acknowledged to increase longevity and vitality (Mishra et al., 2000). It occurs in Sub-Himalayan tract up to Punjab, Nepal, Rajasthan, Sind, dried region of India, Eastern and Western ghats, Andaman and Nicobar Islands, Sri Lanka, Myanmar, Pakistan, Malaysia, Thailand, Java, Asian and African tropics.

The roots of Ashwagandha are primarily used in Ayuverdic medicine, although both the leaves and seeds contain medicinal properties. The three main groups of active constituents are steroidal lactones, alkaloids, and flavonoids, in addition to several amino acids and iron. The presence of withanolides and sitoindosides occur rarely in nature and only to the family Solanaceae. Withaferin A and withanolide D are the two main withanolides contribute to the most of the biological actions of *Withania* (Matsuda et al., 2001). The plant has been found useful in the treatment of burns, wounds, dermatological disorders, gastrointestinal

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diseases, dysfunctions of the respiratory system, asthma, bronchitis and cancer (Bone, 1996; Grierson & Afolayan, 1999).

It is a superior class herb with multiple benefits. The bruised leaves of this plant are used in the treatment of tumors and as an anti-inflammatory agent (Jayaprakasam et al., 2003). The methanolic extracts of different parts of *W. somnifera* exhibit therapeutic potential against various types of cardiovascular problems and are also effective against hyperlipidemia, obesity, (Mary et al., 2003) and aging. In the traditional system of medicine Ayurveda, this plant is claimed to have potent aphrodisiac rejuvenative and life prolonging properties. It has general animating and regenerative qualities and is used among others for the treatment of nervous exhaustion, learning ability, memory capacity, insomnia, tiredness potency issues and coughing.

Pharmacological activities of this plant includes physiologic and metabolic restoration, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders like convulsions, tardive dyskinesia etc (Dhuley, 2000; Bhattacharya et al., 2002). Other investigations indicated that *W. somnifera* also has antistrees (Bhattacharya & Muruganandam, 2003) immunomodulatory, cytotoxic, antibacterial and antifungal (Atta-ur-Rahman et al., 1998). This plant is reputed to promote vitality during recovery from chronic illnesses (Singh et al., 2002) and is useful for pain management in arthritic conditions (Bandyopadhyay & Jha, 2003).

Analysis of genetic diversity in crop species is an important component of crop improvement programs and it is useful in constructing genetic maps. Study of genetic diversity is the process by which variation among individuals or groups of individuals or populations is analyzed by a specific method or a combination of methods (Mohammadi & Prasanna, 2003). Genetic diversity studies can be done at different levels such as morphological, cytological, biochemical and DNA marker systems. Molecular markers have been reported to be useful in genotype identification and introgression of desirable traits from diverse or wild germplasm into the available cultivars to broaden the genetic base (Thompson et al., 1998). Molecular markers have proven useful for assessment of genetic variation in germplasm collections (Mohammadi & Prasanna, 2003).

SSRs are the markers of choice in many plant breeding programs because they are multi-allelic co-dominant markers, PCR-based, easily reproducible, randomly and widely distributed along the genome and their analysis may be automated. Microsatellite polymorphism is based on the different numbers of a short repeated motif at a given locus (Rafalski et al., 1996). Microsatellite sequences are mainly expressed as variation in the copy number of tandem repeats at a particular locus, especially suited to distinguish closely related genotypes; because of their high degree of variability, they are, therefore, favored in population studies (Smith & Devey, 1994) and for the identification of closely related cultivars (Vosman et al., 1992). SSR markers are now considered to be among the most powerful genetic marker as they detect the variation in allele frequency at many unlinked loci. Simple sequence repeat (SSR) markers have been proposed as one of the most suitable markers for the assessment of genetic diversity of plants and animals. Expansion and contraction of SSR repeats in genes of known function can be tested for association with phenotypic variation or, more desirably, biological function (Ayers et al., 1997). Several studies have found that genic SSRs are useful for estimating genetic relationship and at the same time provide opportunities to examine functional diversity in relation to adaptive variation (Eujay et al., 2001; Russell et al., 2004).

Development of SSR markers through genomic library construction involves enormous cost and time. However, identification of transferable markers from the related species has proved to be an important way to develop SSR markers and it was successfully achieved in the other crops (Peakall et al., 1998; Somta et al., 2009; Datta et al., 2010 a,b; Datta et al., 2011; Chandra, 2011). The aim of this study was to develop and characterize microsatellite markers in *Withania somnifera*.

Materials and Methods

Plant material and DNA isolation

Fourteen *Withania* accessions available at medicinal garden of Indian Institute of Integrative Medicine (IIIM), Jammu were used as experimental material (Table 1). Approximately 2 g of young leaf material of each was used to extract and purify genomic DNA through the protocol developed by Doyle & Doyle (1990) with slight modifications as described by Sharma et al. (2003). Approximate DNA yields were calculated by a UV–visible spectrophotometer and also quantified in 0.8% (w/v) agarose gels by comparison with standard 1 kb DNA ladder.

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Table 1. *Ashwagandha germplasm selection and its source.*

No.	Accession Number	Origin
1[a]	AGB001	Manasa (M.P.)
2	AGB002	Bikaner (Rajasthan)
3	AGB003	Jammu (J&K)
4	AGB009	Amritsar (Punjab)
5	AGB012	Joginder Nagar (HP)
6[a]	AGB015	Dabur Research Foundation (Ghaziabad)
7	AGB017	Lucknow (UP)
8	AGB019	Lucknow (UP)
9[a]	AGB025	Neemuch (MP)
10	AGB030	Bhopal (MP)
11[a]	AGB036	Pune (Maharashtra)
12	AGB042	Jodhpur (Rajasthan)
13[a]	AGB053	Hyderabad (AP)
14[a]	AGB055	Hyderabad (AP)

'a' means cultivated accession, while other are wild.

Recording of morphological diversity

Based on 10 randomly selected plants from each plot, observations were recorded on following 13 traits: plant height (cm), plant dry biomass (gm), number of shoots plant^{-1}, leaf apex, leaf margin, leaf thickness, leaf hairyness, root type, root texture, root colour, root bark, dry root yield (gm), dry root yield hectare^{-1} (quintal).

Chemical marker analysis

The examination of marker withanolides (withanolide A, withanone, withaferin A and 12-deoxy-withastramonolode) content in root was carried out by HPLC method (Khajuria et al., 2004).

SSR analysis

All SSR primers were synthesized based on published sequence information (Benor et al., 2008; Grover et al., 2009). A total of 14 SSR primer sequences received from Integrated DNA Technologies, Faridabad, India. These primers were used for PCR amplification of *Withania* DNA. The polymerase chain reaction (PCR) was carried out in a final volume of 20 µl containing 10X reaction buffer 2 µl (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM TrisHCl pH 9.0), dNTPs (1.25 µM each) 2 µl, forward microsatellite primer 0.15 µl (200 µM), reverse microsatellite primer 0.15 µl (200 µM), Taq polymerase (3 U µl^{-1}) 0.15 µl and DNA 50-100 mg. PCR protocol consists of one cycle of a pre-denaturation step at 94ºC followed by 35 amplification cycles (1 minute denaturation at 95ºC, 1 minute annealing, 2 minute extension at 72ºC) and ending with a final 10 minute elongation at 72ºC. The PCR products were separated on 3% agarose gel in 1X TBE buffer containing 0.01% of ethidium bromide. Electrophoresis was carried out at a constant voltage of 80 V for 3 hrs and the amplified product was visualized under ultra-violet (UV) light and photographed using gel documentation system "Biorad". Annealing temperature was adjusted based on the specific requirement of each primer combinations. All the accessions were screened for presence/absence of SSR bands.

SSR data scoring and statistical analysis

All the accessions were scored for the presence (1) and absence (0) of the SSR bands and a binary matrix was obtained. These data were used to assess genetic relationships based on Jaccard's similarity index. Cluster analysis was conducted with the unweighted pair group method based on arithmetic averages to generate a dendrogram using free online software (http://genomes.urv.cat/UPGMA) by Garcia-Vallve et al. (1999).

Results

The fourteen *Withania* accessions were evaluated for plant height (cm), plant dry biomass (gm), number of shoots per plant, leaf apex, leaf margin, leaf thickness, leaf hairyness, root type, root texture, root colour, root bark, dry root yield (gm) and dry root yield per hectare (quintal). Variation in morphological characters of *Withania* accessions are summarized in Table 2. The wild accessions were very prolific based on quantitative traits of plant, leaf and root characters, viz., plant height (129–135 cm), plant dry biomass (65.4 gm), number of shoots per plant (6–7), root length (33–36.6 cm), root diameter (2.5–3.4 cm), dry root yield (15.6 gm) and dry root yield per hectare (10-12.4 quintal). Both wild and cultivated accessions portrayed a wide range of diversity in qualitative and quantitative characters however root texture, root colour and root bark were constant. Therefore morphologically, the wild accession could be separated easily from cultivated accessions.

The success of genetic improvement in this medicinal crop strongly depends on diversity of chemical metabolite

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content along with high potential for root yield. The accessions differed quantitatively for withanolides estimated by HPLC method. The total withanolide content ranged from 0.004 to 0.079% on dry root basis. The chemotypes with high content of specific compound are very important from pharmaceutical point of view. Chemical profiling of roots of wild population showed that withanolide A and withanone increases gradually with the advancement of age but decreases in tertiary roots afterwards. Both withanolide A and withanone in cultivated population have the same pattern of increase initially but decrease in contents in secondary roots, followed by increase in tertiary roots. Leaf is the site of synthesis of withaferin A and translocated later on to primary, secondary and tertiary roots. Withaferin A is detected only in tertiary roots (0.004 gm) of wild type whereas in secondary (0.027 gm) and tertiary roots (0.018 gm) of cultivated type (Table 3). Interestingly, the wild accessions had the maximum withanolide-A (0.079 gm) in secondary roots and withanone (0.038 gm) in secondary roots.

DNA fingerprinting is a powerful tool as it provides sharply defined and repeatable genotypic descriptors than characterization based on morphological characteristics. SSR markers are a powerful tool for cultivar identification and analysis of genetic variation. In this study, fourteen microsatellite markers were used to test the genetic diversity of 14 accessions.

Table 2. *Morphological characteristics of Withania somnfera (L.) Dunal.*

Characteristics	Cultivated accession	Wild accession
Plant height (cm)	44-51 cm	129-135 cm
Plant dry biomass (gm)	32.4	65.4
No. of shoots per plant	4-5	6-7
Leaf apex	Obtuse tapering	Sharply acute
Leaf margin	Wavy	Entire
Leaf thickness	Subcoriaceous	Membranous
Leaf hairyness	Dense	Slight
Root type	Unbranched, erect, 11-16.8 cm long, 1.7 – 2.5 cm diameter	Unbranched, rarely branched at lower portion, 33-36.6 cm long, 2.5 – 3.4 cm diameter
Root texture	Very soft, tuberous	Soft, tuberous
Root colour	Creamish yellow	Light creamish
Root bark	Smooth, thin creamish colour	Smooth thick pale brown
Dry root yield (gm)	4.5	15.6
Estimated dry root yield Hectare^{-1}(quintal)	4-4.8	10-12.4

Table 3. *Chemical profile of roots of wild and cultivated accessions based on 3 marker compounds (g100g^{-1}) at flowering stage.*

Accession	Root type	Withanolide A	Withanone	Withaferin A
Cultivated	Primary	0.047	0.11	-
	Secondary	0.023	0.021	0.027
	Tertiary	0.030	0.031	0.018
Wild	Primary	0.054	0.19	-
	Secondary	0.079	0.038	-
	Tertiary	0.042	0.034	0.004

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Ten (71.42%) primers did not show polymorphism and were not considered for further analysis. The remaining four (28.57%) markers produced polymorphic banding pattern and it was possible to differentiate all the accessions used in the present study. The amplification band number differs from primer to primer, ranging from 2 to 9 (polymorphism band number: minimum of 2 and maximum of 9). Among all the selected primers, P27 and P31 have more amplification and polymorphism bands than the others. In order to demonstrate the potentiality of the SSR markers, PIC and H_e values were used as parameter, which varied with a range between 0-0.40. Our results indicate that markers with large number of alleles are informative for population studies. The high number of alleles per locus per plant in *Withania* could be due to heterozygosity and allogamous nature of cultivated *Withania*.

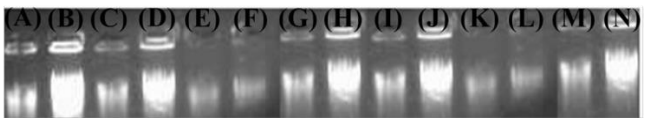

Figure 1. *Withania genomic DNA isolated from different accessions: Lane (A) AGB-001, (B) AGB-002, (C) AGB-003, (D) AGB-009, (E) AGB-012, (F) AGB-015, (G) AGB-017, (H) AGB-019, (I) AGB-025, (J) AGB-030, (K) AGB-036, (L) AGB-042, (M) AGB-053 and (N) AGB-055.*

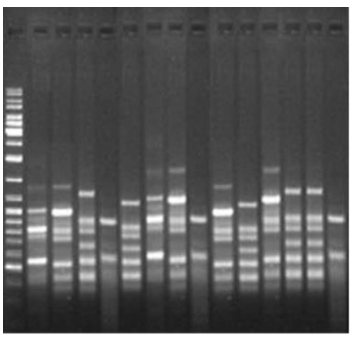

Figure 2. *Amplification profile of 14 accessions of Withania with P27 primer; M: Marker 1 kb ladder; Lanes 2-15: Withania accessions.*

Basing on SSR markers an UPGMA analysis was performed. All 14 *Withania* accessions were discriminated successfully by SSR markers (Figure 3). The accessions were classified into three major clusters with Jaccard's similarity coefficient ranging from 0.125 to 1 (Table 5). The vast range of similarity coefficient between any two accessions indicated the presence of wide genetic variability among the accessions studied. The first group gathered 3 cultivated accessions AGB015, AGB036 and AGB055, while Group II contains three accessions which involve AGB-001, AGB-025 and AGB-053. All the cultivated accessions were included in both the above groups. Group III was further divided into two subgroups (Subgroups 3a and 3b) containing 5 (AGB003 and AGB002; AGB019, AGB017 and AGB009) and 3 (AGB030, AGB042 and AGB012) accessions, respectively of wild group.

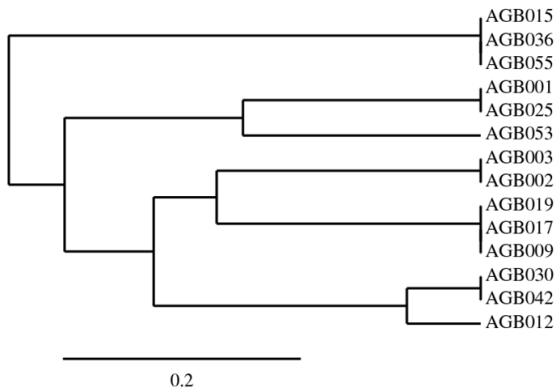

Figure 3. *Dendrogram for the 14 accessions derived from cluster analysis of SSR data.*

Simple sequence repeat (SSR) DNA markers have been shown to be highly polymorphic in soybean (Akkaya et al., 1992) used several types of SSRs to analyze the diversity of 43 soybean genotypes including ancestral and domestic cultivars representing the northern and southern U.S. gene pools. They determined that SSRs with (AT) and (ATT) repeat motifs were highly polymorphic in soybean and identified up to eight alleles at a single locus. Rongwen et al. (1995) identified 11 to 26 alleles at each of seven SSR loci in a diverse sample of soybean genotypes that included U.S. cultivars, *G. max* and *G. soja*. Maughan et al. (1995) detected 79 alleles across five SSR loci in a sample of 94 soybean accessions of *G. max* and *G. soja* genotypes. With 20 SSR markers, Diwan & Cregan (1997) were able to distinguish the 35 soybean genotypes that accounted for about 95% of the alleles present in North American soybean. They detected an average of 10.1 alleles per locus and an average marker diversity of 0.80.

SSR markers are ideal for distinguishing and identification between accessions that are genetically very similar (Saker et al., 2005). Their potential for automation and their inheritance in a codominant manner

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are additional advantages when compared to other types of molecular markers. Up to 37 different alleles for the one SSR locus have been found in barley (Saghai Maroof et al., 1994). The Polymorphism Information Content (PIC) value depends on the diversity of the varieties tested (Pillen et al., 2000; Hamza et al., 2004).

Table 4. *Simple sequence repeats (SSR) primers, their sequence and some information about generated bands in this study.*

Primer name	Forward primer (5'-3') Reverse primer (5'-3')	Microsatellite motif	Allele size range (bp)	Scorable bands	PIC	H
P27	CCGCCTCTTTCACTTGAAC CCAGCGATACGATTAGATACC	$(CAG)_7$	130–175	9	0.370	0.365
P29	CGGGTGACACACGAGATTTT CCGCGAAAAGAAGTGGTAAG	$(AG)_7$	150-162	2	0.0	0.0
P31	TGTGTTGCGTCATTACCACTAAAC CCCAACCACCAATACTTTCC	$(AT)_9$	200-300	7	0.40	0.39
P32	TGGGTATGGGATTTACACCAA AAACGAAGGCAACAACGAAG	$(AGA)_2$ $(GAA)_7$	180-275	8	0.187	0.184

Table 5. *Jaccard similarity matrix for 14 Withania accessions based on SSR data.*

	AGB012	AGB042	AGB009	AGB055	AGB002	AGB053	AGB025	AGB036	AGB030	AGB003	AGB001	AGB017	AGB019	AGB015
AGB012	1	0.875	0.400	0.286	0.400	0.273	0.333	0.286	0.875	0.400	0.333	0.400	0.400	0.286
AGB042		1	0.500	0.250	0.500	0.250	0.417	0.250	1.000	0.500	0.417	0.500	0.500	0.250
AGB009			1	0.286	0.556	0.167	0.455	0.286	0.500	0.556	0.455	1.000	1.000	0.286
AGB055				1	0.125	0.125	0.222	1.000	0.250	0.125	0.222	0.286	0.286	1.000
AGB002					1	0.167	0.333	0.125	0.500	1.000	0.333	0.556	0.556	0.125
AGB053						1	0.600	0.125	0.250	0.167	0.600	0.167	0.167	0.125
AGB025							1	0.222	0.417	0.333	1.000	0.455	0.455	0.222
AGB036								1	0.250	0.125	0.222	0.286	0.286	1.000
AGB030									1	0.500	0.417	0.500	0.500	0.250
AGB003										1	0.333	0.556	0.556	0.125
AGB001											1	0.455	0.455	0.222
AGB017												1	1.000	0.286
AGB019													1	0.286
AGB015														1

Cophenetic correlation coefficient (CP): 0.95

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Discussion

On the basis of banding pattern, SSRs were effectively used for molecular characterization of *Withania* accessions and establishing genetic relatedness among genetic stock evaluated. High information content of the markers enables differentiation and discrimination of *Withania* accessions. The results are in accordance with those of Ben Hmida-Ben Salem (2000) who found that SSR markers seemed to be effective to discriminate local barleys defined as accessions or populations geographically based. Yu et al. (2012) examined the genetic diversity of 80 inbred waxy maize lines using 22 SSR molecular markers that could be used to achieve heterosis in waxy maize and found the values genetic distance from (0.233 to 0.505). An SSR marker used for diversity studies has recorded genetic distance range of 16-53% among 14 genotypes of maize using four SSR markers (Bibi et al., 2010). These results are in agreement with results of the present study.

SSR data has separated the accessions into two major genetic similarity groups. These findings have direct correlation with chemical profiling data. This correlation indicate that the independent sets of data reflect the same pattern of genetic diversity and validate the use of these methods for diversity estimation and also in grouping of accessions. The results also indicate that the combination of morphological and molecular markers may be useful in studying genetic diversity as reported by Cortese et al. (2010). Our results are also in conformity with Roldan-Ruiz et al. (2001) reported that when varieties with shared genepools were examined using molecular markers, extremely high similarity measures were produced and were also linked to morphological similarities. SSRs are relatively quick and easy to use, but refractory to many environmental influences. However, they can be generated in large numbers and can complement traditional character that may be limited in availability. SSRs also provide a valuable new resource for phylogenetic studies. The results obtained in this study may assist *Withania* cultivation and in *Withania* breeding programes.

The combination of microsatellite marker profiles, chemical and the morphological characters serve as reliable tools for detailed description of accessions. Identification of superior genotypes by assessing genetic diversity is an important prerequisite for a successful crop improvement program.

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