INTERNATIONAL JOURNAL OF HIGHER EDUCATION AND RESEARCH
IJHER, Vol., 8(1), 2018, 140-152.
www.ijher.com

IJHER
(ISSN 2277 260X)

CODEN:IJHER ORIGINAL ARTICLE

MOLECULAR DIVERSITY ANALYSIS AMONG *WITHANIA SOMNIFERA* GENOTYPES USING INTER SIMPLE SEQUENCE REPEATS (ISSR) MARKERS

Arpita Awasthi, Niraj Tripathi
Thakur Ranmat Singh College,
Awadhesh Pratap Singh University, Rewa, Madhya Pradesh, India
Email: tripathi.niraj@gmail.com

ABSTRACT

Ashwagandha is the popular drug of more than 200 Ayurvedic and other herbal formulation in India. The medicinal properties of *Withania somnifera* have been attributed to several classes of withanolides, steroidal lactones such as withaferin, and other alkaloids. A subset of 55 ISSR primers was used to diversity analysis. These primers containing di, tri, tetra and penta nucleotides repeats of 16-20 bases. 55 ISSR primers amplified 399 ISSR markers loci. The size of the amplified markers ranged from100-4000 bp. Out of these 399 loci, 252 loci were found polymorphic (63.15%) across all the Ashwagandha genotypes. Based on ISSR markers, pairwise genetic similarity among 21 *W. somnifera* genotypes were estimated and a dendrogram was generated by UPGMA cluster analysis based on similarity coefficient. The cluster analysis grouped the *W. somnifera* accessions into two major groups. The first major group contained seventeen accessions. The present study demonstrates considerable level of genetic diversity present among ashwagandha genotypes.

Key words: *W. somnifera*, ISSR, Genetic diversity, molecular markers, phylogenetic relationship

Introduction

The angiospermic genus Withania is a member of family Solanaceae and consists of 23 species. It grows in dry and sub-tropical regions. Being hardy and drought tolerant species with its enormous biocompounds, its usage is forever regarded and continuous to enjoy the monopoly in many parts of India. The major Ashwagandha cultivating states are Madhya Pradesh, Rajasthan,

Punjab, Uttar Pradesh, Haryana, Gujarath and Maharashtra among which Madhya Pradesh alone is having more than 4000 ha area (Misra et al., 1997).

Ashwagandha root contains 0.4 – 1.2% alkaloids, 40-65% starch, 40-65% fibres and minor quantity of oil. The important chemical constituents are alkaloids (Withanolides) that are present in roots, leaf and berries.Various compounds isolated from Ashwagandha (Withanolides) are now being investigated as highly promising and non- toxic antitumerol drugs. The Great variability has been found for phytochemicals in different accessions of Ashwagandha (Dhar et al., 2006; Scartezzini et al., 2007).

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. The present study was carried out with the aim to assess genetic diversity among *W. somnifera* genotypes using ISSR markers.

Materials and Methods

Fresh leaves of twenty onegenotypes (Table 1) of *W. somnifera* were collected from different locations of Madhya Pradesh for the isolation of genomic DNA. Inter Simple Sequence Repeats (ISSR) markers were used for the analysis of genetic diversity in *W. somnifera*.

The total genomic DNA from individual samples was extracted from seedling tissue using CTAB method (Saghai-Maroof et al., 1984). DNA was quantified and diluted (50ng/µL) for further RAPD amplification. The polymerase chain reactions (PCR) were performed in a 20 µL mixture containing 50 ng of genomic DNA, 0.2 µM RAPD decamer primer, 0.25 mMdNTPs, 0.02 U *Taq* polymerase, 10X assay buffer and 2.5 mM $MgCl_2$. Amplifications were performed on thermal cycler. The standardized amplification profile was: initial denaturation temperature 94 °C for 2 min followed by 45 cycles of denaturation 94 °C for 1 min; primer annealing 50 °C for 1 min; primer extension 72 °C for 1 min; and a final primer extension at 72 °C for 5 min. Twenty µL of the reaction mixtures were size fractionated through 1.2% agarose gel electrophoresis and stained with ethidium bromide. These agarose gels were visualized under UV light source, documented with the gel documentation system and analyzed for band presence

or absence. The amplified products were scored as 1 for presence and 0 for absence respectively. Only those gels that showed amplicons upon repetition with each of the primers tested were scored in order to overcome problems of poor repeatability. The resulting data matrix of the ISSR primers was analyzed using NTSYS-pc software version 2.02 (Exeter Software, NewYork, USA). A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard 1908) using the marker data for all the *W. somnifera*genotypes following unweighted pair group method (UPGMA).

Results and discussion

Identification and characterization of germplasm diversityis an essential prerequisite for formulating strategies for

plant improvement and conservation of genetic resources.Molecular markers represent a powerful and rapid

tool for characterizing diversity within the target species.A subset of 100 ISSR primers (UBC primers set # 9. 801-900) were screened from a set of 100 primers in 21 genotypes of *W.somnifera* out of them, 66 primers successfully amplified but good amplification and clear banding profile was obtained only in 55 primers. These primers containing di, tri, tetra and penta nucleotides repeats of 16-22 bases.

Majority of ISSR primers which showed clear amplification and sharp resolution had di-nucleotide repeat motifs than tri or penta nucleotide primers. 55 ISSR primers amplified 399 ISSR markers loci. The size of the amplified markers ranged from100-4000 bp. Maximum numbers of bands i.e. 12 were amplified by primer 811, 812 and 859, while minimum number of bands i.e. 3 was obtained with primer 850, 856 and 875. Out of these 399 loci, 252 loci were found polymorphic (63.15%) across all the *W.somnifera*genotypes. Percentage polymorphism ranged from 0 to as high as 100 (primer 822, 842, 856 and 875). Average number of total bands per primer was 7.25, while average number of polymorphic bands per primer was 4.58 (Table 2)

Specific bands were amplified by primers 807, 808, 840, 853, 854, 885 and 899, which separated specific *Withaniasomnifera* accessions from remaining accessions. Primer 807 amplified 3 specific bands in WS-3, WS-4 and WS-13 accessions at molecular weight of about ~2500 bp, ~2100bp and ~500bp respectively. Another specific band of molecular weight ~700 bp was amplified by the primer 840 in WS-7 accessions. Another primer 853 amplified a unique

band in WS-3 at a molecular weight of ~1500 bp. Similarly, primer 854 amplified a specific band in WS-15 at a molecular weight of ~3500 bp, primer 873 amplified a specific band in WS-1 at a molecular weight of ~500 bp. Another specific primer 885 amplified a specific band in WS-11 at a molecular weight of ~1500 bp. Similarly, primer 899 amplified a specific band in WS-7 at a molecular weight of ~1400 bp.

Based on ISSR markers, pairwise genetic similarity among 21 *W.somnifera*genotypes were estimated and a dendrogram was generated by UPGMA cluster analysis based on similarity coefficient. The cluster analysis grouped the *W.somnifera*genotypes into two major groups. The first major group contained seventeen accessions. The first major group divided into two subgroups. First subgroup contained three accessions namely WS-1, WS-4 and WS-8 while, second subgroup consisted fourteen accessions. Among fourteen accessions WS-15 andWS-21 grouped together and remaining twelve accessions namely WS-3, WS-5, WS-7, WS-12, WS-9, WS-6, WS-10, WS-13, WS-16, WS-19 and WS-18 formed separate group. The second major group consisted of four accessions WS-11, WS-14, WS-17 and WS-20.

Majority of ISSR primers used in the study which showed clear amplification and sharp resolution had di-nucleotide repeat motifs than tri or penta nucleotide primers. During the selection of ISSR primer, most of AT repeat primers showed smear on the gels and did not amplify sharp bands. Kochieva et al. (2002) also reported that di nucleotides repeats motifs amplified more number of bands as compare to other repeats in Solanaceae family members. Several studies also showed the similar result in other species of Solanaceae family (Kumar et al., 2007; Danilova and Karlov 2006). The genetic similarity coefficients values among 21 Ashwagandha accessions based on ISSR analysis were ranged from 0.194 to 0.982. Similar results were obtained by Negi et al. (2006) when analysed 25 genotypes, showed similar genetic similarity. A high similarity was observed among different accession of *Withania*analyzed by AFLP techniques (Negi et al., 2000) although in another experiment lately conducted by Negi et al. (2006) observed 79% polymorphic bands among 25 *Withania* genotypes.

Five distinct morphological forms of *W. somnifera* are present in India and also have genetic variation (Dhar et al., 2006). In this study, ISSRs were used for assessment of phylogenetic relationship. The amount of polymorphism produced by ISSR was 63.15%. Such a high degree of polymorphism within *W. somnifera* may be explained that there are different chemotypesprevelant with in *W. somnifera* population based on their chemical composition.

Similarly Scartezzini et al. (2007) also observed a high level of polymorphism between and among Indian and Italian genotypes of *W. somnifera* when analyzed by PCR-RAPD and whitaferinA phytochemical. It has also been observed that Indian samples were more diverged for withaferinA content as compared to Sardinian samples. Thesestudies indicate that either the species is highly polymorphic or the name W. somnifera has been indiscriminately applied to a wide variety of dissimilar forms.

Chaurasiya et al. (2008) showed that *W. somnifera* accessions can be differentiated at metabolomic, proteiomic, isozymic and molecular levels. Dharmar and De Britto (2011) found higher percentage (83.78%) of polymorphism among *W. somnifera* accessions collected from different geographical areas. On the contrary, Mir et al., (2011) reported low levels of genetic diversity revealed with RAPD (37.82%) and AFLP (43.94%) primers. ISSR markers proved to be marginally more informative than RAPD in the assessment of genetic diversity of *W. somnifera*. Tripathi et al., (2012) found ISSR markers better in comparison with RAPD markers during diversity analysis among 12 *W. somnifera* genotypes. However, microsatellites based markers have been reported to be more informative as compared to other types of markers in many plant species.

The present study demonstrates the efficiency of ISSR markers in genetic diversity analysis of *W. somnifera*genotypes. The considerable level of genetic diversity was found among studied genotypes. These results may provide a base for the improvement of *W. somnifera* genotypes at genetic level.

References

Dhar, R.S., Verma, V., Suri, K.A., Sangwan, R.S., Satti, N.K., Kumar, A., Tuli, R., Qazi, G.N., 2006. Phytochemical and genetic analysis in selected chemotypes of *Withaniasomnifera*.Phytochemistry 67, 2269-2276.

Scartezzini, P., Antognoni, F., Conte, L., Maxia, A., Troia, A., Poli, F., 2007.Genetic and phytochemical differences between some Indian and Italian plants of *Withaniasomnifera* (L.)Dunal. Natl. Prod. Res. 21, 923–932

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW. 1984. Ribosomal DNA spacer – length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proceedings of the National Academy of Sciences (USA) 81:8014-8018.

Jaccard P (1908) Nouvellesrecherchessur la distribution florale. Bull Soc Vaud Sci Nat 44:223–270

Kochieva, E.Z., N. N. Ryzhova, , I. A. Khrapalova , and V. A. Pukhalskyi (2002). Genetic Diversity and Phylogenetic relationships in the genus Lycopersicon (Tourn.) Mill. as

Revealed by Inter-Simple Sequence Repeat (ISSR) analysis. Russian Journal of Genetics, 38(8): 958–966.

Kumar, A., M. Kaul, M. Bhan, Khanna, Punit and K. Suri (2007). Morphological and chemical variation in 25 collections of the Indian medicinal plant, Withaniasomnifera (L.)Dunal (Solanaceae).Genet. Res. and Crop Evol., 54(3): 655-660.

Danilova, T. and Karlov, G. (2006) Application of inter simple sequence repeat (ISSR) polymorphism for detection of sex-specific molecular markers in hop (Humuluslupulus L.). Euphytica, 151(1): 15-2.

Negi, M.S., A. Singh and M. Lakshmikumaran (2000). Genetic variation and relationship among and within Withania species as revealed by AFLP markers. Genome, 43: 975–980.

Negi, M.S., V. Sabharwal, N. Wilson and M. S. Lakshmi kumaran (2006).Comparative analysis of the efficiency of SAMPL and AFLP in assessing genetic relationships among Withaniasomnifera genotypes. Current Sci,91(4):464-471.

Chaurasiya, N.D., N.S. Sangwan, L.N. Misra, R. Tuli and R.S. Sangwan (2008).Metabolomic, proteomic, isozymic and genomic diversity analysis in WithaniasomniferaDunal of different accessions. L. Souvenir/ National Interactive meet/NIM/CIMAP/ Lucknow(India).

Dharmar K. and A. John De Britto (2011) RAPD Analysis of Genetic Variability in Wild Populations of Withaniasomnifera(L.) Dunal. International Journal of Biological Technology 2(1): 21-25

Mir, B.A., Koul, S., Kumar, A., Kaul, M.K., Soodan, A.S., Raina, S.N. 2011.Assessment and characterization of genetic diversity in Withaniasomnifera (L.)Dunal using RAPD and AFLP markers.Afr J Biotechnol 10,14746–14756.

Tripathi, N., Saini, N., Mehto, V., Kumar, S., Tiwari, S., 2012. Assessment of genetic diversity among Withaniasomnifera collected from central India using RAPD and ISSR analysis. Med. Aromatic Plant Sci. Biotechnol. 6,33–39

Table 1 *Withaniasomnifera* (L.) Dunal genotypes used in the present study

S.	Collection sites	Label	Lattitide	Longitude
1.	Hoshangabad	WS-1	22° 46'	77° 45'
2.	Dewas	WS-2	22° 58'	78° 06'
3.	Khandwa	WS-3	21° 82'	76° 34'
4.	Indore	WS-4	22° 44'	75° 50'
5.	Bhopal	WS-5	23° 16'	77° 36'
6.	Seoni	WS-6	22° 06'	79° 35'
7.	Rewa	WS-7	24° 32'	81° 18'
8.	Badwani	WS-8	22° 03'	74° 57'
9.	Amarkantak	WS-9	22° 67'	81° 75'
10.	Shivpuri	WS-10	25° 4'	77° 44'
11.	Satna	WS-11	24° 34'	80° 55'
12.	Singrauli	WS-12	24° 12'	82° 39'
13.	TFRI, Jabalpur	WS-13	23° 10'	79° 59'
14.	JNKVV, Jabalpur	WS-14	23° 10'	79° 59'
15.	Sehore	WS-15	23° 12'	77° 00'
16.	Shahdol	WS-16	23° 00'	81° 3'
17.	Gwalior	WS-17	26° 14'	78° 10'
18.	Pachmarhi	WS-18	22° 3'	78° 22'
19.	Maihar	WS-19	24° 16'	80° 49'
20.	Katni	WS-20	23° 47'	80° 27'
21.	Dindori	WS-21	22° 57'	81° 41'

Table 2 Numbers of bands obtained using ISSR primers

S.	Primer	TB	MB	PB	PP	PIC
1.	UBC801	6	2	4	66.67	0.133
2.	UBC 803	7	3	4	55.55	0.125
3.	UBC 807	8	2	6	75.0	0.601
4.	UBC 808	7	2	5	71.43	0.401
5.	UBC 810	8	1	7	88.88	0.125
6.	UBC 811	12	6	6	50.00	0.123
7.	UBC 812	12	4	8	66.67	0.147
8.	UBC 813	8	1	7	88.88	0.332
9.	UBC 814	7	1	6	85.71	0.381
10.	UBC 815	8	8	0	0.00	0.000
11.	UBC 820	6	1	5	83.33	0.340
12.	UBC 822	5	0	5	100.0	0.508
13.	UBC 823	8	3	5	62.50	0.184
14.	UBC 824	9	4	5	55.55	0.355
15.	UBC 825	7	2	5	71.43	0.285
16.	UBC 826	7	7	0	0.00	0.000
17.	UBC 827	8	4	4	50.00	0.113
18.	UBC 830	4	3	1	25.00	0.185
19.	UBC 834	5	2	3	60.00	0.135
20.	UBC 835	10	4	6	60.00	0.138
21.	UBC 836	8	2	6	75.00	0.238
22.	UBC 838	7	1	6	85.71	0.142
23.	UBC 839	5	3	2	40.00	0.189
24.	UBC 840	9	1	8	88.88	0.105
25.	UBC 842	4	0	4	100.0	0.709
26.	UBC 843	6	1	5	83.33	0.640
27.	UBC 844	5	2	3	60.00	0.166
28.	UBC 850	3	0	3	100	0.388
29.	UBC 851	8	2	6	75.00	0.386
30.	UBC 853	8	3	5	62.50	0.244
31.	UBC 854	9	2	7	77.77	0.206
32.	UBC 856	3	0	3	100.00	0.287
33.	UBC 857	7	1	6	85.71	0.224
34.	UBC 859	12	4	8	66.66	0.476
35.	UBC 860	4	4	0	0.00	0.000
36.	UBC 862	6	0	6	100.0	0.422
37.	UBC 865	6	2	4	66.67	0.255
38.	UBC 866	7	2	5	71.43	0.285
39.	UBC 870	7	2	5	0.00	0.000
40.	UBC 873	9	6	3	33.33	0.179
41.	UBC 875	3	0	3	100.00	0.276
42.	UBC 876	8	8	0	0.0	0.000
43.	UBC 878	7	3	4	57.14	0.142

44.	UBC 880	10	2	8	80.00	0.242
45.	UBC 881	5	5	0	0.00	0.000
46.	UBC 885	10	5	5	50.00	0.138
47.	UBC 886	8	2	6	75.00	0.244
48.	UBC 887	9	3	6	64.44	0.206
49.	UBC 888	8	1	7	87.50	0.259
50.	UBC 890	6	2	4	66.66	0.135
51.	UBC 891	10	4	6	60.00	0.228
52.	UBC 895	5	5	0	0.00	0.000
53.	UBC 896	10	6	4	40.00	0.138
54.	UBC 899	8	2	6	75.00	0.238
55.	UBC 900	7	1	6	85.71	0.142
Total		**399**	**147**	**252**	**-**	**13.225**
Average		**7.25**	**2.67**	**4.58**	**-**	**0.241**

TB-Total Bands, MP-Monommorphic Bands, PB-Polymorphic Bands, PP-Percentage Polymorphism, PIC-Polymorphism Information Content

Table 3 Genetic distance among *Withaniasomnifera*accessions based on ISSR analysis

	WS-1	**WS-2**	**WS-3**	**WS-4**	**WS-5**	**WS-6**	**WS-7**	**WS-8**	**WS-9**	**WS-10**	**WS-11**	**WS-12**	**WS-13**	**WS-14**	**WS-15**	**WS-16**	**WS-17**	**WS-18**	**WS-19**	**WS-20**	**WS-21**
WS-1	1.00																				
WS-2	0.567	1.00																			
WS-3	0.226	0.564	1.00																		
WS-4	0.763	0.537	0.233	1.00																	
WS-5	0.240	0.520	0.848	0.287	1.00																
WS-6	0.366	0.658	0.683	0.393	0.799	1.00															
WS-7	0.273	0.558	0.752	0.323	0.892	0.813	1.00														
WS-8	0.629	0.495	0.266	0.754	0.278	0.364	0.304	1.00													
WS-9	0.235	0.515	0.814	0.283	0.867	0.771	0.812	0.308	1.00												
WS-10	0.250	0.533	0.803	0.297	0.858	0.794	0.790	0.305	0.971	1.00											
WS-11	0.282	0.257	0.200	0.300	0.199	0.217	0.183	0.285	0.206	0.221	1.00										
WS-12	0.247	0.518	0.794	0.284	0.850	0.798	0.782	0.297	0.950	0.978	0.207	1.00									
WS-13	0.251	0.520	0.796	0.287	0.839	0.788	0.761	0.300	0.923	0.950	0.210	0.971	1.00								
WS-14	0.268	0.239	**0.194**	0.293	0.205	0.212	0.194	0.278	0.211	0.226	0.761	0.212	0.210	1.00							
WS-15	0.324	0.410	0.412	0.372	0.444	0.490	0.445	0.366	0.492	0.494	0.328	0.502	0.497	0.319	1.00						
WS-16	0.306	0.541	0.690	0.357	0.731	0.714	0.688	0.375	0.808	0.831	0.252	0.813	0.792	0.267	0.514	1.00					
WS-17	0.338	0.274	0.212	0.362	0.211	0.227	0.201	0.362	0.212	0.226	0.847	0.213	0.216	0.721	0.356	0.347	1.00				
WS-18	0.304	0.527	0.682	0.345	0.724	0.716	0.681	0.367	0.790	0.813	0.239	0.828	0.807	0.254	0.521	**0.982**	0.334	1.00			
WS-19	0.307	0.529	0.684	0.348	0.715	0.708	0.664	0.370	0.770	0.792	0.242	0.807	0.830	0.252	0.516	0.958	0.337	0.975	1.00		
WS-20	0.304	0.257	0.205	0.336	0.209	0.220	0.204	0.334	0.210	0.224	0.685	0.211	0.209	0.876	0.328	0.345	0.823	0.331	0.329	1.00	
WS-21	0.357	0.427	0.410	0.413	0.433	0.470	0.428	0.409	0.470	0.473	0.337	0.480	0.475	0.334	0.892	0.600	0.433	0.609	0.603	0.409	1.00

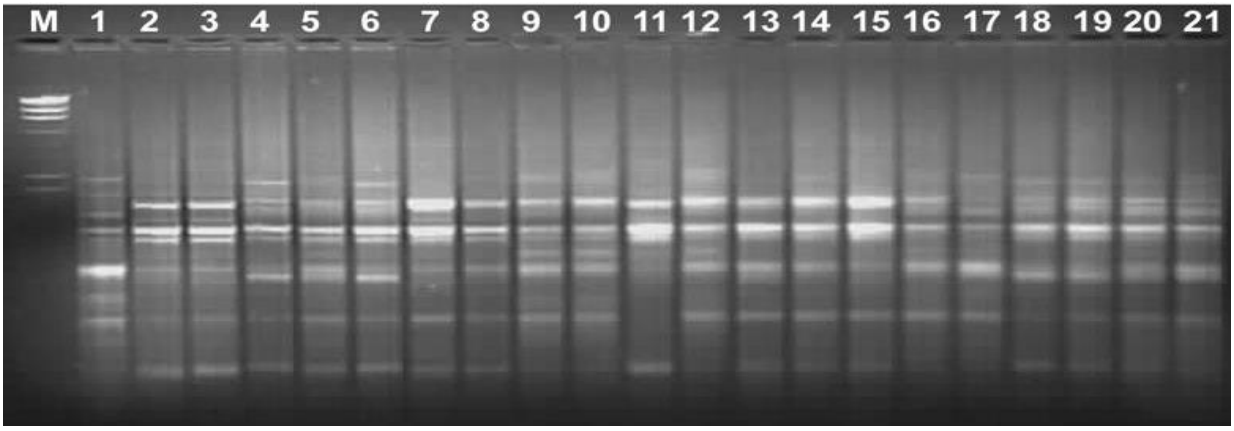

Fig. 1 Electrophoretic banding pattern of ISSR marker UBC873 resolved on 1.2% Agarose gel. M: 1kb DNA ladder, Lane 1-21: *W. somnifera*genotypes as described in Table 1.

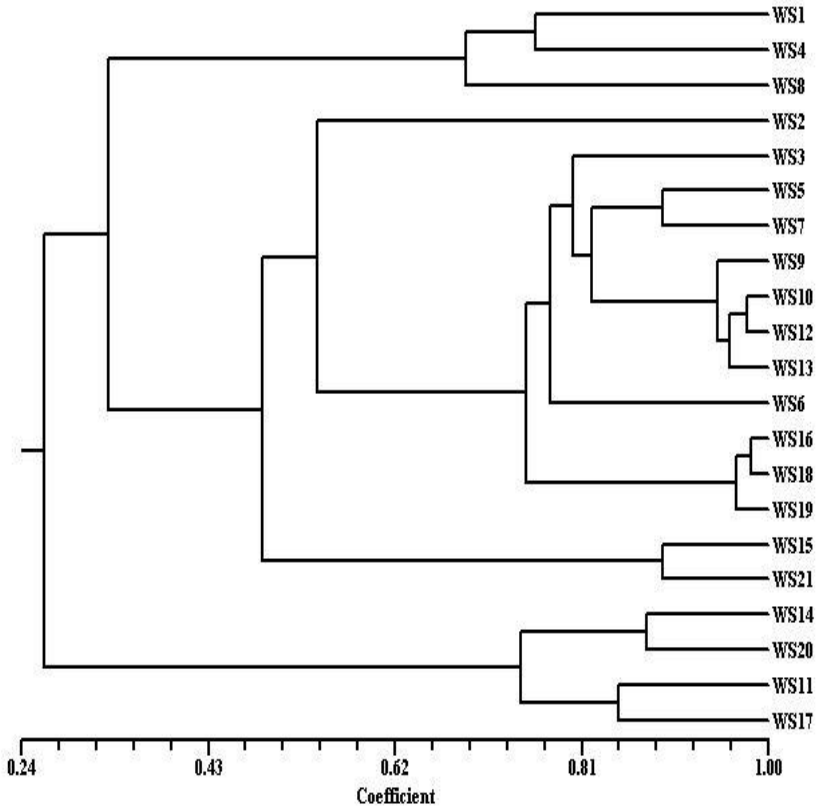

Fig. 2 Dendrogram generated using UPGMA analysis showing relationship among *W. somnifera*genotypes using ISSR markers

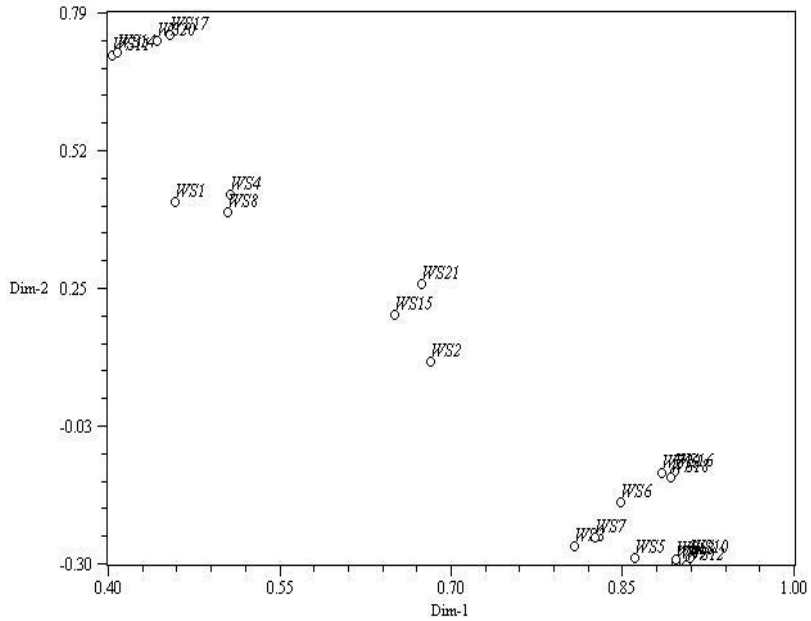

Fig. 3 Two dimensional representation of genetic similarity among *W. somnifera*genotypes using ISSR markers

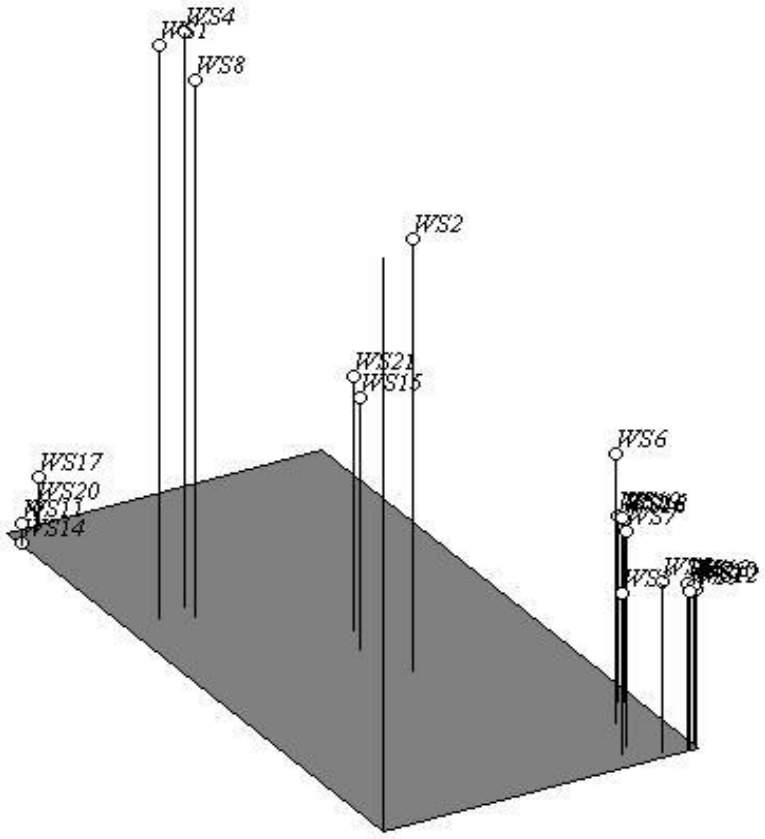

Fig. 4 Three dimensional representation of genetic similarity among *W. somnifera*genotypesusing ISSR markers