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ORIGINAL ARTICLE **OPEN ACCESS**

Development of DNA based molecular markers for *Withania somnifera* and *Withania coagulans*

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ABSTRACT
The medicinal plant Withania somnifera (ashwagandha) of family Solanaceae has immense therapeutic value and have a wide geographical distribution across India. It is well documented in ayurvedic texts having several applications, such as an anabolic, a tonic and a nutrient. The other species of Withania, i.e. W. coagulans (paneer dodi) has been widely used in folklore medicines, widely distributed across India and south Asia. In the backdrop of immense therapeutic value of these two species of Withania and the scarcity of data on genetic diversity, the information on intraspecific relatedness is important for selection of divergent genotypes for several agricultural applications. In the present study, a comparative analysis of W. somnifera and W. coagulans had been carried out using PCR-RAPD analysis.
Key words: *Medicinal plants, Ayurvedic formulation, Genotype, RAPD, SCAR marker*

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INTRODUCTION

Withania somnifera (Ashwagandha) and *Withania* coagulans (Paneer booti/Ashutoshbooti) are economically significant, and are cultivated in several regions for their use in Ayurveda. It is claimed to help control diabetes. The berries contain a rennet-like protease that can be used to clot milk for cheese production. *Withania somnifera* is one of the most popular medicinal plants of great importance, used in India, Africa and the world at large. This is an ecologically and economically important plant used in Ayurvedic formulations [1]. *Withania somnifera* (Ashwagandha) is a multipurpose plant of immense therapeutic value having wide geographic distribution. *Withania somnifera*, a common herbal plant, is often contaminated in medicines with *Withania coagulans*.

The medicinal plant *Withania somnifera* (ashwagandha) of family Solanaceae has immense therapeutic value and have a wide geographical distribution across India. It is well documented in ayurvedic texts having several applications, such as an anabolic, a tonic and a nutrient.The other species of *Withania, i.e. W. coagulans* (paneer dodi) has been widely used in folklore medicines, widely distributed across India and south Asia. In the backdrop of immense therapeutic value of these two species of Withania and the scarcity of data on genetic diversity, the information on intraspecific relatedness is important for selection of divergent genotypes for several agricultural applications. In the present study, a comparative analysis of *W. somnifera* and *W. coagulan s*had been carried out using PCR-RAPD analysis.

At present there are no molecular markers available for identifying *W. sominifera* from other closely related species such as *W. coagulans*. The other objective of present study is to identify some markers that could be used to identify *W. sominifera* species. With the advent of Polymerase chain reaction (PCR), genetic markers are widely used for molecular detection as well as genetic variation in several species. These marker systems take advantage of the analyses of random amplified polymorphic DNA (RAPD) [7]. RAPDs are polymorphic DNA sequences that can be amplified using PCR; the resultant products can be separated using electrophoresis as discrete bands on an agarose gel. Decamers (10 nucleotide sequence) are generally used as primer in a PCR system to amplify a locus of a polymorphic template DNA.

Although RAPD markers are usually dominant markers, they are sensitive to minor changes in reaction conditions during PCR amplification, which can result in irreproducible results. To improve the reliability of RAPDs and to convert them to co-dominant markers, a technique known as sequence-characterized

amplified regions (SCAR) has been developed. SCAR markers are more specific because they usually detect only a single locus. Their PCR amplification is less sensitive to reaction conditions, they are more likely to be co-dominant markers, and are therefore reproducible.

MATERIAL AND METHODS

DNA extraction and quantification:

The leaf tissue of *W. somnifera* and dried powder of *W. coagulans* were used for DNA extraction carried out using CTAB-PVP method. Approximately 0.5x0.5cm tissue (washed) or 20mg of powder was ground in 0.5ml extraction buffer (10% 1M Tris-HCl, 10% 200mM EDTA, 40% 5M NaCl, 3% CTAB and 1g PVP) and incubated for 15min at 65^0C. 100µl of SDS (20%) was added and re-incubated for 30 minutes. 150µl of 7.5M Ammonium acetate was then added to the above solution and mixed by inverting. The tubes were incubated at 4^0C for 15 minutes. DNA was extracted using 1 volume of chloroform: isoamyl alcohol mixture (24:1) and centrifuging at 10000rpm for 5mins. Double volume of ethanol (96-100%) was added to the aqueous phase in a new tube, inverted twice and allowed to stand at 4^0C for 30 minutes. The mixture was then centrifuged at 10000 rpm for 15 minutes. After drying for few seconds pellet was dissolved in 50µl elution buffer (10mM Tris-HCl, 1mM EDTA). Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at 4^0C till further use.

PCR amplification:

The DNA isolated from tissues of *W. somnifera* and *W. coagulans* was subjected to polymerase chain reaction (PCR) amplification using 10 random 10-mer primers (Table 1). PCR amplification was performed using Biometra thermal cycler with 5 µl of 5X qARTATaq Master Mix from Qartabio, 1µl of primer, 1µl Template DNA and 18µl of Nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94 °C; 35 cycles of 1min at 94 °C, 1 min at 40 °C, 2 min at 72 °C; and additionally 1 cycle of 7 min at 72 °C.

Gel electrophoresis:

Gel electrophoresis was performed using 1.4% agarose to analyse the size of amplified PCR product.

Selection of unique band

Two bands unique to *W. somnifera* labelled as OPK01 (800bp) and OPK08 (750bp) (figure 2) were cut and purified using Quick Gel Extraction Kit (K2100-12).

DNA sequencing and primer design

The two products (OPK01 and OPK08) were sequenced at Eurofins Genomics India Pvt. Ltd. For each of the two sequences obtained, two primers had been designed using Primer 3 software.

Primer synthesis

Four sets of designed primers were synthesized by Sigma-Aldrich Company.

PCR amplification of band specific to W. somnifera

In order to check the efficacy of primers synthesized, the DNA isolated from tissues of *W. somnifera* and *W. coagulans* was subjected to PCR amplification. The PCR amplification was performed using Biometra thermal cycler with 5 µl of 5X qARTATaq Master Mix from Qartabio, 1µl of each primer, 1µl Template DNA and 18µl of Nuclease free water. The PCR amplification cycle consisted of, a cycle of 5 min at 94 °C; 30 cycles of 1min at 94 °C, 1 min
at 55 °C, 2 min at 72 °C; and additionally, 1 cycle of 7 min at 72 °C.

Gel electrophoresis:

Gel electrophoresis was performed using 1.0% agarose to analyse the size of amplified PCR product.

RESULTS AND DISCUSSION

Rapid amplification of polymorphic DNA (RAPD):

RAPD data were scored for presence (1), absence (0) or as a missing observation. These bands were considered as polymorphic when they were absent in one among those two samples in a frequency greater than 1% [5] and changes in band intensity were not considered as polymorphism. The band size generated by ten primers ranged from 250 to 1600 bp [Table 1].

Comparative RAPD analysis of W. sominifera and W. coagulans:

The RAPD analysis between the two species is based on 111 loci. Out of these 111 loci the two species 27 loci are common (i.e.24.32%), while 57 bands are polymorphic i.e. 51.35%. (Table 2).

Species specific diagnostic marker for W. Somnifera

In order to develop a PCR-based identification scheme for *W. somnifera*, a total of four primers were screened to generate RAPDs from the 2 different *Withania*species. Out of these four primers, primer pair CBL_WSF1/R1 did not produce a PCR product for both samples while CBL_WSF2/R2 and CBL_WSF4/R4 gave a PCR product for both samples.

Therefore these primers could not be considered as they are not specific to *somnifera* species. Primer pair CBL_WSF3/R3 gave a PCR product for *W. somnifera* but no product was obtained for *W. coagulans*. Therefore CBL_WS F3/R3 was chosen as the SCAR primer to amplify band unique to the *somnifera* species. The amplified PCR product CBL_WS3 was chosen as SCAR marker for *W. somnifera*as it is specific to *somnifera*species (Fig 3).

In recent years, molecular DNA markers have been used extensively for biodiversity analysis and identification purposes. The RAPD technique is simple yet efficient technique for the biodiversity analysis. It has been widely used for studying genetic polymorphism, DNA fingerprinting, varietal identification, classification and population genetics [4-7]. In the present study, RAPD analysis shows that the percentage of genetic polymorphism is low between two species of *Withania (W. somnifera* and *W. coagulans)*. The low genetic polymorphism indicates close genetic similarity between these two species. Interestingly, RAPD analysis of *W. somnifera* collected from different locations in India revealed 37.82 % among 163 loci [2].

Although the RAPD technique is simple, the short primer size and low annealing temperature affects amplification reproducibility, so it may be inappropriate for use in diagnostic purposes. Among the molecular markers that have been developed thus far, SCAR markers appear to be one of the most reliable methods for molecular identification purposes. Also the high sensitivity of the SCAR markers together with their accuracy makes them reliable tools for the identification. In the present study, the Primer CBL_WS F3/R3 was chosen as the SCAR primer to amplify band unique to the *somnifera* species, and the sequenced PCR product CBL_WS3 was chosen as SCAR marker for *W. somnifera* as it is specific to *somnifera* species (Fig 3).

Table 1: RAPD analysis of *W. somnifera and W. coagulans* samples

Appr Band size	OPH 02		OPH 12		OPH 16		OPC 03		OPC 13		OPC 20		OPK 01		OPK 03		OPK 08		OPK 18	
	s	*c*	*s*	*c*	*s*	*c*	*s*	*c*	*s*	*c*	*s*	*c*	*s*	*c*	*s*	*c*	*s*	*c*	*s*	*c*
1600	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
1500	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1400	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
1300	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0
1200	0	1	0	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	0
1100	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
1000	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0
950	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0
900	0	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
850	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
800	0	0	0	0	1	0	0	0	1	1	1	0	1	0	1	0	1	0	0	0
750	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
700	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1	1
650	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0
600	0	1	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0
550	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	1
520	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
500	1	1	0	0	0	1	0	0	1	1	0	0	0	1	1	1	0	1	0	0
450	1	0	0	0	1	0	0	1	0	0	1	0	0	0	1	1	1	0	0	0
420	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0
350	0	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	1	1	0	1
300	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
250	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*s= *Withania somnifera,* *c=*Withania coagulans*

Table: 2 Comparative RAPD analysis of *W. sominifera and W. coagulans*

Primer name	sample	Total bands	Molecular wt. range bp	Common bands	Total polymorphic bands
OPH-02	*W.somnifera*	6	1600-450	2	7
	W.coagulans	5	1200-500		
OPH-12	*W.somnifera*	9	1300-250	6	4
	W.coagulans	7	750-250		
OPH-16	*W.somnifera*	10	1500-450	1	14
	W.coagulans	6	950-350		
OPC-03	*W.somnifera*	1	1200	1	3
	W.coagulans	4	1200-450		
OPC-13	*W.somnifera*	6	1100-500	4	3
	W.coagulans	5	1100-500		
OPC-20	*W.somnifera*	8	1600-350	4	5
	W.coagulans	5	1200-350		
OPK-01	*W.somnifera*	6	1200-300	2	7
	W.coagulans	5	1000-350		
OPK-03	*W.somnifera*	5	1300-450	3	3
	W.coagulans	4	1300-350		
OPK-08	*W.somnifera*	7	1400-350	2	6
	W.coagulans	3	1000-350		
OPK-18	*W.somnifera*	6	1200-400	2	5
	W.coagulans	3	700-350		
Total		111		27	57

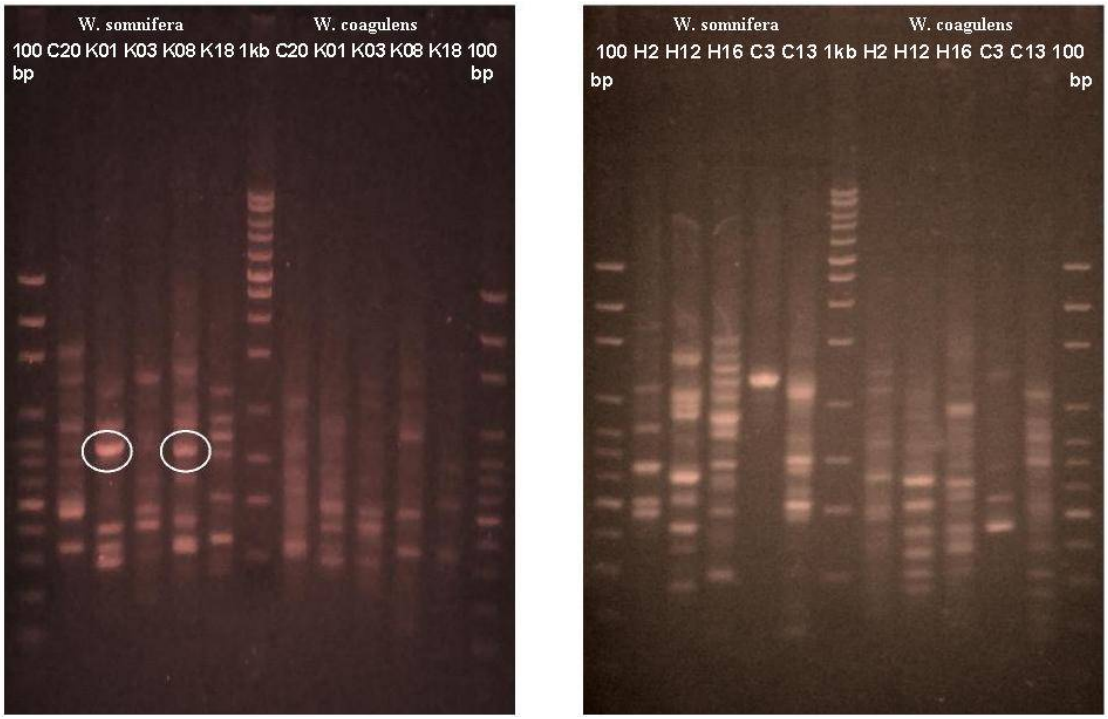


Figure1: RAPD pattern using 10 random primers for *W. somnifera*and *W. coagulans*

Gel I:

Lane 1: 100 bp DNA marker, Lane 2: RAPD pattern of *W. somnifera*using primer OPC20, Lane 3: RAPD pattern of *W. somnifera*using primer OPK01, Lane 4: RAPD pattern of *W. somnifera*using primer OPK03, Lane 5: RAPD pattern of *W. somnifera*using primer OPK08, Lane 6: RAPD pattern of *W. somnifera*using primer OPK18, Lane 7: 1 Kb DNA marker, Lane 8: RAPD pattern of *W. coagulans*using primer OPC20, Lane 9: RAPD pattern of *W. coagulans*using primer OPK01, Lane 10: RAPD pattern of W. coagulans using

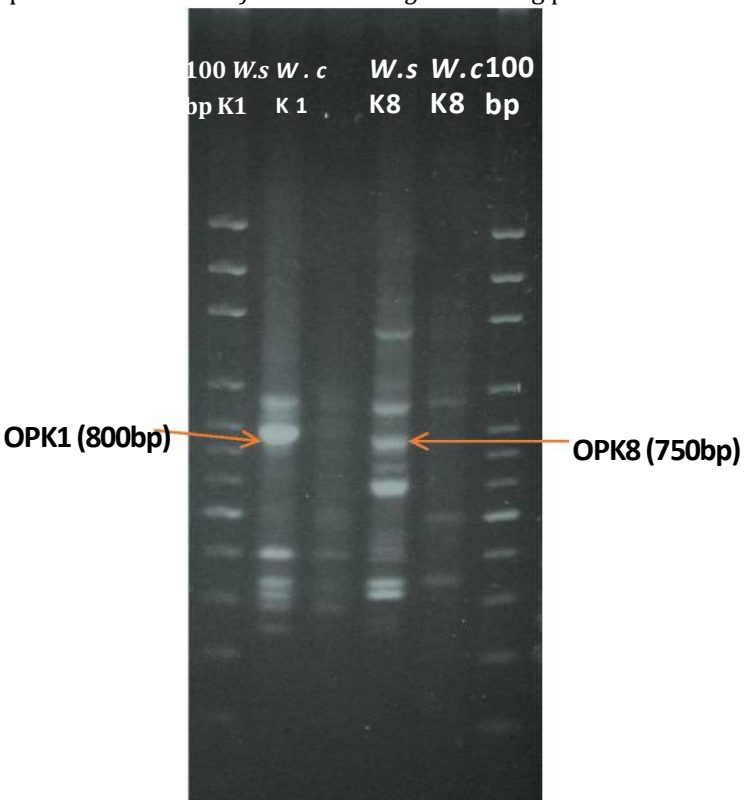
primer OPK03, Lane 11: RAPD pattern of W. coagulans using primer OPK08, Lane 12: RAPD pattern of W. coagulans using primer OPK18, lane 13: 100 bp DNA marker.

Gel II:

Lane 1: 100 bp DNA marker, Lane 2: RAPD pattern of *W. somnifera* using primer OPH02, Lane 3: RAPD pattern of *W. somnifera* using primer OPH12, Lane 4: RAPD pattern of *W. somnifera* using primer OPH16, Lane 5: RAPD pattern of *W. somnifera* using primer OPC03, Lane 6: RAPD pattern of *W. somnifera* using primer OPC13, Lane 7: 1 Kb DNA marker, Lane 8: RAPD pattern of *W. coagulans* using primer OPH02, Lane 9: RAPD pattern of W. *coagulans* using primer OPH12, Lane 10: RAPD pattern of *W. coagulans* using primer OPH16, Lane 11: RAPD pattern of *W. coagulans* using primer OPC03, Lane 12: RAPD pattern of *W. coagulans* using primer OPC13. Lane 13: 100bp DNA marker.

As shown in Fig 1, encircled band was selected for DNA sequencing.

Figure 2: RAPD patterns of *W. sominifera*and *W. coagulans* using primer OPK01 and OPK08



Lane 1: 100 bp DNA marker, Lane 2: RAPD pattern for *W. somnifera*using OPK01, Lane 3: RAPD pattern for *W. coagulans*using OPK01, Lane 4: RAPD pattern for *W. somnifera*using OPK08, Lane 5: RAPD pattern for *W. coagulans*using OPK08, Lane 6: 100 bp DNA marker.

100bp DNA marker (top to bottom): 3000, 2000, 1500, 1000, 800, 700, 600, 500, 400, 300, 200, 100bp.

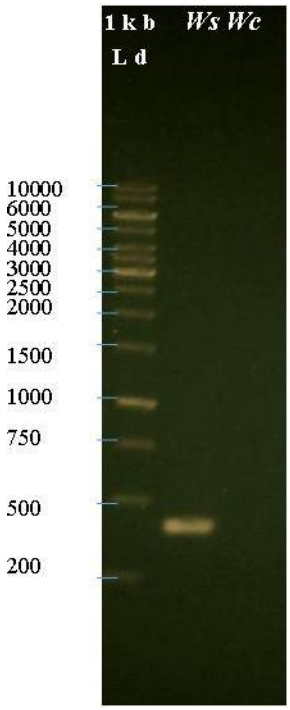


Figure 3: SCAR marker for *Withaniasomnifera*
Lane 1: 1Kb DNA marker (Fermentas)
Lane 2: PCR product with primer designed (CBL_WSF3/R3) for *Withaniasomnifera*
Lane 3: No PCR product with primer designed (CBL_WSF3/R3) seen for *Withaniacoagulans*
1Kb DNA marker (top to bottom): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250bp.

CONCLUSION

In conclusion, the utilization of DNA-based molecular markers, specifically PCR-RAPD analysis, has provided valuable insights into the genetic diversity and relationship between *Withania somnifera* and *Withania coagulans*. This comparative analysis has offered significant findings that contribute to our understanding of the genetic characteristics of these two medicinal plant species.This information holds promise for enhancing our understanding of these valuable medicinal plants and can guide efforts in their conservation, breeding, and utilization for therapeutic purposes.

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CONFLICT OF INTEREST

There is no conflict of interest to publish this date**.**

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